PTO-1390 (01-18)
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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A SUBMISSION UNDER 35 U.S.C. 371

Attorney Docket No.	U.S. Application No. (if known, see 37 CFR 1.5)
19472-0070001	Not yet assigned

International Application No.	International Filing Date	Priority Date Claimed
PCT/IB2017/057476	November 29, 2017	October 4, 2016

Title of Invention
METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

First Named Inventor
Hyun Ho JUNG

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. [x] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). NOTE: The express request under 35 U.S.C. 371(f) will not be effective unless the requirements under 35 U.S.C. 371(c)(1), (2), and (4) for payment of the basic national fee, copy of the International Application and English translation thereof (if required), and the oath or declaration of the inventor(s) have been received.
2. [x] A copy of the International Application (35 U.S.C. 371(c)(2)) is attached hereto (not required if the International Application was previously communicated by the International Bureau or was filed in the United States Receiving Office (RO/US)).
3. An English language translation of the International Application (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
4. An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4))
   a. [ ] is attached.
   b. [ ] was previously filed in the international phase under PCT Rule 4.17(iv).

Items 5 to 8 below concern amendments made in the international phase.

PCT Article 19 and 34 amendments

5. [ ] Amendments to the claims under PCT Article 19 are attached (not required if communicated by the International Bureau) (35 U.S.C. 371(c)(3)).
6. [ ] English translation of the PCT Article 19 amendment is attached (35 U.S.C. 371(c)(3)).
7. [ ] English translation of annexes (Article 19 and/or 34 amendments only) of the International Preliminary Examination Report is attached (35 U.S.C. 371(c)(5)).

Cancellation of amendments made in the international phase

8a. [ ] Do not enter the amendment made in the international phase under PCT Article 19.

8b. [ ] Do not enter the amendment made in the international phase under PCT Article 34.

NOTE: A proper amendment made in English under Article 19 or 34 will be entered in the U.S. national phase application absent a clear instruction from applicant not to enter the amendment(s).

The following items 9 to 17 concern a document(s) or information included.

9. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
10. [ ] A preliminary amendment.
11. [x] An Application Data Sheet under 37 CFR 1.76.
12. [ ] A substitute specification. NOTE: A substitute specification cannot include claims. See 37 CFR 1.125(b).
13. [x] A power of attorney and/or change of address letter.
14. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13*ter*.3 and 37 CFR 1.821-1.825 (not required if sequence listing in text format was indicated on the PCT Request as part of the International Application and the sequence listing was published as part of the international application).
15. [ ] Assignment papers *(cover sheet and document(s))*. Name of Assignee: _____
16. [ ] 37 CFR 3.73(c) Statement *(when there is an Assignee)*.

This collection of information is required by 37 CFR 1.414 and 1.491-1.492. The information is required to obtain or retain a benefit by the public, which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 15 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450

U.S. APPLN. No. (if known – see 37 CFR 1.5)	INTERNATIONAL APPLICATION No.	ATTORNEY DOCKET No.
Not yet assigned	PCT/IB2017/057476	19472-0070001

17. [x] Other items or information:

PCT Request 101; PCT/IB/304; PCT/RO/159;
International Search Report; and
Written Opinion of the International Search Authority

The following fees have been submitted.	CALCULATIONS
18. [x] Basic national fee (37 CFR 1.492(a)) **$300**	$ 300
19. [x] Examination fee (37 CFR 1.492(c)) • If the written opinion prepared by ISA/US or the international preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4)..... **$0** • All other situations **$760**	$ 760
20. [x] Search fee (37 CFR 1.492(b)) • If the written opinion prepared by ISA/US or the international preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4)..... **$0** • Search fee (37 CFR 1.445(a)(2)) has been paid on the international application to the USPTO as an International Searching Authority ..... **$140** • International Search Report prepared by an ISA other than the US and provided to the Office or previously communicated to the US by the IB..... **$520** • All other situations **$680**	$ 520
TOTAL OF 18, 19, and 20 =	$ 1580

[ ] Additional fee for specification and drawings filed in paper over 100 sheets (excluding sequence listing in compliance with 37 CFR 1.821(c) or (e) in an electronic medium or computer program listing in an electronic medium) (37 CFR 1.492(j)).

Fee for each additional 50 sheets of paper or fraction thereof **$400**

Total Sheets	Extra Sheets	Number of each additional 50 or fraction thereof (round **up** to a whole number)	RATE	
- 100 =	/ 50 =		x **$400**	$

Surcharge for furnishing any of the search fee, examination fee, or the oath or declaration after the date of commencement of the national stage (37 CFR 1.492(h))..... **$140**	$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	- 20 =		x **$100**	$
Independent claims	- 3 =		x **$480**	$
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$820**	$

Fee for submission of Sequence Listing text file of 300 MB to 800 MB (37 CFR 1.21(o)(1))..... **$1,000**	$
Fee for submission of Sequence Listing text file of more than 800 MB (37 CFR 1.21(o)(2))..... **$10,000**	$
Processing fee for furnishing the English translation later than 30 months from the earliest claimed priority date (37 CFR 1.492(i))..... **$140** +	$
TOTAL OF ABOVE CALCULATIONS =	$ 1580
[ ] **Applicant asserts small entity status.** See 37 CFR 1.27. Fees above are reduced by ½.	
[ ] **Applicant certifies micro entity status.** See 37 CFR 1.29. Fees above are reduced by ¾. Applicant must attach form PTO/SB/15A or B or equivalent.	
TOTAL NATIONAL FEE =	$ 1580
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31)..... **$50.00** per property +	$
TOTAL FEES ENCLOSED =	$ 1580

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees.

c. ☒ The Director is hereby authorized to charge additional fees which may be required, or credit any overpayment, to Deposit Account No. 60-2219 as follows:

i. ☐ any required fee.

ii. ☒ any required fee except for excess claims fees required under 37 CFR 1.492(d) and (e) and multiple dependent claim fee required under 37 CFR 1.492(f).

d. ☒ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038. The PTO-2038 should only be mailed or faxed to the USPTO. However, when paying the basic national fee, the PTO-2038 may NOT be faxed to the USPTO.

ADVISORY: If filing by EFS-Web, do **NOT** attach the PTO-2038 form as a PDF along with your EFS-Web submission. Please be advised that this is **not** recommended and by doing so your **credit card information may be displayed via PAIR**. To protect your information, it is recommended to pay fees online by using the electronic payment method.

NOTE: Where an appropriate time limit under 37 CFR 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the International Application to pending status.

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013, and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.

NOTE 1: By providing this statement under 37 CFR 1.55 or 1.78, **this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA**.

NOTE 2: A U.S. national stage application may not claim priority to the international application of which it is the national phase. The filing date of a U.S. national stage application is the international filing date. See 35 U.S.C. 363.

Correspondence Address

☒ The address associated with Customer Number: 141404 **OR** ☐ Correspondence address below

Name					
Address					
City		State		Zip Code	
Country		Telephone			
Email					

Signature	Judith U. Kim	Date	April 1, 2019
Name (Print/Type)	Judith U. Kim	Registration No. (Attorney/Agent)	40,679

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	19472-0070001
	Application Number	
Title of Invention	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Hyun Ho		JUNG	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Seoul	Country of Residence i	KR

Mailing Address of Inventor:

Address 1	101-2402, 32-14, Seoulsup 2-gil, Seongdong-gu		
Address 2			
City	Seoul	State/Province	
Postal Code	04769	Country i	KR

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Gi Hyeok		YANG	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Cheonan-si	Country of Residence i	KR

Mailing Address of Inventor:

Address 1	603-904, 82, Buldang 11-ro Seobuk-gu		
Address 2	Chungcheongnam-do		
City	Cheonan-si	State/Province	
Postal Code	31165	Country i	KR

Inventor 3 Remove

Legal Name

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	19472-0070001
	Application Number	
Title of Invention	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION	

Prefix	Given Name	Middle Name	Family Name	Suffix
	Chang Hoon		RHEE	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Seoul	Country of Residence i	KR

Mailing Address of Inventor:

Address 1	105-812, 400, Cheonggyecheon-ro, Jung-gu		
Address 2			
City	Seoul	State/Province	
Postal Code	04572	Country i	KR

Inventor 4 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Hack Woo		KIM	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Cheongju-si	Country of Residence i	KR

Mailing Address of Inventor:

Address 1	201-904, 55, Daenong-ro Heungdeok-gu		
Address 2	Chungcheongbuk-do		
City	Cheongju-si	State/Province	
Postal Code	28425	Country i	KR

Inventor 5 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Gi Hyuck		RYU	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Daejeon	Country of Residence i	KR

Mailing Address of Inventor:

Address 1	104-1202, 70, Cheongsa-ro, Seo-gu		
Address 2			
City	Daejeon	State/Province	
Postal Code	35218	Country i	KR

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	19472-0070001
	Application Number	
Title of Invention	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION	

Inventor 6 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Hyung Pyo		HONG	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Cheongju-si	Country of Residence i	KR

Mailing Address of Inventor:

Address 1	405-1501, 103, Yuliyang-ro Cheongwon-gu		
Address 2	Chungcheongbuk-do		
City	Cheongju-si	State/Province	
Postal Code	28317	Country i	KR

Inventor 7 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Jung Hyun		SONG	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Gunsan-si	Country of Residence i	KR

Mailing Address of Inventor:

Address 1	Rm. 201, 42, Namsusong 5-gil, Jeollabuk-do		
Address 2			
City	Gunsan-si	State/Province	
Postal Code	54102	Country i	KR

Inventor 8 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Joon Chan		KONG	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Cheongju-si	Country of Residence i	KR

Mailing Address of Inventor:

Address 1	Rm. 506, 200, Osongsaengmyeong-ro, Osong-eup		
Address 2	Heungdeok-gu, Chungcheongbuk-do		

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	19472-0070001
	Application Number	
Title of Invention	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION	

City	Cheongju-si	State/Province	
Postal Code	28166	Country i	KR

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	141404		
Email Address	USPat@rubinrudman.com	Add Email	Remove Email

Application Information:

Title of the Invention	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION		
Attorney Docket Number	19472-0070001	Small Entity Status Claimed	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	2	Suggested Figure for Publication (if any)	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

PTO/AIA/14 (02-18)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	19472-0070001
	Application Number	
Title of Invention	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION	

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	(●) Customer Number	( ) US Patent Practitioner	( ) Limited Recognition (37 CFR 11.9)
Customer Number	141404		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	a 371 of international	PCT/IB2017/057476	2017-11-29

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[1] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

			Remove
Application Number	Country[1]	Filing Date (YYYY-MM-DD)	Access Code[1] (if applicable)
10-2016-0127540	KR	2016-10-04	00EE

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button.

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	19472-0070001
	Application Number	
Title of Invention	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION	

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	19472-0070001
	Application Number	
Title of Invention	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION	

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

PTO/AIA/14 (02-18)
Approved for use through 11/30/2020. OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	19472-0070001
	Application Number	
Title of Invention	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION	

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

○ Assignee	○ Legal Representative under 35 U.S.C. 117	○ Joint Inventor
◉ Person to whom the inventor is obligated to assign.	○ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name	MEDYTOX INC.		
Mailing Address Information For Applicant:			
Address 1	78, Gangni 1-gil, Ochang-eup Cheongwon-gu		
Address 2	Chungcheongbuk-do		
City	Cheongju-si	**State/Province**	
Country	KR	Postal Code	28126
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button.

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

PTO/AIA/14 (02-18)
Approved for use through 11/30/2020. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	19472-0070001
	Application Number	
Title of Invention	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION	

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		**State/Province**	
Country i		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.

Signature:

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	Judith U. Kim			Date (YYYY-MM-DD)	2019-04-01
First Name	Judith	Last Name	Kim	Registration Number	40,679

Additional Signature may be generated within this form by selecting the Add button.

PTO/AIA/14 (02-18)
Approved for use through 11/30/2020. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	19472-0070001
	Application Number	
Title of Invention	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION	

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent CooperationTreaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82A (07-13)
Approved for use through 01/31/2018. OMB 0651-0035
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TRANSMITTAL FOR POWER OF ATTORNEY TO ONE OR MORE REGISTERED PRACTITIONERS

NOTE: This form is to be submitted with the Power of Attorney by Applicant form (PTO/AIA/82B) to identify the application to which the Power of Attorney is directed, in accordance with 37 CFR 1.5, unless the application number and filing date are identified in the Power of Attorney by Applicant form. If neither form PTO/AIA/82A nor form PTO/AIA82B identifies the application to which the Power of Attorney is directed, the Power of Attorney will not be recognized in the application.

Application Number	Not yet assigned
Filing Date	Concurrently herewith
First Named Inventor	Hyun Ho JUNG
Title	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
Art Unit	Not yet assigned
Examiner Name	Not yet assigned
Attorney Docket Number	19472-0070001

SIGNATURE of Applicant or Patent Practitioner

Signature	Judith U. Kim	Date (Optional)	April 1, 2019
Name	Judith U. Kim	Registration Number	40,679
Title (if Applicant is a juristic entity)			
Applicant Name (if Applicant is a juristic entity)	MEDYTOX INC.		

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. If more than one applicant, use multiple forms.

☐ *Total of _____ forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82B (07-13)
Approved for use through 11/30/2014. OMB 0651-0051
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

☑ I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 141404

OR

☐ I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

☑ The address associated with the above-mentioned Customer Number

OR

☐ The address associated with Customer Number:

OR

Firm or Individual Name	Rubin and Rudman LLP				
Address	800 Connecticut Avenue, N.W., Suite 400				
City	Washington	State	DC	Zip	20006
Country	United States of America				
Telephone	202-794-6300	Email	USPat@rubinrudman.com		

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

Medytox Inc.

☐ Inventor or Joint Inventor (title not required below)

☐ Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

☑ Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

☐ Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature		Date (Optional)	Dec. 19th, 2016
Name	Hyun Ho JUNG		
Title	CEO		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

☑ Total of forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2017/057476

A. CLASSIFICATION OF SUBJECT MATTER

C07K 1/18(2006.01)i, C07K 14/33(2006.01)i, B01D 15/36(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K 1/18; C07K 14/33; C12P 21/04; C12P 1/00; C07H 21/00; C12N 1/20; B01D 15/36

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: botulinum toxin complex, anion exchange chromatography, cation exchange chromatography

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 9206409 B2 (ALLERGAN, INC.) 08 December 2015 See columns 2-3, 6-9, 31, and 38-39.	1-9
A	EISELE, KARL-HEINZ et al., "Studies on the dissociation of botulinum neurotoxin type A complexes", Toxicon, 2011, Vol.57, No.4, pp.555-565 See abstract; and pages 556-558.	1-9
A	US 2011-0171226 A1 (JOHNSON, ERIC A. et al.) 14 July 2011 See the whole document.	1-9
A	KR 10-2003-0060150 A (MEDYTOX INC.) 16 July 2003 See the whole document.	1-9
A	HASEGAWA, KIMIKO et al., "Characterization of toxin complex produced by a unique strain of Clostridium botulinum serotype D 4947", The Protein Journal, 2004, Vol.23, No.6, pp.371-378 See the whole document.	1-9

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 2018 (22.02.2018)	**22 February 2018 (22.02.2018)**

Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea
Facsimile No. +82-42-481-8578

Authorized officer
KIM, Sun Hee
Telephone No. +82-42-481-5405

Form PCT/ISA/210 (second sheet) (January 2015)

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 9206409 B2	08/12/2015	AU 2010-273601 A1	20/01/2011
		AU 2010-273601 B2	12/05/2016
		CA 2767760 A1	20/01/2011
		CN 102482332 A	30/05/2012
		CN 102482332 B	03/08/2016
		CN 106117325 A	16/11/2016
		EP 2454275 A1	23/05/2012
		EP 2454275 B1	12/07/2017
		EP 3252070 A1	06/12/2017
		JP 2012-532932 A	20/12/2012
		JP 2016-028601 A	03/03/2016
		JP 2017-070291 A	13/04/2017
		JP 5887268 B2	16/03/2016
		KR 10-1667174 B1	17/10/2016
		KR 10-1784532 B1	11/10/2017
		KR 10-2012-0105417 A	25/09/2012
		KR 10-2015-0085140 A	22/07/2015
		KR 10-2017-0019489 A	21/02/2017
		MX 2012-000615 A	26/03/2012
		MX 340718 B	22/07/2016
		RU 2012-104778 A	20/08/2013
		RU 2561459 C2	27/08/2015
		US 2011-0008843 A1	13/01/2011
		US 2012-0108792 A1	03/05/2012
		US 2012-0123095 A1	17/05/2012
		US 2012-0156756 A1	21/06/2012
		US 2012-0245324 A1	27/09/2012
		US 2015-0184141 A1	02/07/2015
		US 2016-0097045 A1	07/04/2016
		US 2017-0327811 A1	16/11/2017
		US 8129139 B2	06/03/2012
		US 8324349 B2	04/12/2012
		US 8357541 B2	22/01/2013
		US 8927229 B2	06/01/2015
		US 8932827 B2	13/01/2015
		US 9719076 B2	01/08/2017
		WO 2011-008713 A1	20/01/2011
US 2011-0171226 A1	14/07/2011	CA 2759797 A1	04/11/2010
		EP 2424884 A1	07/03/2012
		EP 2424884 B1	22/07/2015
		US 8440204 B2	14/05/2013
		WO 2010-127258 A1	04/11/2010
KR 10-2003-0060150 A	16/07/2003	None	

PCT REQUEST

Print Out (Original in Electronic Form)

0	**For receiving Office use only**	
0-1	International Application No.	PCT/IB2017/057476
0-2	International Filing Date	29 November 2017 (29.11.2017)
0-3	Name of receiving Office and "PCT International Application"	RO/IB

0-4	**Form PCT/RO/101 PCT Request**	
0-4-1	Prepared Using	ePCT-Filing Version 4.1.015 MT/FOP 20151028/0.20.5.24
0-5	**Petition** The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	International Bureau of the World Intellectual Property Organization (RO/IB)
0-7	**Applicant's or agent's file reference**	GN-55281-PCT
I	**Title of invention**	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
II	**Applicant**	
II-1	This person is	Applicant only
II-2	Applicant for	All designated States
II-4	Name	MEDYTOX INC.
II-5	Address	78, Gangni 1-gil, Ochang-eup Cheongwon-gu, Cheongju-si Chungcheongbuk-do 28126 Republic of Korea
II-6	State of nationality	KR
II-7	State of residence	KR
II-10	e-mail	iplaw@leemock.com
II-10(a)	E-mail authorization The receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority are authorized to use this e-mail address, if the Office or Authority so wishes, to send notifications issued in respect of this international application:	exclusively in electronic form (no paper notifications will be sent)
II-11	Applicant's registration No. with the Office	1-2002-000134-8

PCT REQUEST

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III-1	Applicant and/or inventor	
III-1-1	This person is	Inventor only
III-1-3	Inventor for	All designated States
III-1-4	Name (LAST, First)	JUNG, Hyun Ho
III-1-5	Address	101-2402, 32-14, Seoulsup 2-gil Seongdong-gu Seoul 04769 Republic of Korea
III-2	Applicant and/or inventor	
III-2-1	This person is	Inventor only
III-2-3	Inventor for	All designated States
III-2-4	Name (LAST, First)	YANG, Gi Hyeok
III-2-5	Address	603-904, 82, Buldang 11-ro Seobuk-gu, Cheonan-si Chungcheongnam-do 31165 Republic of Korea
III-3	Applicant and/or inventor	
III-3-1	This person is	Inventor only
III-3-3	Inventor for	All designated States
III-3-4	Name (LAST, First)	RHEE, Chang Hoon
III-3-5	Address	105-812, 400, Cheonggyecheon-ro Jung-gu Seoul 04572 Republic of Korea
III-4	Applicant and/or inventor	
III-4-1	This person is	Inventor only
III-4-3	Inventor for	All designated States
III-4-4	Name (LAST, First)	KIM, Hack Woo
III-4-5	Address	201-904, 55, Daenong-ro Heungdeok-gu, Cheongju-si Chungcheongbuk-do 28425 Republic of Korea
III-5	Applicant and/or inventor	
III-5-1	This person is	Inventor only
III-5-3	Inventor for	All designated States
III-5-4	Name (LAST, First)	RYU, Gi Hyuck
III-5-5	Address	104-1202, 70, Cheongsa-ro Seo-gu Daejeon 35218 Republic of Korea

PCT REQUEST

Print Out (Original in Electronic Form)

III-6	Applicant and/or inventor	
III-6-1	This person is	Inventor only
III-6-3	Inventor for	All designated States
III-6-4	Name (LAST, First)	HONG, Hyung Pyo
III-6-5	Address	405-1501, 103, Yullyang-ro Cheongwon-gu, Cheongju-si Chungcheongbuk-do 28317 Republic of Korea
III-7	Applicant and/or inventor	
III-7-1	This person is	Inventor only
III-7-3	Inventor for	All designated States
III-7-4	Name (LAST, First)	SONG, Jung Hyun
III-7-5	Address	Rm.201, 42, Namsusong 5-gil Gunsan-si Jeollabuk-do 54102 Republic of Korea
III-8	Applicant and/or inventor	
III-8-1	This person is	Inventor only
III-8-3	Inventor for	All designated States
III-8-4	Name (LAST, First)	KONG, Joon Chan
III-8-5	Address	Rm.506, 200, Osongsaengmyeong-ro, Osong-eup Heungdeok-gu, Cheongju-si Chungcheongbuk-do 28166 Republic of Korea

PCT REQUEST

Print Out (Original in Electronic Form)

IV-1	**Agent or common representative; or address for correspondence** The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	Agent
IV-1-1	Name	Y.P.LEE, MOCK & PARTNERS
IV-1-2	Address	12F Daelim Acrotel, 13 Eonju-ro 30-gil Gangnam-gu, Seoul 06292 Republic of Korea
IV-1-3	Telephone No.	82-2-588-8585
IV-1-4	Facsimile No.	82-2-588-8586
IV-1-5	e-mail	iplaw@leemock.com
IV-1-5(a)	E-mail authorization The receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority are authorized to use this e-mail address, if the Office or Authority so wishes, to send notifications issued in respect of this international application:	as advance copies followed by paper notifications
IV-1-6	Agent's registration No.	9-2005-100002-8
V	**DESIGNATIONS**	
V-1	**The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.**	
V-2	**Item V-2 may only be used to exclude (irrevocably) the designations concerned if, at the time of filing or subsequently under Rule 26bis.1, the international application contains in Box No. VI a priority claim to an earlier national application filed in the particular State concerned, in order to avoid the ceasing of the effect, under the national law, of this earlier national application.**	KR
VI-1	**Priority claim of earlier national application**	
VI-1-1	Filing date	04 October 2016 (04.10.2016)
VI-1-2	Number	10-2016-0127540
VI-1-3	Country or Member of WTO	KR
VI-1-5	Restore the right of priority	The receiving Office is requested to restore the right of priority

PCT REQUEST

Print Out (Original in Electronic Form)

VI-2	**Priority document request** The International Bureau is requested to obtain from a digital library a certified copy of the earlier application(s) identified above as item(s), using, where applicable, the access code(s) indicated:	VI-1 Access code: 00EE	
VI-3	**Incorporation by reference :** where an element of the international application referred to in Article 11(1)(iii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(iii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.		
VII-1	**International Searching Authority Chosen**	Korean Intellectual Property Office (ISA/KR)	
VIII	**Declarations**	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	-	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	-	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	-	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	-	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-	
IX	**Check list**	Number of sheets	Electronic file(s) attached
IX-1	Request (including declaration sheets)	6	✓
IX-2	Description	12	✓
IX-3	Claims	2	✓
IX-4	Abstract	1	✓
IX-5	Drawings	1	✓
IX-7	TOTAL	22	

PCT REQUEST

Print Out (Original in Electronic Form)

	Accompanying Items	Paper document(s) attached	Electronic file(s) attached
IX-8	Fee calculation sheet	-	✓
IX-9	Original separate power of attorney	-	✓
IX-13(A)	Statement for restoration of the right of priority	-	✓
IX-20	Figure of the drawings which should accompany the abstract	1	
IX-21	Language of filing of the international application	English	
X-1	Signature of applicant, agent or common representative	/LEE, Young Pil/	
X-1-1	Name	Y.P.LEE, MOCK & PARTNERS	
X-1-2	Name of signatory	LEE, Young Pil	
X-1-3	Capacity (if such capacity is not obvious from reading the request)	CEO	

FOR RECEIVING OFFICE USE ONLY

10-1	Date of actual receipt of the purported international application	29 November 2017 (29.11.2017)
10-2	Drawings:	
10-2-1	Received	
10-2-2	Not received	
10-3	Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application	
10-4	Date of timely receipt of the required corrections under PCT Article 11(2)	
10-5	International Searching Authority	ISA/KR
10-6	Transmittal of search copy delayed until search fee is paid	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	Date of receipt of the record copy by the International Bureau	

ADVANCE E-MAIL

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION, OBTENTION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

Y.P.LEE, MOCK & PARTNERS
12F Daelim Acrotel, 13 Eonju-ro 30-gil
Gangnam-gu,
Seoul 06292
RÉPUBLIQUE DE CORÉE

Date of mailing *(day/month/year)* 14 December 2017 (14.12.2017)	
Applicant's or agent's file reference GN-55281-PCT	**IMPORTANT NOTIFICATION**
International application No. PCT/IB2017/057476	International filing date *(day/month/year)* 29 November 2017 (29.11.2017)
International publication date *(day/month/year)* **Not yet published**	Priority date *(day/month/year)* 04 October 2016 (04.10.2016)
Applicant MEDYTOX INC.	

The applicant is hereby notified of the date of receipt (or of obtaining by the International Bureau) of the priority document(s) relating to all earlier application(s) whose priority is claimed. Unless otherwise indicated by the letters "NR", in the right-hand column or by an asterisk appearing next to the date of receipt, **the priority document concerned was submitted or transmitted to or obtained by the International Bureau in compliance with Rule 17.1(a), (b) or (b-*bis*)**. This Form replaces any previously issued notification concerning submission, transmittal or obtaining of priority documents.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
04 October 2016 (04.10.2016)	10-2016-0127540	KR	30 November 2017 (30.11.2017)

The letters "NR" denote a priority document which, on the date of mailing of this Form, had not yet been received or obtained by the International Bureau in compliance with Rule 17.1(a), (b) or (b-*bis*). Where the applicant has failed to either submit, request to prepare and transmit, or to request the International Bureau to obtain the priority document within the applicable time limit under that Rule, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

An asterisk "*" next to a date of receipt, denotes a priority document submitted or transmitted to or obtained by the International Bureau but not in compliance with Rule 17.1(a), (b) or (b-*bis*) (the priority document was received after the time limit prescribed in Rule 17.1(a); the request to prepare and transmit the priority document was submitted to the receiving Office after the applicable time limit under Rule 17.1(b) or the request to the International Bureau to obtain the priority document was made after the applicable time limit under Rule 17.1(b-*bis*)). Even though the priority document was not furnished in compliance with Rule 17.1(a), (b) or (b-*bis*), the International Bureau will nevertheless transmit a copy of the document to the designated Offices, for their consideration. In case such a copy is not accepted by the designated Office as the priority document, Rule 17.1(c) provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. +41 22 910 06 10

Authorized officer
Cécile Chatel
e-mail ro.ib@wipo.int
Telephone No. +41 22 338 92 22

PATENT COOPERATION TREATY

ADVANCE EMAIL

PCT

NOTIFICATION OF DECISION ON REQUEST TO RESTORE RIGHT OF PRIORITY

(PCT Rule 26*bis*.3(h)(iii))

From the RECEIVING OFFICE

To:

Y.P.LEE, MOCK & PARTNERS
12F Daelim Acrotel, 13 Eonju-ro 30-gil
Gangnam-gu,
Seoul 06292
RÉPUBLIQUE DE CORÉE

Facsimile No.: 82-2-588-8586

Date of mailing *(day/month/year)* **20 December 2017 (20.12.2017)**	
Applicant's or agent's file reference **GN-55281-PCT**	**IMPORTANT NOTIFICATION**

International application No.	International filing date/Date of receipt *(day/month/year)*	Priority date *(day/month/year)*
PCT/IB2017/057476	**29 November 2017 (29.11.2017)**	**04 October 2016 (04.10.2016)**

Applicant: **MEDYTOX INC.**

Upon the request of the applicant:

☐ included in Form PCT/RO/101 at the time of filing of this international application, or

☑ received on **29 November 2017 (29.11.2017)**

to restore the right of priority in respect of the following priority claim(s) **KR 10-2016-0127540 (04 October 2016 (04.10.2016))**

this receiving Office has decided:

☑ **to restore** the right of priority, based on the finding by this receiving Office that the criterion for restoration applied by **it is satisfied**, namely that the failure to file the international application within the priority period:

- ☐ occurred in spite of due care required by the circumstances having been taken for the reasons set out in the Annex.
- ☑ was unintentional ☑ The reasons are set out in the Annex.

☐ **to refuse** the request to restore the right of priority, following issuance of the Notification of Intended Refusal of Request to Restore Right of Priority (Form PCT/RO/158) dated _____ for the following reason(s) set out below (and, where necessary, more fully in the Annex):

1. ☐ the request to restore the right of priority was not received within the applicable time limit under Rule 26*bis*.3(e).
2. ☐ the international application has an international filing date which is beyond two months from the expiration of the priority period (Rule 26*bis*.3(a)).
3. ☐ a priority claim in respect of an earlier application is not contained in the international application (Rule 26*bis*.3(c)).
4. ☐ the statement of reasons for the failure to file the international application within the priority period is missing (Rule 26*bis*.3(b)(ii)).
5. ☐ a required declaration and/or evidence in support of the statement of reasons for the failure to file the international application within the priority period is missing or insufficient (Rule 26*bis*.3(f)).
6. ☐ lack of or late payment of the fee for restoration of the priority right (Rule 26*bis*.3(d)).
7. ☐ the criterion for restoring the priority right, as applied by this receiving Office (the due care and/or the unintentional criterion), is not met for the reasons set out in the Annex .

☐ The applicant is hereby notified that this receiving Office has decided not to transmit the following document(s) or part(s) thereof to the International Bureau (Rule 26*bis*.3(h-*bis*)):

☐ The applicant is hereby notified that this receiving Office has considered the applicant's request not to transmit (a) document(s) or part(s) thereof to the International Bureau (Rule 26*bis*.3(h-*bis*)), and has nevertheless decided to transmit this/these document(s) or part(s) thereof to the International Bureau.

A copy of this Notification is being sent to the International Bureau.

Name and mailing address of the receiving Office	Authorized officer
The International Bureau of WIPO **PCT RO/IB Team** **34, chemin des Colombettes,** **1211 Geneva 20, Switzerland** Facsimile No. **+41 22 910 06 10**	**VIVET, Jean-Marc** Telephone No. **+4122 338 9895** Email: **ro.ib@wipo.int**

Form PCT/RO/159 (July 2016)

ANNEX TO FORM PCT/RO/159

International application No.
PCT/IB2017/057476

This receiving Office has made the above decision(s) for the following reasons:

The applicant has submitted a request for restoration of the right of priority under Rule 26bis.3 of the Regulations under the PCT, asking to restore the priority right based on the unintentionality criterion. In this request for restoration of the priority right the applicant stated that the failure to file the international application within the priority period was strictly unintentional, as a result of a mistake by an employee who had failed to check the e-mail reminder by the agent, despite the wish of the applicant to file a PCT application claiming priority on the Korean national application.

The criterion of unintentionality is complied with if the applicant can show that the failure to file the international application within the priority period was not on purpose and that he/she had the intent to file when the priority period expired. In view of the above, the applicant stated that the failure to timely file this international application within the priority period was unintentional.

This receiving Office is therefore satisfied that the failure was unintentional and hence decides to restore the priority right under this criterion.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference GN-55281-PCT	FOR FURTHER ACTION	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. **PCT/IB2017/057476**	International filing date *(day/month/year)* **29 November 2017 (29.11.2017)**	(Earliest) Priority Date *(day/month/year)* 04 October 2016 (04.10.2016)

Applicant
MEDYTOX INC.

This International search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language**, the international search was carried out on the basis of :
      ☒ the international application in the language in which it was filed
      ☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.
2. ☐ **Certain claims were found unsearchable** (See Box No. II)
3. ☐ **Unity of invention is lacking** (See Box No. III)
4. With regard to the **title,**
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established by this Authority to read as follows:
5. With regard to the **abstract,**
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
6. With regard to the drawings,
   a. the figure of the **drawings** to be published with the abstract is Figure No. 1
      ☒ as suggested by the applicant.
      ☐ as selected by this Authority, because the applicant failed to suggest a figure.
      ☐ as selected by this Authority, because this figure better characterizes the invention.
   b. ☐ none of the figures is to be published with the abstract.

Form PCT/ISA/210 (first sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2017/057476

A. CLASSIFICATION OF SUBJECT MATTER

C07K 1/18(2006.01)i, C07K 14/33(2006.01)i, B01D 15/36(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K 1/18; C07K 14/33; C12P 21/04; C12P 1/00; C07H 21/00; C12N 1/20; B01D 15/36

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: botulinum toxin complex, anion exchange chromatography, cation exchange chromatography

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 9206409 B2 (ALLERGAN, INC.) 08 December 2015 See columns 2-3, 6-9, 31, and 38-39.	1-9
A	EISELE, KARL-HEINZ et al., `Studies on the dissociation of botulinum neurotoxin type A complexes`, Toxicon, 2011, Vol.57, No.4, pp.555-565 See abstract and pages 556-558.	1-9
A	US 2011-0171226 A1 (JOHNSON, ERIC A. et al.) 14 July 2011 See the whole document.	1-9
A	KR 10-2003-0060150 A (MEDYTOX INC.) 16 July 2003 See the whole document.	1-9
A	HASEGAWA, KIMIKO et al., `Characterization of toxin complex produced by a unique strain of Clostridium botulinum serotype D 4947`, The Protein Journal, 2006, Vol.23, No.6, pp.371-378 See the whole document.	1-9

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 2018 (22.02.2018)	**22 February 2018 (22.02.2018)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
International Application Division
189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea
Facsimile No. +82-42-481-8578

Authorized officer
KIM, Sun Hee
Telephone No. +82-42-481-5405

Form PCT/ISA/210 (second sheet) (January 2015)

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 9206409 B2	08/12/2015	AU 2010-273601 A1	20/01/2011
		AU 2010-273601 B2	12/05/2016
		CA 2767760 A1	20/01/2011
		CN 102482332 A	30/05/2012
		CN 102482332 B	03/08/2016
		CN 106117325 A	16/11/2016
		EP 2454275 A1	23/05/2012
		EP 2454275 B1	12/07/2017
		EP 3252070 A1	06/12/2017
		JP 2012-532932 A	20/12/2012
		JP 2016-028601 A	03/03/2016
		JP 2017-070291 A	13/04/2017
		JP 5887268 B2	16/03/2016
		KR 10-1667174 B1	17/10/2016
		KR 10-1784532 B1	11/10/2017
		KR 10-2012-0105417 A	25/09/2012
		KR 10-2015-0085140 A	22/07/2015
		KR 10-2017-0019489 A	21/02/2017
		MX 2012-000615 A	26/03/2012
		MX 340718 B	22/07/2016
		RU 2012-104778 A	20/08/2013
		RU 2561459 C2	27/08/2015
		US 2011-0008843 A1	13/01/2011
		US 2012-0108792 A1	03/05/2012
		US 2012-0123095 A1	17/05/2012
		US 2012-0156756 A1	21/06/2012
		US 2012-0245324 A1	27/09/2012
		US 2015-0184141 A1	02/07/2015
		US 2016-0097045 A1	07/04/2016
		US 2017-0327811 A1	16/11/2017
		US 8129139 B2	06/03/2012
		US 8324349 B2	04/12/2012
		US 8357541 B2	22/01/2013
		US 8927229 B2	06/01/2015
		US 8932827 B2	13/01/2015
		US 9719076 B2	01/08/2017
		WO 2011-008713 A1	20/01/2011
US 2011-0171226 A1	14/07/2011	CA 2759797 A1	04/11/2010
		EP 2424884 A1	07/03/2012
		EP 2424884 B1	22/07/2015
		US 8440204 B2	14/05/2013
		WO 2010-127258 A1	04/11/2010
KR 10-2003-0060150 A	16/07/2003	None	

PATENT COOPERATION TREATY

ADVANCE E-MAIL

From the INTERNATIONAL BUREAU

PCT

SECOND AND SUPPLEMENTARY NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION (TO DESIGNATED OFFICES WHICH APPLY THE 30 MONTH TIME LIMIT UNDER ARTICLE 22(1))

(PCT Rule 47.1(c))

To:

Y.P.LEE, MOCK & PARTNERS
12F Daelim Acrotel, 13 Eonju-ro 30-gil
Gangnam-gu
Seoul 06292
RÉPUBLIQUE DE CORÉE

Date of mailing *(day/month/year)*	
07 February 2019 (07.02.2019)	
Applicant's or agent's file reference	IMPORTANT NOTICE
GN-55281-PCT	

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IB2017/057476	29 November 2017 (29.11.2017)	04 October 2016 (04.10.2016)

Applicant

MEDYTOX INC.

1. **ATTENTION**: For any designated Office(s), for which the time limit under Article 22(1), as in force from 1 April 2002 (30 months from the priority date), **does not apply**, please see Form PCT/IB/308(First Notice) issued previously.

2. Notice is hereby given that the following designated Office(s), for which the time limit under Article 22(1), as in force from 1 April 2002, **does apply**, has/have requested that the communication of the international application, as provided for in Article 20, be effected under Rule 93*bis*.1. The International Bureau has effected that communication on the date indicated below:
12 April 2018 (12.04.2018)

AZ, BY, CN, EP, HU, KG, KP, MD, MK, MZ, NA, NG, PG, RU, TM

In accordance with Rule 47.1(*c-bis*)(i), those Offices will accept the present notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

3. The following designated Offices, for which the time limit under Article 22(1), as in force from 1 April 2002, **does apply**, have not requested, as at the time of mailing of the present notice, that the communication of the international application be effected under Rule 93*bis*.1 :

AE, AG, AL, AM, AO, AP, AT, AU, BA, BB, BG, BH, BN, BR, BW, BZ, CA, CH, CL, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EA, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, ID, IL, IN, IR, IS, JO, JP, KE, KH, KN, KW, KZ, LA, LC, LK, LR, LS, LY, MA, ME, MG, MN, MW, MX, MY, NI, NO, NZ, OA, OM, PA, PE, PH, PL, PT, QA, RO, RS, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TN, TR, TT, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW

In accordance with Rule 47.1(*c-bis*)(ii), those Offices accept the present notice as conclusive evidence that the Contracting State for which that Office acts as a designated Office does not require the furnishing, under Article 22, by the applicant of a copy of the international application.

4. **TIME LIMITS for entry into the national phase**

For the designated or elected Office(s) listed above, the applicable time limit for entering the national phase will, **subject to what is said in the following paragraph,** be **30 MONTHS** from the priority date.

In practice, **time limits other than the 30-month time limit** will continue to apply, for various periods of time, in respect of certain of the designated or elected Office(s) listed above. For **regular updates on the applicable time limits** (30 or 31 months, or other time limit), Office by Office, refer to the *PCT Gazette*, the *PCT Newsletter* and the *PCT Applicant's Guide*, Volume II, National Chapters, all available from WIPO's Internet site, at http://www.wipo.int/pct/en/index.html.

It is the applicant's **sole responsibility** to monitor all these time limits.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. +41 22 338 82 70

Authorized officer

Cécile Chatel

e-mail: ro.ib@wipo.int

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
Y.P.LEE, MOCK & PARTNERS

12F Daelim Acrotel, 13 Eonju-ro 30-gil Gangnam-gu, Seoul
06292 Republic of Korea

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

Date of mailing *(day/month/year)*	**22 February 2018 (22.02.2018)**
Applicant's or agent's file reference GN-55281-PCT	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IB2017/057476	**29 November 2017 (29.11.2017)**	04 October 2016 (04.10.2016)

International Patent Classification (IPC) or both national classification and IPC

C07K 1/18(2006.01)i, C07K 14/33(2006.01)i, B01D 15/36(2006.01)i

Applicant

MEDYTOX INC.

1. This opinion contains indications relating to the following items:

- [x] Box No. I Basis of the opinion
- [ ] Box No. II Priority
- [ ] Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- [ ] Box No. IV Lack of unity of invention
- [x] Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- [ ] Box No. VI Certain documents cited
- [ ] Box No. VII Certain defects in the international application
- [ ] Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.
For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/KR	Date of completion of this opinion	Authorized officer
International Application Division Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea Facsimile No. +82-42-481-8578	22 February 2018 (22.02.2018)	KIM, Sun Hee Telephone No. 182-42-481-5405

Form PCT/ISA/237 (cover sheet) (January 2015)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/IB2017/057476

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of :

   ☒ the international application in the language in which it was filed

   ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:
      ☐ in the form of an Annex C/ST.25 text file.
      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/IB2017/057476

Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-9	YES
	Claims	NONE	NO
Inventive step (IS)	Claims	NONE	YES
	Claims	1-9	NO
Industrial applicability (IA)	Claims	1-9	YES
	Claims	NONE	NO

2. Citations and explanations :

Reference is made to the following document:
D1 : US 9206409 B2 (ALLERGAN, INC.) 08 December 2015

1. Novelty and Inventive Step

1.1. Claim 1

D1, which is considered to be the closest prior art to the subject matter of claim 1, discloses a two column chromatographic process for obtaining a botulinum neurotoxin type A complex, the process comprising:

fermenting *Clostridium botulinum* bacteria in a fermentation medium; and recovering the biologically active botulinum neurotoxin from a fermentation medium by contacting the fermentation medium with an anion exchange chromatography media followed by contacting an eluent from the anion exchange chromatography medium with a cation exchange chromatography media (see column 6, line 60 to column 7, line 3; and column 9, lines 32-45). D1 also discloses that the botulinum neurotoxin type A complex is eluted from the anion exchange column using a pH step change where more negatively charged components such as nucleic acids and other host cell proteins remain bound to the anion exchange column (see column 31, lines 41-51).

Claim 1 differs from D1 in using chromatography mediums at a pH lower than an isoelectric point (PI) of botulinum toxin. However, such a difference can be easily conceived by a person skilled in the art from D1 disclosing that botulinum toxin type A is known to be soluble in dilute aqueous solutions at pH 4-6.8, wherein at pH above about 7 stabilizing non-toxin proteins dissociate from a neurotoxin, resulting in a gradual loss of toxicity (see column 2, line 66 to column 3, line 7 in D1). Accordingly, claim 1 would have been obvious in view of D1.

Continued on Supplemental Box

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of : Box No. V

Therefore, claim 1 is considered to be novel under PCT Article 33(2), but does not involve an inventive step under PCT Article 33(3).

1.2. Claims 2-9

The additional feature of claim 2, dependent on claim 1, would be obvious over D1 disclosing that botulinum toxin type A is known to be soluble in dilute aqueous solutions at pH 4-6.8, wherein at pH above about 7 stabilizing non-toxin proteins dissociate from a neurotoxin, resulting in a gradual loss of toxicity (see column 2, line 66 to column 3, line 7 in D1).

The additional features of claims 3-7, directly or indirectly dependent on claim 1, would be obvious over D1 disclosing solutions and buffers for use in a purification process to obtain a purified botulinum neurotoxin type A complex, the solutions and buffers comprising: 10 mM sodium phosphate, pH 6.5; 50 mM sodium phosphate, pH 6.5; 50 mM sodium acetate, pH 4.8; 50 mM sodium acetate, pH 4.0; and 10 mM citrate, pH 6.5 (see column 38, line 63 to column 39, line 9).

Concerning the additional features of claims 8-9, dependent on claim 1, D1 discloses a POROS® 50HQ anion exchange chromatography resin and a POROS® 20HS cation exchange chromatography resin (see column 31, lines 30-37). Accordingly, claims 8-9 would have been obvious in view of D1.

Therefore, claims 2-9 are considered to be novel under PCT Article 33(2), but do not involve an inventive step under PCT Article 33(3).

2. Industrial Applicability

Claims 1-9 meet the requirements of industrial applicability under PCT Article 33(4).

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
First Named Inventor/Applicant Name:	Hyun Ho JUNG
Filer:	Judith Unjin Kim/Jessica LaBossiere
Attorney Docket Number:	19472-0070001

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
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NATIONAL STAGE FEE	1631	1	300	300
NATL STAGE SEARCH FEE - REPORT PROVIDED	1642	1	520	520
NATIONAL STAGE EXAM - ALL OTHER CASES	1633	1	760	760
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
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1		19472-0070001_NatlStage_Filing.pdf	4342555 7afa1e6cb82957d0949f7cdda41570a1b87eb6d2	yes	53

Multipart Description/PDF files in .zip description

Document Description	Start	End
Transmittal of New Application	1	4
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New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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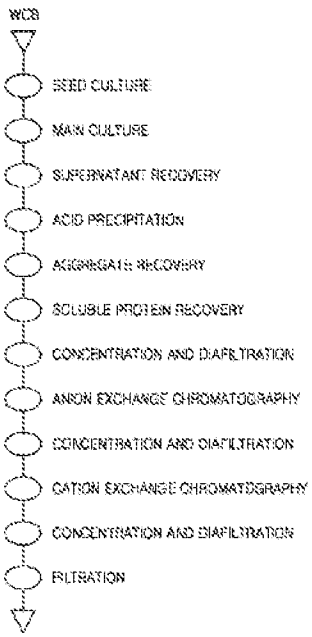
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(54) Title: METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

(57) Abstract: Provided is a method of isolating a botulinum toxin type A macro complex from a botulinum toxin-containing solution, the method including performing anion exchange chromatography and cation exchange chromatography.

[Fig. 1]



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[Continued on next page]

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Description

Title of Invention: METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

Technical Field

[1] CROSS-REFERENCE TO RELATED APPLICATION

[2] This application claims the benefit of Korean Patent Application No. 10-2016-0127540, filed on October 4, 2016, in the Korean Intellectual Property Office, the disclosure of which is incorporated herein in its entirety by reference.

[3] The present disclosure relates to a method of isolating botulinum toxin from a botulinum toxin-containing solution.

Background Art

[4] *Clostridium* strains which secrete toxins having neurotoxicity have been discovered from 1890 until the present day, and research into the properties of toxins secreted by these strains has been conducted for the past 70 years (Schant, E. J. et al., Microbiol. Rev. 56; 80; 1992).

[5] The genus *Clostridium* includes more than 127 species, which are divided according to their morphology and functions. *Clostridium botulinum* is an anaerobic and Gram-positive bacterium which produces a strong polypeptide neurotoxin that causes a neuroparalytic illness, known as botulism, in humans and animals. Spores of *Clostridium botulinum* are found in soil, and a large amount of these spores may even cultured even be cultured in foods which have not been processed properly. These spores may cause many forms of botulism.

[6] Botulinum toxin is divided into seven types of A through G according to their serological characteristics. Each toxin includes about 150 kDa of toxin protein, and naturally is composed of a complex with a variety of non-toxic proteins bound thereto. An intermediate complex (300 kDa) is composed of a toxin protein and a non-toxic non-hemaglutinin (HA) protein, and a large complex (500 kDa) and a macro complex (900 kDa), each have a structure in which the intermediate complex is bound to hemaglutinin (Sugiyama, H., Microbiol. Rev., 44, 419; 1980). Such non-toxic non-hemaglutinin proteins are known to protect the toxin from low pH and various kinds of protein hydrolytic enzymes in intestines (Sugiyama, H., Microbiol. Rev., 44, 419; 1980).

[7] Botulinum toxin is synthesized in a cell as a single-chain polypeptide with a molecular weight of approximately 150 kDa, and then cleaved into two subunits of a light chain (a molecular weight of 50 kDa) and a heavy chain (a molecular weight of 100 kDa) at a position that is one-third the distance from the N-terminus by action of

intracellular protease or by artificial treatment with an enzyme such as trypsin. Such a cleaved toxin has highly increased toxicity in comparison with the single-chain polypeptide. The two subunits are bound to each other by a disulfide bond, and each subunit has a different function. The heavy chain binds with a receptor of a target cell (FEMS Microbiol. Lett. 72, 243; 1990), and reacts with a bio-membrane at a low pH (pH 4.0) to form a channel (Mantecucco, C. et al., TIBS 18, 324; 1993), and the light chain has pharmacological activities, and thus interferes with secretion of neurotransmitters, when cells are provided with permeability by a surfactant or the light chain is introduced into cells by electroporation, (Poulain, B. et al., Proc. Natl. Acad. Sci. USA. 85, 4090; 1988).

[8] Botulinum toxin type A is a natural biological agent which is known to be the most lethal to humans. On a molar basis, botulinum toxin type A is 1.8 billion times more lethal than diphtheria toxin, 600 million times more lethal than sodium cyanide, 30 million times more lethal than cobratoxin, and 12 million times more lethal than cholera toxin (Singh, Critical Aspects of Bacterial protein Toxins, page 63-84 of natural toxins II, edited by B. R. Sigh et al., Plenum Press, New York (1976)).

[9] Botulinum toxin inhibits exocytosis of acetylcholine at a cholinergic presynapse of a neuromuscular junction, thereby causing asthenia. Taking into account that toxicity is exhibited even with exposure to a very small amount of botulinum toxin, it has been considered that this toxin may have enzymatic activity (Simpson, L. L. et al., Ann. Rev. Pharmacol. Toxicol. 26, 427; 1986). According to recent reports, botulinum toxin has metallopeptidase activity, and substrates thereof are unit proteins of an exocytosis machinery complex, such as synaptobrevin, syntaxin, synaptosomal associated protein of 25 kDa (SNAP 25), etc. Each type of botulinum toxin uses one of the three proteins as a substrate, and it is known that botulinum toxin types B, D, F, and G cleave synaptobrevin at a specific site, botulinum toxin types A and E cleave SNAP25 at a specific site, and botulinum toxin type C cleaves syntaxin at a specific site (Binz, T. et al., J. Biol. Chem. 265, 9153; 1994).

[10] Korean Patent Publication NO. 10-2012-0105417 discloses a method of isolating botulinum toxin from a culture of *Clostridium botulinum*, the method including recovering the biologically active botulinum neurotoxin from a fermentation medium by contacting the fermentation medium with an anion exchange chromatography medium and then contacting an eluent from the anion exchange chromatography medium with a cation exchange chromatography medium. In this method, elution is performed after binding the toxin to the anion exchange chromatography medium, but the method does not disclose flowing of the toxin without binding it to the medium.

[11] According to the method, there is a problem in that during purification of the botulinum toxin protein, a neurotoxic component (i.e., a neurotoxic molecule of ap-

proximately 150 kDa), an intermediate complex (300 kDa), or a large complex (500 kDa) is dissociated and removed from the botulinum toxin protein.

Disclosure of Invention

Technical Problem

[12] An aspect provides a method of isolating a botulinum toxin type A macro complex from a botulinum toxin-containing solution.

Solution to Problem

[13] An aspect provides a method of isolating a botulinum toxin type A macro complex from a botulinum toxin-containing solution, the method including contacting the botulinum toxin-containing solution with an anion exchange chromatography medium at a pH lower than an isoelectric point (PI) of botulinum toxin; contacting the solution, which is not bound to the anion exchange chromatography medium, with a cation exchange chromatography medium at a pH lower than PI of botulinum toxin; and separating the botulinum toxin type A macro complex from the cation exchange chromatography medium; or

[14] contacting the botulinum toxin-containing solution with the cation exchange chromatography medium at a pH lower than PI of botulinum toxin; separating botulinum toxin from the cation exchange chromatography medium; contacting the solution containing the toxin separated from the cation exchange chromatography medium, with the anion exchange chromatography medium at a pH lower than PI of botulinum toxin; and separating the botulinum toxin type A macro complex from the solution which is not bound to the anion exchange chromatography medium.

[15] The method includes contacting the botulinum toxin-containing solution with the anion exchange chromatography medium at a pH lower than an isoelectric point (PI) of botulinum toxin; contacting the solution, which is not bound to the anion exchange chromatography medium, with the cation exchange chromatography medium at a pH lower than PI of botulinum toxin; and separating the botulinum toxin type A macro complex from the cation exchange chromatography medium.

[16] As used herein, the "botulinum toxin type A macro complex" may be a dimer of two 16S toxins linked via HA1 which is one of HA proteins, each 16S toxin, i.e., a toxin of about 500 kDa, is composed of botulinum toxin (BoNT), non-toxic non-HA (NTNHA), and HA components.

[17] The method includes contacting the botulinum toxin-containing solution with the anion exchange chromatography medium at a pH lower than PI of botulinum toxin.

[18] In the above process, the botulinum toxin-containing solution may be a culture of *Clostridium botulinum* or a solution derived therefrom. The solution derived therefrom may be a solution obtained by partially purifying the toxin from the culture. Culturing

of *Clostridium botulinum* may be performed by a known method. The culturing may be performed in a medium containing plant-derived components. The culturing is performed by using the medium containing plant-derived components, thereby expressing the toxin outside cells, unlike the prior art. As used herein, the "expressing the toxin outside cells" indicates that, after the cells produce the toxin therein, the cells excrete the toxin spontaneously, and the cells containing the toxin expressed therein are also lysed, whereby the toxin is exposed to a solution of the culture, not inside the cells of the culture.

[19] The medium containing plant-derived components may include phytone peptone, and preferably, phytone peptone, yeast extract, and glucose. In addition, the medium containing plant-derived components may further include one or more components selected from the group consisting of vegetable tryptone, soytone, and sodium thioglycolate. Further, the medium containing plant-derived components may be a medium selected from the group consisting of a medium containing glucose, yeast extract, and phytone peptone; a medium containing glucose, yeast extract, phytone peptone, and vegetable tryptone; a medium containing glucose, yeast extract, phytone peptone, and soytone; a medium containing glucose, yeast extract, soytone, and vegetable tryptone; a medium containing glucose, yeast extract, sodium thioglycolate, phytone peptone, vegetable tryptone, and soytone; and a medium containing glucose, yeast extract, phytone peptone, vegetable tryptone, and soytone.

[20] Further, amounts of glucose, yeast extract, sodium thioglycolate, phytone peptone, vegetable tryptone, and soytone in each medium described as the medium containing plant-derived components may be 0.2 % to 2 %, 0.5 % to 5 %, 0.05 % to 2 %, 0.5 % to 2 %, 0.5 % to 2 %, and 0.5 % to 2 %, respectively, based on a total weight of the medium. The medium containing plant-derived components may be selected from the group consisting of a medium containing glucose, yeast extract, and phytone peptone; a medium containing glucose and yeast extract; a medium containing glucose, yeast extract, phytone peptone, and vegetable tryptone; a medium containing glucose, yeast extract, phytone peptone, and soytone; a medium containing glucose, yeast extract, soytone, and vegetable tryptone; a medium containing glucose, yeast extract, sodium thioglycolate, phytone peptone, vegetable tryptone, and soytone; and a medium containing glucose, yeast extract, phytone peptone, vegetable tryptone, and soytone. Amounts of glucose, yeast extract, sodium thioglycolate, phytone peptone, vegetable tryptone, and soytone in each medium described above are 0.2 % to 2 %, 0.5 % to 5 %, 0.05 % to 2 %, 0.5 % to 2 %, 0.5 % to 2.0 %, and 0.5 % to 2 %, respectively, based on a total weight of the medium, and the rest of the medium may be composed of water.

[21] The toxin may be selected from the group consisting of botulinum toxin A, B, C, D, E, F and G, and preferably, the toxin may be type A toxin. An isoelectric point of

botulinum toxin A may be about 6.06. Therefore, the "pH lower than the isoelectric point" may differ depending on the type of the toxin to be isolated. For example, in the case of type A toxin, the pH may be 6.06 or lower, 6.05 or lower, 6.00 or lower, 5.7 or lower, 5.5 or lower, 5.0 or lower, 4.5 or lower, 4.0 or lower, or 3.5 or lower. In the case of type A toxin, the pH may be 2.5 to 6.05, 3.0 to 6.00, 3.5 to 5.5, 3.5 to 5.0, 3.5 to 4.5, 3.5 to 4.0, 3.5 to 6.00, 4.0 to 6.0, 4.5 to 6.0, 5.5 to 6.0, 4.0 to 5.5, or 4.5 to 5.5.

[22] An anion exchange resin used in the anion exchange chromatography may be a resin substituted with a diethylaminoethyl (DEAE) group or a quaternary ammonium (Q) group, but is not limited thereto. For example, DEAE-Sephadex as described in US Patent NO. 5,696,077, International Patent Publication NO. WO96/05222, and US Patent NO. 5,846,929 may be used. Preferably, any one selected from anion exchange resins having a strong basic quaternary ammonium group or a weak basic diethylaminoethyl (DEAE) group may be used.

[23] For example, the strong basic anion exchange group may be Q Sepharose Fast Flow, Q Sepharose High Performance, Resource Q, Source 15Q, Source 30Q, Mono Q, Mini Q, Capto Q, Capto Q ImpRes, Q HyperCel, Q Ceramic HyperD F, Nuvia Q, UNOsphere Q, Macro-Prep High Q, Macro-Prep 25 Q, Fractogel EMD TMAE(S), Fractogel EMD TMAE Hicap (M), Fractogel EMD TMAE (M), Eshmono Q, Toyopearl QAE-550C, Toyopearl SuperQ-650C, Toyopearl GigaCap Q-650M, Toyopearl Q-600C AR, Toyopearl SuperQ-650M, Toyopearl SuperQ-650S, TSKgel SuperQ-5PW (30), TSKgel SuperQ-5PW (20), TSKgel SuperQ-5PW, etc., but is not limited thereto. Any anion exchange resin known in the art may be used.

[24] By the anion exchange chromatography, *Clostridium botulinum* toxin may flow out without binding to the medium, and impurities bind to the medium. The liquid flowing out of the chromatography may be used in further separation.

[25] In the above process, the contacting may include flowing of the botulinum toxin-containing solution into the medium, for example, flowing of the botulinum toxin-containing solution into the medium packed in a column under predetermined conditions. Conditions such as a flow rate, temperature, time, etc. may be controlled. In the contacting, a phosphate buffer solution, a citrate buffer solution, or an acetate buffer solution may be used as a column buffer solution. A concentration of the column buffer solution may be controlled from 5 mM to 30 mM, for example, from 7 mM to 27 mM, from 10 mM to 25 mM, or from 15 mM to 20 mM. A flow rate of a mobile phase may be 0.5 ml/min to 5.0 ml/min, 1.0 ml/min to 30.0 ml/min, 10 ml/min to 30.0 ml/min, or 15 ml/min to 25.0 ml/min. In this regard, after conductivity of the buffer solution may be adjusted at 3 mS/cm to 30 mS/cm and column equilibration is finished, a sample may be injected.

[26] Further, the method may include contacting the solution, which is not bound to the

medium, with the cation exchange chromatography medium at a pH lower than PI of botulinum toxin. The "pH lower than PI" and "contacting" are the same as described above.

[27] In the above process, the cation exchange chromatography medium may be a medium having a strong acidic cation exchange group such as sulfopropyl (SP) and methyl sulfonate (S) or a weak acidic cation exchange group such as carboxymethyl (CM).

[28] Further, the method includes separating the botulinum toxin from the cation exchange chromatography medium. Separating of the toxin may include recovering of the botulinum toxin from an elution solvent by contacting the medium with the elution solvent. Any elution solvent may be used, as long as it may separate the toxin from the medium. The elution solvent may be, for example, a buffer solution used in the buffering of the column or the buffer solution of which a salt concentration or conductivity may be properly controlled. The salt may be a salt of Na or K. The column buffer solution may be a phosphate buffer solution, a citrate buffer solution, or an acetate buffer solution. A concentration of the column buffer solution may be controlled from 5 mM to 100 mM, for example, from 10 mM to 100 mM, from 20 mM to 100 mM, or from 30 mM to 70 mM. A flow rate of the elution solvent which is a mobile phase may be 0.5 ml/min to 5.0 ml/min, 1.0 ml/min to 150.0 ml/min, 50.0 ml/min to 150.0 ml/min, or 75.0 ml/min to 125.0 ml/min. In this regard, conductivity of the elution solvent may be 3 mS/cm to 30 mS/cm. The elution solvent may be a phosphate buffer (50 mM, pH 5.0) containing 0.5 M to 2.0 M NaCl, for example, 1.0 M NaCl.

[29] The method may further include adjusting the pH of the botulinum toxin or the botulinum toxin-containing solution to a pH lower than PI of botulinum toxin, before contacting with the anion exchange chromatography medium, before contacting with the cation exchange chromatography medium, or before each of these two processes. The adjusting may include mixing the botulinum toxin or the botulinum toxin-containing solution with a buffer having a pH lower than PI of botulinum toxin. The buffer may be a phosphate buffer, a citrate buffer, an acetate buffer, or a mixture thereof. The buffer may have pH of 3.5 to 6.5, or pH of 3.5 to 5.5. The buffer may include 10 mM to 100 mM phosphate, citrate, or acetate.

[30] The method may further include performing ultrafiltration of the botulinum toxin-containing solution, before contacting with the anion exchange chromatography medium, before contacting with the cation exchange chromatography medium, or before each of these two processes. The ultrafiltration may be performed by using a membrane with a molecular weight cut-off of 25 kDa to 125 kDa. The ultrafiltration may be adjusted such that a molecular weight cut-off before contacting with the cation

exchange chromatography medium, for example, a molecular weight cut-off of 50 kDa may be lower than a molecular weight cut-off before contacting with the anion exchange chromatography medium, for example, a molecular weight cut-off of 100 kDa.

[31] The method includes expressing *Clostridium botulinum* toxin outside cells by culturing *Clostridium botulinum* in a medium, before contacting with the anion exchange chromatography medium, and the botulinum toxin-containing solution may be a portion containing *Clostridium botulinum* toxin expressed outside cells by removing the cells from the obtained culture.

[32] The method includes expressing *Clostridium botulinum* toxin outside cells by culturing *Clostridium botulinum* in a medium, before contacting with the anion exchange chromatography medium; and precipitating the toxin by adding an acid to a culture solution which is obtained by removing the cells from the culture, and then separating precipitates; and dissolving the precipitates in a medium and performing ultrafiltration, wherein the botulinum toxin-containing solution may be a solution obtained by the ultrafiltration.

[33] In the method, removing of the cells may be performed by one or more filtrations selected from the group consisting of depth filtration and microfiltration, precipitation of the toxin may be performed by maintaining the culture at pH 3 to pH 4 with an acid, and ultrafiltration may be performed by using a membrane with a molecular weight cut-off of 100 kDa or less.

[34] Another aspect provides a method of isolating botulinum toxin from the botulinum toxin-containing solution, the method including contacting the botulinum toxin-containing solution with the cation exchange chromatography medium at a pH lower than PI, separating the botulinum toxin from the cation exchange chromatography medium, contacting the solution containing the toxin which is separated from the medium, with the anion exchange chromatography medium at a pH lower than PI, and isolating the botulinum toxin from the solution which is not bound to the medium.

[35] The contacting of the botulinum toxin-containing solution with the cation exchange chromatography medium at a pH lower than PI and the separating of the botulinum toxin from the cation exchange chromatography medium are the same as described above, except that the botulinum toxin-containing solution is used instead of "the solution which is not bound to the medium". Further, "the contacting of the solution containing the toxin which is separated from the medium, with the anion exchange chromatography medium at a pH lower than PI, and the isolating of the botulinum toxin from the solution which is not bound to the medium" are the same as described above, except that "the solution containing toxin which is separated from the medium" is used instead of "the botulinum toxin-containing solution".

[36] Reference will now be made in detail to embodiments, examples of which are illustrated in the accompanying drawings, wherein like reference numerals refer to like elements throughout. In this regard, the present embodiments may have different forms and should not be construed as being limited to the descriptions set forth herein. Accordingly, the embodiments are merely described below, by referring to the figures, to explain aspects.

[37]

Advantageous Effects of Invention

[38] According to the method of isolating botulinum toxin from the botulinum toxin-containing solution of an aspect, botulinum toxin type A macro complex, i.e., 900 kDa may be efficiently isolated from the botulinum toxin-containing solution.

Brief Description of Drawings

[39] These and/or other aspects will become apparent and more readily appreciated from the following description of the embodiments, taken in conjunction with the accompanying drawings in which:

[40] FIG. 1 is a schematic view showing a process of purifying *Clostridium botulinum* type A toxin from a culture of *Clostridium botulinum*; and

[41] FIG. 2 is a schematic view showing results of SEC-HPLC of a sample obtained by cation exchange chromatography.

Best Mode for Carrying out the Invention

[42] Hereinafter, the present invention will be described in more detail with reference to Examples. However, these Examples are for illustrative purposes only, and the scope of the invention is not intended to be limited by these Examples.

[43] **Example 1: Culture of *Clostridium botulinum* and Purification of *Clostridium botulinum* toxin**

[44] **1. Culture of *Clostridium botulinum***

[45] In this Example, a large amount of *Clostridium botulinum* type A toxin was expressed outside cells by culturing *Clostridium botulinum* in an animal product-free medium.

[46] The used medium was a medium containing 4 % yeast extract and 0.5 % glucose, based on a total weight of the medium. The components of the medium were dissolved in water for injection (WFI), and pH was adjusted with NaOH to 7.2.

[47] 200 ml of the medium thus prepared was filtered through a sterile single use microfiltration system (0.2 μm Sartorius 2, manufactured by Sartorius), and injected into Cellbag ™ (manufactured by GE healthcare, a single use fermentation bag). Then, the medium was inoculated with 1 ml of a seed culture of *Clostridium botulinum* at a density of 10^6 cells/ml ~ 10^7 cells/ml through a sample injection port. Cellbag ™ is a

disposable bioreactor that uses rocking motion in order to cause mixing under low shear stress. Cellbag ™ is a bag including an air inlet and outlet and a sample port, and is composed of a flexible material, and the bag is air impermeable. In addition, the bag is equipped with a pump combined thereto, which is used for bag inflation and cell supply. The used bag has a volume of 2 L.

[48] Culturing was performed such that 2 L Cellbag ™ containing 200 ml of the species culture was species-cultured at 37 ℃ for 12 hours to 30 hours under rocking at 10 rpm by using a rocker unit in Cellbag ™, and the resultant was inoculated in a bag containing 20 L of a main culture medium through a sterile single use connector (Kleenpack connector PALL) made of polycarbonate, and then cultured for 48 hours to 72 hours while maintaining anaerobic conditions. A sterile filter (0.2 μm) was installed at the sample injection port of the bag, and 200 ml of the species culture medium was injected into the bag through the sterile filter. The injection of the medium was performed using a peristaltic pump.

[49] The species culture medium was injected to the bag without a headspace so as to maintain anaerobic conditions. The species culture was also injected in the same manner. In addition, the main culture was performed by mounting the bag on a platform of the rocker unit of Cellbag ™ and rocking the bag at 10 rpm at a temperature of 37 ℃. During the main culture, 1800 L of nitrogen was purged for 6 hours in order to maintain anaerobic conditions inside the bag, and the culture temperature may be 34 ℃, 35 ℃, or 36 ℃, in addition to 37 ℃.

[50] After the culturing was terminated, a QDC connector (manufactured by Satorious) which is a sterile connector was connected to the sample port of the bag, and the culture was transferred to another disposable bag (flexible disposable bag) through the sterile connector. Then, a sterile disposable depthfilter (Sartoclear or SupraCap, PALL) and microfilter (0.2 μm, Sartopore 2, Sartorius) were connected to the disposable container containing the culture, and the culture was filtered therethrough to remove the cells. Then, the obtained supernatant was put in another disposable bag. From the supernatant, *Clostridium botulinum* toxin was purified.

[51] **2. Purification of *Clostridium botulinum* toxin from culture**

[52] Hereinbelow, a method of producing type A toxin from the cell-removed culture will be described in detail.

[53] **(1) Acid precipitation**

[54] 1 N sulfuric acid was added to a bag containing 20 L of the culture supernatant obtained as above to adjust pH from 3.0 to 4.5, and as a result, toxin protein was precipitated. All procedures of this process were also performed in a disposable sample bag without being in contact with the outside.

[55] Then, when the precipitation was completed, the resulting solution was filtered

through a sterile disposable filtration system (disposable slice 200 0.2 μm filter, manufactured by Sartorius) without being in contact with the outside, thereby removing the supernatant, and the precipitate was collected in a disposable sample bag. 5 L of WFI was added to the collected precipitate to wash sulfuric acid, and a sterile disposable microfiltration system (disposable slice 200 0.2 μm filter) was connected to the bag and the supernatant was removed. As a result, the precipitate was collected into a disposable bag.

[56] **(2) Concentration and Filtration**

[57] 10 L of citrate buffer (25 mM, pH 5.5) was added to the collected precipitate to re-dissolve toxin protein existing in the precipitate. This process was also performed in a disposable bag without being in contact with the outside. A sterile disposable filtration system (0.2 μm, ULTA Cap HC, GE, USA) was connected to the bag containing the re-dissolved sample, and filtration was performed without being in contact with the outside. As a result, the supernatant was collected in a sterile disposable bag and insoluble impurities were removed.

[58] A sterile disposable ultrafiltration membrane (disposable slice 200 100 kDa, manufactured by Sartorius) having a molecular weight cut-off of 100 kDa was connected to the bag containing the sample from which the insoluble impurities were removed. Then, the sample was filtered without being in contact with the outside to be concentrated. Even in this process, toxin protein was in contact with an inner surface of the sterile container, and not in contact with the outside.

[59] **(3) Anion exchange chromatography**

[60] Next, a disposable DEAE anion exchange chromatography system (DEAE BPG column ™; GE Healthcare) was connected to the bag containing the sample after the concentration and buffer exchange were completed, and DEAE anion exchange chromatography was performed without being in contact with the outside. In detail, the citrate buffer (25 mM pH 5.5) was passed through a column packed with a DEAE anion exchange resin to equilibrate the column, and the toxin-containing citrate buffer (25 mM pH 5.5) was passed through the column. In this process, *Clostridium botulinum* type A toxin was not adsorbed to the anion exchange resin, and most impurities were adsorbed thereto and removed. A flow rate was 20 ml/min, and pH was maintained at 5.5.

[61] **(4) Concentration and Filtration**

[62] Next, a fraction containing *Clostridium botulinum* type A toxin was collected from the flow-through solutions which were not bound to the anion exchange resin during the anion exchange chromatography.

[63] A sterile disposable ultrafiltration membrane (disposable slice 200 100 kDa, manufactured by Sartorius) having a molecular weight cut-off of 50 kDa was connected to

the bag containing the fraction, and concentration was performed by filtration without being in contact with outside, and buffer exchange was performed by using a phosphate buffer (50 mM pH 5.0). Even in this process, toxin protein was in contact with an inner surface of the sterile container, and not in contact with the outside. As the buffer used in the buffer exchange, 10 mM to 75 mM phosphate buffer, citrate buffer, or acetate buffer having pH of 3.5 to 6.5, or pH of 3.5 to 5.5 lower than pI of *Clostridium botulinum* toxin may be also used, in addition to the phosphate buffer (50 mM pH 5.0).

[64] **(5) cation exchange chromatography**

[65] Next, a disposable SP cation exchange chromatography system (GE healthcare, SP sepharose FF) was connected to the bag containing the sample obtained by buffer exchange, and cation exchange chromatography was performed without being in contact with outside. In detail, the phosphate buffer (75 mM pH 5.0) was passed through a column packed with a SP cation exchange resin to equilibrate the column, and the sample was passed through the column. In this process, *Clostridium botulinum* type A toxin was adsorbed to the cation exchange resin. This procedure was performed for 25 minutes at a flow rate of 100 ml/min, and pH was maintained at 5.0. Then, elution was performed by using a phosphate buffer (75 mM pH 5.0) containing 1 M NaCl.

[66] The obtained sample was subjected to size exclusion chromatography (SEC-HPLC) and purity of the toxin was analyzed. As a mobile phase, a 40 mM phosphate solution of pH 6.5 was used. SRT SEC-500 (manufactured by Sepax Technologies, P/N 215500) column was connected, and 40 μL of botulinum type A toxin protein was loaded and passed through the column for 20 minutes at 0.8 mL/min.

[67] FIG. 1 is a schematic view showing the process of purifying *Clostridium botulinum* type A toxin from the culture of *Clostridium botulinum*.

[68] FIG. 2 is a schematic view showing results of SEC-HPLC of the sample obtained by cation exchange chromatography.

[69] [Table 1]

Name	Retention time (min)	Area	%Area
12S	12.306	77163	0.19
19S	9.459	40065228	99.64
HMW1	7.000	-	-
HMW2	8.578	127860	0.32

[70] Table 1 shows data of peaks of FIG. 2, and purity of type A toxin was 99.64 %. In

particular, HMW1 appearing at a retention time (RT) was 0 %, indicating absence. In Table 1, HMW1 and HMW2 represent aggregate 1 and aggregate 2, respectively and 12S(300kDa) and 19S(900kDa) are regarded as 12S toxin (also called "M toxin" consisting of BoNT toxin and non-toxic non-HA(NTNHA) and a dimer of two 16S toxins linked via HA1 which is one of HA proteins, each 16S(500kDa) toxin consisting of BoNT, NTNHA, and HA components. The HA component has three different proteins, i.e., HA1, HA2 and HA3. Toxin type A has forms of 12S, 16S, and 19S.

[71] Therefore, according to the purification method, a macro complex of botulinum toxin type A which is not dissociated may be isolated with high purity.

[72] It should be understood that embodiments described herein should be considered in a descriptive sense only and not for purposes of limitation. Descriptions of features or aspects within each embodiment should typically be considered as available for other similar features or aspects in other embodiments.

[73] While one or more embodiments have been described with reference to the figures, it will be understood by those of ordinary skill in the art that various changes in form and details may be made therein without departing from the spirit and scope as defined by the following claims.

Industrial Applicability

[74] Therefore, according to the purification method, a macro complex of botulinum toxin type A which is not dissociated may be isolated with high purity.

[75]

[76]

Claims

[Claim 1] A method of isolating a botulinum toxin type A macro complex from a botulinum toxin-containing solution, the method comprising:
contacting the botulinum toxin-containing solution with an anion exchange chromatography medium at a pH lower than an isoelectric point (PI) of botulinum toxin;
contacting the solution, which is not bound to the anion exchange chromatography medium, with a cation exchange chromatography medium at a pH lower than the PI of botulinum toxin; and
separating the botulinum toxin type A macro complex from the cation exchange chromatography medium; or
contacting the botulinum toxin-containing solution with the cation exchange chromatography medium at a pH lower than the PI of botulinum toxin;
separating botulinum toxin from the cation exchange chromatography medium;
contacting the solution containing the toxin separated from the cation exchange chromatography medium, with the anion exchange chromatography medium at a pH lower than the PI; and
separating the botulinum toxin type A macro complex from the solution which is not bound to the anion exchange chromatography medium.

[Claim 2] The method of claim 1, wherein the pH lower than the PI of botulinum toxin is pH 3.5 to 6.0.

[Claim 3] The method of claim 1, further comprising adjusting the pH of the botulinum toxin or the botulinum toxin-containing solution to a pH lower than the PI of botulinum toxin, before the contacting of the solution with the anion exchange chromatography medium, before the contacting of the solution with the cation exchange chromatography medium, or before each of these two processes.

[Claim 4] The method of claim 3, wherein the adjusting comprises mixing the botulinum toxin or the botulinum toxin-containing solution with a buffer having a pH lower than the PI of botulinum toxin.

[Claim 5] The method of claim 4, wherein the buffer is a phosphate buffer, a citrate buffer, or an acetate buffer.

[Claim 6] The method of claim 4, wherein the buffer has pH of 3.5 to 6.0.

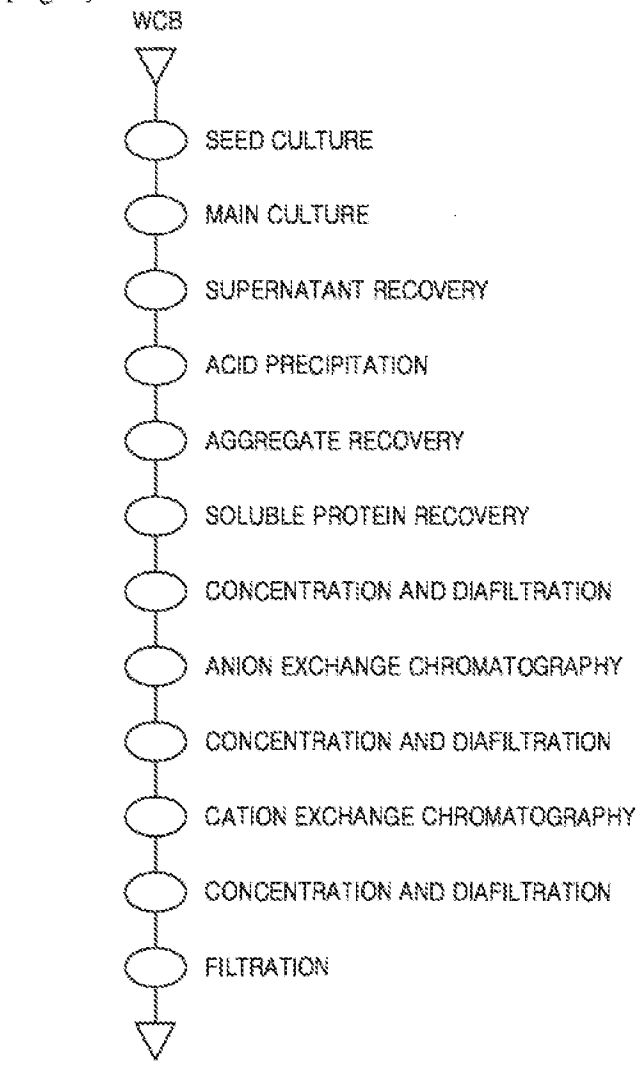
[Claim 7] The method of claim 4, wherein the buffer comprises a 10 mM to 100 mM phosphate buffer, a 10 mM to 100 mM citrate buffer, or a 10 mM

to 100 mM acetate buffer.

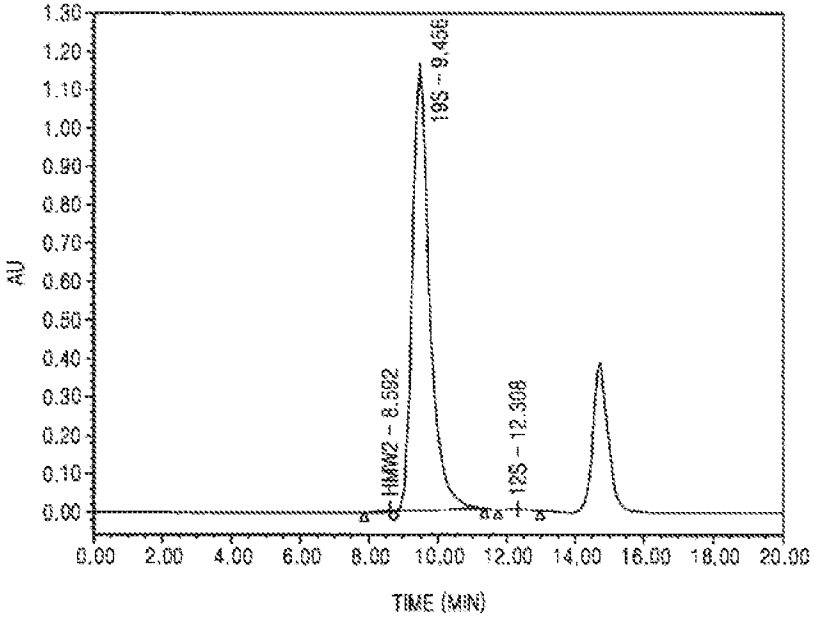
[Claim 8] The method of claim 1, wherein the anion exchange chromatography is DEAE or Q anion exchange chromatography.

[Claim 9] The method of claim 1, wherein the cation exchange chromatography is CM, S, or SP cation exchange chromatography.

[Fig. 1]


WCB
SEED CULTURE
MAIN CULTURE
SUPERNATANT RECOVERY
ACID PRECIPITATION
AGGREGATE RECOVERY
SOLUBLE PROTEIN RECOVERY
CONCENTRATION AND DIAFILTRATION
ANION EXCHANGE CHROMATOGRAPHY
CONCENTRATION AND DIAFILTRATION
CATION EXCHANGE CHROMATOGRAPHY
CONCENTRATION AND DIAFILTRATION
FILTRATION
PURIFIED BOTULINUM TOXIN

[Fig. 2]


AU
TIME (MIN)
HMW2 – 8.592
185 – 9.438
185 – 12.308
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
1.00
1.10
1.20
1.30
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00

PATENT COOPERATION TREATY

ADVANCE EMAIL

PCT

From the RECEIVING OFFICE

NOTIFICATION OF DECISION ON REQUEST TO RESTORE RIGHT OF PRIORITY

(PCT Rule 26*bis*.3(h)(iii))

To:

Y.P.LEE, MOCK & PARTNERS
12F Daelim Acrotel, 13 Eonju-ro 30-gil
Gangnam-gu,
Seoul 06292
RÉPUBLIQUE DE CORÉE

Facsimile No.: 82-2-588-8586

Date of mailing *(day/month/year)* **20 December 2017 (20.12.2017)**	
Applicant's or agent's file reference **GN-55281-PCT**	**IMPORTANT NOTIFICATION**

International application No.	International filing date/Date of receipt *(day/month/year)*	Priority date *(day/month/year)*
PCT/IB2017/057476	**29 November 2017 (29.11.2017)**	**04 October 2016 (04.10.2016)**

Applicant: **MEDYTOX INC.**

Upon the request of the applicant:

☐ included in Form PCT/RO/101 at the time of filing of this international application, or

☑ received on **29 November 2017 (29.11.2017)**

to restore the right of priority in respect of the following priority claim(s) **KR 10-2016-0127540 (04 October 2016 (04.10.2016))**

this receiving Office has decided:

☑ **to restore** the right of priority, based on the finding by this receiving Office that the criterion for restoration applied by **it is satisfied**, namely that the failure to file the international application within the priority period:

- ☐ occurred in spite of due care required by the circumstances having been taken for the reasons set out in the Annex.
- ☑ was unintentional ☑ The reasons are set out in the Annex.

☐ **to refuse** the request to restore the right of priority, following issuance of the Notification of Intended Refusal of Request to Restore Right of Priority (Form PCT/RO/158) dated _____ for the following reason(s) set out below (and, where necessary, more fully in the Annex):

1. ☐ the request to restore the right of priority was not received within the applicable time limit under Rule 26*bis*.3(e).
2. ☐ the international application has an international filing date which is beyond two months from the expiration of the priority period (Rule 26*bis*.3(a)).
3. ☐ a priority claim in respect of an earlier application is not contained in the international application (Rule 26*bis*.3(c)).
4. ☐ the statement of reasons for the failure to file the international application within the priority period is missing (Rule 26*bis*.3(b)(ii)).
5. ☐ a required declaration and/or evidence in support of the statement of reasons for the failure to file the international application within the priority period is missing or insufficient (Rule 26*bis*.3(f)).
6. ☐ lack of or late payment of the fee for restoration of the priority right (Rule 26*bis*.3(d)).
7. ☐ the criterion for restoring the priority right, as applied by this receiving Office (the due care and/or the unintentional criterion), is not met for the reasons set out in the Annex .

☐ The applicant is hereby notified that this receiving Office has decided not to transmit the following document(s) or part(s) thereof to the International Bureau (Rule 26*bis*.3(h-*bis*):

☐ The applicant is hereby notified that this receiving Office has considered the applicant's request not to transmit (a) document(s) or part(s) thereof to the International Bureau (Rule 26*bis*.3(h-*bis*), and has nevertheless decided to transmit this/these document(s) or part(s) thereof to the International Bureau.

A copy of this Notification is being sent to the International Bureau.

Name and mailing address of the receiving Office	Authorized officer
The International Bureau of WIPO **PCT RO/IB Team** **34, chemin des Colombettes,** **1211 Geneva 20, Switzerland** Facsimile No. **+41 22 910 06 10**	**VIVET, Jean-Marc** Telephone No. **+4122 338 9895** Email: **ro.ib@wipo.int**

Hugel Ex. 1006 - Page 39 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

This receiving Office has made the above decision(s) for the following reasons:

The applicant has submitted a request for restoration of the right of priority under Rule 26bis.3 of the Regulations under the PCT, asking to restore the priority right based on the unintentionality criterion. In this request for restoration of the priority right the applicant stated that the failure to file the international application within the priority period was strictly unintentional, as a result of a mistake by an employee who had failed to check the e-mail reminder by the agent, despite the wish of the applicant to file a PCT application claiming priority on the Korean national application.

The criterion of unintentionality is complied with if the applicant can show that the failure to file the international application within the priority period was not on purpose and that he/she had the intent to file when the priority period expired. In view of the above, the applicant stated that the failure to timely file this international application within the priority period was unintentional.

This receiving Office is therefore satisfied that the failure was unintentional and hence decides to restore the priority right under this criterion.

PCT REQUEST

Print Out (Original in Electronic Form)

0	**For receiving Office use only**	
0-1	International Application No.	PCT/IB2017/057476
0-2	International Filing Date	29 November 2017 (29.11.2017)
0-3	Name of receiving Office and "PCT International Application"	RO/IB

0-4	**Form PCT/RO/101 PCT Request**	
0-4-1	Prepared Using	ePCT-Filing Version 4.1.015 MT/FOP 20151028/0.20.5.24
0-5	**Petition** The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	International Bureau of the World Intellectual Property Organization (RO/IB)
0-7	**Applicant's or agent's file reference**	GN-55281-PCT
I	**Title of Invention**	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
II	**Applicant**	
II-1	This person is	Applicant only
II-2	Applicant for	All designated States
II-4	Name	MEDYTOX INC.
II-5	Address	78, Gangni 1-gil, Ochang-eup Cheongwon-gu, Cheongju-si Chungcheongbuk-do 28126 Republic of Korea
II-6	State of nationality	KR
II-7	State of residence	KR
II-10	e-mail	iplaw@leemock.com
II-10(a)	E-mail authorization The receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority are authorized to use this e-mail address, if the Office or Authority so wishes, to send notifications issued in respect of this international application:	exclusively in electronic form (no paper notifications will be sent)
II-11	Applicant's registration No. with the Office	1-2002-000134-8

PCT REQUEST

Print Out (Original in Electronic Form)

III-1	**Applicant and/or inventor**	
III-1-1	This person is	Inventor only
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III-4	**Applicant and/or inventor**	
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III-5	**Applicant and/or inventor**	
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III-5-3	Inventor for	All designated States
III-5-4	Name (LAST, First)	RYU, Gi Hyuck
III-5-5	Address	104-1202, 70, Cheongsa-ro Seo-gu Daejeon 35218 Republic of Korea

PCT REQUEST

Print Out (Original in Electronic Form)

III-6	**Applicant and/or inventor**	
III-6-1	This person is	Inventor only
III-6-3	Inventor for	All designated States
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III-7	**Applicant and/or inventor**	
III-7-1	This person is	Inventor only
III-7-3	Inventor for	All designated States
III-7-4	Name (LAST, First)	SONG, Jung Hyun
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III-8	**Applicant and/or inventor**	
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III-8-4	Name (LAST, First)	KONG, Joon Chan
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PCT REQUEST

Print Out (Original in Electronic Form)

IV-1	**Agent or common representative; or address for correspondence**	
	The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	Agent
IV-1-1	Name	Y.P.LEE, MOCK & PARTNERS
IV-1-2	Address	12F Daelim Acrotel, 13 Eonju-ro 30-gil Gangnam-gu, Seoul 06292 Republic of Korea
IV-1-3	Telephone No.	82-2-588-8585
IV-1-4	Facsimile No.	82-2-588-8586
IV-1-5	e-mail	iplaw@leemock.com
IV-1-5(a)	E-mail authorization The receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority are authorized to use this e-mail address, if the Office or Authority so wishes, to send notifications issued in respect of this international application:	as advance copies followed by paper notifications
IV-1-6	Agent's registration No.	9-2005-100002-8
V	**DESIGNATIONS**	
V-1	**The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.**	
V-2	**Item V-2 may only be used to exclude (irrevocably) the designations concerned if, at the time of filing or subsequently under Rule 26bis.1, the international application contains in Box No. VI a priority claim to an earlier national application filed in the particular State concerned, in order to avoid the ceasing of the effect, under the national law, of this earlier national application.**	KR
VI-1	**Priority claim of earlier national application**	
VI-1-1	Filing date	04 October 2016 (04.10.2016)
VI-1-2	Number	10-2016-0127540
VI-1-3	Country or Member of WTO	KR
VI-1-5	Restore the right of priority	The receiving Office is requested to restore the right of priority

PCT REQUEST

Print Out (Original in Electronic Form)

VI-2	**Priority document request** The International Bureau is requested to obtain from a digital library a certified copy of the earlier application(s) identified above as item(s), using, where applicable, the access code(s) indicated:	VI-1 Access code: 00EE	
VI-3	**Incorporation by reference :** where an element of the international application referred to in Article 11(1)(iii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(iii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.		
VII-1	**International Searching Authority Chosen**	Korean Intellectual Property Office (ISA/KR)	
VIII	**Declarations**	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	–	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	–	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	–	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	–	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	–	
IX	**Check list**	Number of sheets	Electronic file(s) attached
IX-1	Request (including declaration sheets)	6	✓
IX-2	Description	12	✓
IX-3	Claims	2	✓
IX-4	Abstract	1	✓
IX-5	Drawings	1	✓
IX-7	TOTAL	22	

PCT REQUEST

Print Out (Original in Electronic Form)

	Accompanying Items	Paper document(s) attached	Electronic file(s) attached
IX-8	Fee calculation sheet	–	✓
IX-9	Original separate power of attorney	–	✓
IX-13(A)	Statement for restoration of the right of priority	–	✓
IX-20	**Figure of the drawings which should accompany the abstract**	1	
IX-21	**Language of filing of the international application**	English	
X-1	**Signature of applicant, agent or common representative**	/LEE, Young Pil/	
X-1-1	Name	Y.P.LEE, MOCK & PARTNERS	
X-1-2	Name of signatory	LEE, Young Pil	
X-1-3	Capacity (if such capacity is not obvious from reading the request)	CEO	

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10-1	**Date of actual receipt of the purported international application**	29 November 2017 (29.11.2017)
10-2	**Drawings:**	
10-2-1	Received	
10-2-2	Not received	
10-3	**Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application**	
10-4	**Date of timely receipt of the required corrections under PCT Article 11(2)**	
10-5	**International Searching Authority**	ISA/KR
10-6	**Transmittal of search copy delayed until search fee is paid**	

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출 원 번 호 : 10-2016-0127540
Application Number

출 원 년 월 일 : 2016년 10월 04일
Filing Date OCT. 04, 2016

출 원 인 : (주)메디톡스
Applicant(s) Medytox Inc.

2017년 12월 04일

특 허 청
COMMISSIONER

제출 일자 : 2016-10-04

【서지사항】

【서류명】 특허출원서

【참조번호】 3

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【발명의 영문명칭】 Method for isolating botulinum toxin from a solution containing botulinum toxin

【발명자】

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제출 일자 : 2016-10-04

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【출원언어】 국어

【취지】 위와 같이 특허청장에게 제출합니다.

대리인 리앤목 특허법인 (서명 또는 인)

【수수료】

【출원료】	0	면	46,000 원
【가산출원료】	26	면	0 원
【우선권주장료】	0	건	0 원
【심사청구료】	0	항	0 원

【합계】 46,000 원

【감면사유】 중기업[1]

【감면후 수수료】 13,800 원

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【발명의 설명】

【발명의 명칭】

보툴리눔 독소 함유 용액으로부터 보툴리눔 독소를 분리하는 방법{Method for isolating botulinum toxin from a solution containing botulinum toxin}

【기술분야】

<1> 보툴리눔 독소 함유 용액으로부터 보툴리눔 독소를 분리하는 방법에 관한 것이다.

【배경기술】

<2> 신경 독성을 지닌 독소를 분비하는 클로스트리디움 균주들이 1890년대에서부터 지금까지 발견되었으며, 지난 70년간 이들 균주들이 분비하는 독소에 대한 특성이 연구되어 왔다(Schant, E. J. 등, Microbiol. Rev. 56; 80; 1992).

<3> 클로스트리디움 속은 127종 이상이며, 형태 및 기능에 따라 구분된다. 혐기성, 그람 양성 박테리아 클로스트리디움 보툴리눔은 사람 및 동물에서 보툴리즘이라는 신경마비 질환을 일으키는, 강력한 폴리펩티드 신경독소를 생산한다. 클로스트리디움 보툴리눔의 포자는 토양에서 발견되며, 제대로 처리되지 않은 식품에서도 다량 배양 될 수 있다. 이러한 것들이 다양하게 보툴리즘의 원인이 되기도 한다.

<4> 보툴리눔 독소는 그 혈청학적 특징에 따라 A에서 G까지의 7가지 형으로 나뉘어 진다. 각 독소는 약 150 kDa 정도의 독소 단백질을 가지고 있는데, 자연적으로는 여러 비독성 단백질들과 결합되어 있는 복합체로 이루어져 있다. 중간 복합체

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(300 kDa)는 독소 단백질과 비독성-비헤마글루티닌(HA) 단백질로 이루어져 있고, 큰 복합체 (500 kDa) 및 거대 복합체 (900 kDa)는 중간 복합체가 헤마글루티닌과 결합되어 있는 형태를 띠고 있다(Sugiyama, H., Microbiol. Rev., 44, 419; 1980). 이러한 비독성 비헤마글루티닌 단백질들은 장내에서 낮은 pH와 각종 단백질 가수분해 효소로부터 독소를 보호하는 기능을 하는 것으로 알려져 있다 (Sugiyama, H., Microbiol. Rev., 44, 419; 1980).

<5> 독소는 세포 내에서 분자량이 약 150 kDa인 하나의 폴리펩티드로 합성된 후, 세포내 단백질 분해 효소의 작용이나 트립신과 같은 인위적인 효소 처리에 의하여 N말단으로부터 1/3되는 위치에서 절단되어 2개의 단위체인 경쇄 (light chain: 분자량 50 kDa)와 중쇄 (heavy chain: 분자량 100 kDa)로 나뉘어 진다. 이렇게 나뉘어진 독소는 단일 폴리펩티드일 때에 비해 그 독성이 크게 증가한다. 두 단위체는 이황화결합에 의해 서로 연결되어 있으며, 각각은 다른 기능을 갖는다. 중쇄는 표적(target)이 되는 세포의 수용체 (receptor)와 결합하고 (FEMS Microbiol.Lett. 72, 243; 1990), 낮은 pH(pH 4.0)에서 생체막과 반응하여 채널 (channel)을 형성하는 기능을 가지며 (Mantecucco, C. 등, TIBS 18, 324; 1993), 경쇄는 약리활성을 가지고 있어 계면활성제를 사용하여 세포에 투과성을 부여하거나, 전기천공(electroporation)등으로 세포내 투입되었을 때 신경전달물질의 분비를 방해한다 (Poulain, B. 등, Proc. Natl. Acad. Sci. USA. 85, 4090 ; 1988).

<6> 보툴리눔 독소 타입 A는 사람에게 알려진 가장 치명적인 천연 생물학제이다. 몰을 기준으로 할때, 보툴리눔 독소 타입 A는 디프테리아 보다 18억배, 시안화 나

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트륨보다 6억배, 코브로톡신 보다 3천만배, 그리고 콜레라 보다 1천 2백만배 더 치명적이다, Singh, Critical Aspects of Bacterial protein Toxins, page 63-84 of natural toxins II, edited by B.R.Sigh et al., Plenum Press, New York (1976)

<7> 독소는 신경근육종말 (neuromuscular junction)의 콜린성 전시냅스 (cholinergic presynapse)에서 아세틸콜린의 엑소시토시스 (exocytosis)를 저해하여 전신 무력증을 일으킨다. 극미량의 독소를 처리해도 독성이 나타나는 것으로 보아 이 독소는 어떤 효소 활성을 가질 것으로 생각되어져 왔다(Simpson, L. L. 등, Ann. Rev. Pharmaeol. Toxicol. 26, 427; 1986). 최근 밝혀진 바에 의하면, 독소는 메탈로펩티다제 활성 (metallopeptidase activity)을 가지고 있으며, 그 기질은 엑소사이토시스 장치 복합체 (exocytosis machinary complex)를 이루는 단위 단백질들인 시냅토브레빈 (synaptobrevin), 신택신 (syntaxin), 25 kDa의 시냅토좀 연계 단백질 (synaptosomal associated protein of 25 kDa) (SNAP 25)등이다. 각 형의 독소는 이 세가지 단백질들 중 하나를 기질로 삼고 있는데, B, D, F 및 G형은 시냅토브레빈을, A와 E형은 SNAP25를, C형은 신택신을 특정부위에서 절단하는 것으로 알려져 있다 (Binz, T. 등, J. Biol. Chem.265, 9153; 1994).

<8> 한국공개특허공보 제10-2012-0105417호는 발효 배지를 음이온 교환 크로마토그래피 매질과 접촉시키고 이어서 음이온 교환 크로마토그래피 매질에서 나온 용리액을 양이온 교환 크로마토그래피와 접촉시켜 발효 배지로부터 생물학적으로 활성인 보툴리눔 신경독소를 회수하는 단계를 포함하는 클로스트리디움 보툴리눔 배양액으로부터 보툴리눔 독소를 분리하는 방법을 개시하고 있다. 그러나, 상기 방법은

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음이온 교환 크로마토그래피 매질에 독소를 결합시킨 후 용리하는 것으로서, 매질에 결합시키지 않고 흘려보내는 것에 대하여는 개시하고 있지 않다.

<9> 상기 방법에 의하면 보툴리눔 독소 단백질의 정제시 보툴리눔 독소로부터 신경독성 성분(즉, 대략 150 kDa 신경독성 분자), 중간복합체(300 kDa) 또는 큰 복합체(500 kDa)가 불순물로부터 해리되어 제거된다는 문제점이 있었다.

【발명의 내용】

【해결하려는 과제】

<10> 일 양상은 보툴리눔 독소 함유 용액으로부터 보툴리눔 독소 A형 거대 복합체를 분리하는 방법을 제공한다.

【과제의 해결 수단】

<11> 일 양상은 보툴리눔 독소 함유 용액을 보툴리눔 독소의 등전점(isoelectric point: PI) 이하의 산도에서 음이온 교환 크로마토그래피 매질과 접촉시키는 단계;상기 매질과 결합하지 않은 상기 용액을 보툴리눔 독소의 등전점 이하의 산도에서 양이온 교환 크로마토그래피 매질과 접촉시키는 단계; 및 상기 양이온 교환 크로마토그래피 매질로부터 보툴리눔 독소 A형 거대 복합체를 분리하는 단계; 또는

<12> 보툴리눔 독소 함유 용액을 등전점 이하의 산도에서 양이온 교환 크로마토그래피 매질과 접촉시키는 단계, 상기 양이온 교환 크로마토그래피 매질로부터 보툴리눔 독소를 분리하는 단계, 상기 매질에서 분리된 독소를 포함하는 용액을 등전점 이하의 산도에서 음이온 교환 크로마토그래피 매질과 접촉시키는 단계, 및 상기 매

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질과 결합하지 않은 상기 용액으로부터 보툴리눔 독소 A형 거대 복합체를 분리하는 단계;를 포함하는, 보툴리눔 독소 함유 용액으로부터 보툴리눔 독소 A형 거대 복합체를 분리하는 방법을 제공한다.

<13> 상기 방법은 보툴리눔 독소 함유 용액을 보툴리눔 독소의 등전점 (isoelectric point: pI) 이하의 산도에서 음이온 교환 크로마토그래피 매질과 접촉시키는 단계;상기 매질과 결합하지 않은 상기 용액을 보툴리눔 독소의 등전점 이하의 산도에서 양이온 교환 크로마토그래피 매질과 접촉시키는 단계; 및 상기 양이온 교환 크로마토그래피 매질로부터 보툴리눔 독소 A형 거대 복합체를 분리하는 단계를 포함한다.

<14> 본 명세서에 있어서, "보툴리눔 독소 A형 거대 복합체"는 보툴리눔 독소 (BoNT), 비-독성 비-HA(NTNHA) 및 HA 성분으로 구성된 16S 즉 약 500kDa의 독소가 HA 단백질 중 하나인 HA1에 의하여 두 16S 독소가 연결된 디머일 수 있다.

<15> 상기 방법은 보툴리눔 독소 함유 용액을 보툴리눔 독소의 등전점 (isoelectric point: pI) 이하의 산도에서 음이온 교환 크로마토그래피 매질과 접촉시키는 단계를 포함한다.

<16> 상기 단계에서, 상기 보툴리눔 독소 함유 용액은 클로스트리디움 보툴리눔 배양액 또는 그로부터 유래된 것일 수 있다. 그로부터 유래된 것은 배양액으로 상

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기 독소가 부분적으로 정제된 것일 수 있다. 클로스트리디움 보툴리눔의 배양은 알려진 방법에 의하여 이루어진 것일 수 있다. 상기 배양은 식물 유래 성분 함유 배지에서 수행된 것일 수 있다. 식물 유래 성분 함유 배지를 사용하여 배양함으로써, 종래 알려진 바와는 달리, 세포 외로 독소를 발현시키는 것일 수 있다. 본 발명에 있어서, "세포 외로 독소를 발현시킨다"라는 표현의 의미는 세포가 독소를 세포 내에서 생산한 후 자발적으로 세포 외부로 배출하는 것뿐만 아니라, 세포 내부에 발현된 독소를 포함하는 세포가 파쇄 (lysis)되어 독소가, 배양물 중의 세포 내부가 아닌, 배양물의 용액 부분에 노출되는 것을 포함한다.

<17> 상기 식물 유래 성분 함유 배지는 피톤 펩톤 (phytone peptone)을 함유하는 것, 바람직하게는, 피톤 펩톤, 효모 추출물 (yeast extract), 및 포도당을 포함하는 것일 수 있다. 이에 대하여, 상기 식물 유래 성분 함유 배지는 식물 트립톤 (vegetable tryptone), 소이톤 (soytone), 및 소듐 티오글리콜레이트 (sodium thioglycolate)로 이루어진 군으로부터 선택되는 하나 이상의 성분을 더 포함하는 것일 수 있다. 또한, 상기 식물 유래 성분 함유 배지는 포도당, 효모 추출물 및 피톤 펩톤을 포함하는 배지; 포도당, 효모 추출물, 피톤 펩톤 및 식물 트립톤을 포함하는 배지; 포도당, 효모 추출물, 피톤 펩톤 및 소이톤을 포함하는 배지; 포도당, 효모 추출물, 소이톤 및 식물 트립톤을 포함하는 배지; 포도당, 효모 추출물, 소듐 티오글리콜레이트, 피톤 펩톤, 식물 트립톤 및 소이톤을 포함하는 배지; 및 포도당, 효모 추출물, 피톤 펩톤, 식물 트립톤 및 소이톤을 포함하는 배지;로 이루어진 군으로부터 선택되는 배지일 수 있다.

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<18> 또한, 상기 식물 유래 성분 함유 배지로서 열거된 각 배지 중의 포도당, 효모 추출물, 소듐 티오글리콜레이트, 피톤 펩톤, 식물 트립톤, 및 소이톤의 함량은 중량 기준으로 각각 0.2% 내지 2%, 0.5% 내지 5%, 0.05% 내지 2%, 0.5% 내지 2%, 0.5% 내지 2%, 및 0.5% 내지 2%인 것일 수 있다. 또한, 상기 식물 유래 성분 함유 배지는 포도당, 효모 추출물 및 피톤 펩톤을 포함하는 배지; 포도당, 및 효모 추출물 함유 배지; 포도당, 효모 추출물, 피톤 펩톤 및 식물 트립톤을 포함하는 배지; 포도당, 효모 추출물, 피톤 펩톤 및 소이톤을 포함하는 배지; 포도당, 효모 추출물, 소이톤 및 식물 트립톤을 포함하는 배지; 포도당, 효모 추출물, 소듐 티오글리콜레이트, 피톤 펩톤, 식물 트립톤 및 소이톤을 포함하는 배지; 및 포도당, 효모 추출물, 피톤 펩톤, 식물 트립톤 및 소이톤을 포함하는 배지;로 이루어진 군으로부터 선택되는 것으로, 상기 열거된 각 배지 중의 포도당, 효모 추출물, 소듐 티오글리콜레이트, 피톤 펩톤, 식물 트립톤, 및 소이톤의 함량은 중량 기준으로 각각 0.2% 내지 2%, 0.5% 내지 5%, 0.05% 내지 2%, 0.5% 내지 2%, 0.5% 내지 2.0%, 및 0.5% 내지 2%이고, 나머지는 물로 구성되는 것일 수 있다.

<19> 상기 독소는 보툴리눔 독소 A, B, C, D, E, F 및 G로 이루어진 군으로부터 선택되는 것일 수 있으며, 바람직하게는 타입 A 독소이다. 상기 보툴리눔 독소 A의 등전점은 약 6.06일 수 있다. 따라서, 상기 "등전점 이하의 산도"는 분리하고자 하는 독소의 타입에 따라 달라질 수 있다. 예를 들면, 타입 A 독소인 경우, 상기 산도는 pH 6.06이하, pH 6.05이하, pH 6.00이하, pH 5.7이하, pH 5.5이하, pH 5.0이하, pH 4.5이하, pH 4.0이하, 또는 pH 3.5 이하일 수 있다. 타입 A 독소인 경우,

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상기 산도는 pH 2.5 내지 6.05, pH 3.0 내지 6.00, pH 3.5 내지 5.5, pH 3.5 내지 5.0, pH 3.5 내지 4.5, pH 3.5 내지 4.0, pH 3.5 내지 6.00, pH 4.0 내지 6.0, pH 4.5 내지 6.0, pH 5.5 내지 6.0, pH 4.0 내지 5.5, 또는 pH 4.5 내지 5.5일 수 있다.

<20> 상기 음이온교환 크로마토그래피에 사용되는, 음이온교환수지로는 디에틸아미노에틸(DEAE) 또는 사차 암모니움(Quaternary ammonium, Q)기들로 치환된 것들을 사용할 수 있지만, 이에 제한되는 것은 아니다. 예를 들어, 미국특허 제5,696,077호, 국제특허공개 제WO96/05222 및 미국특허 제5,846,929호에 기재된 바와 같은 DEAE-Sephadex를 사용할 수 있다. 바람직하게는 강염기성의 사차 암모니움기를 가지는 그룹이나 또는 약염기성의 디에틸아미노에틸(DEAE) 그룹을 가지는 음이온 교환수지 중에서 어느 하나를 선택하여 사용한다.

<21> 예를 들면, 강염기성의 음이온교환 그룹으로는 Q Sepharose Fast Flow, Q Sepharose High Performance, Resource Q, Source 15Q, Source 30Q, Mono Q, Mini Q, Capto Q, Capto Q ImpRes, Q HyperCel, Q Cermic HyperD F, Nuvia Q, UNOsphere Q, Macro-Prep High Q, Macro-Prep 25 Q, Fractogel EMD TMAE(S), Fractogel EMD TMAE Hicap (M), Fractogel EMD TMAE (M), Eshmono Q, Toyopearl QAE-550C, Toyopearl SuperQ-650C, Toyopearl GigaCap Q-650M, Toyopearl Q-600C AR, Toyopearl SuperQ-650M, Toyopearl SuperQ-650S, TSKgel SuperQ-5PW (30), TSKgel SuperQ-5PW (20), TSKgel SuperQ-5PW 등을 사용할 수 있으나, 이에 한정되는 것은

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아니며 당업계에 공지된 음이온교환수지를 사용할 수 있다.

<22> 상기 음이온 교환 크로마토그래피에 의하여 클로스트리디움 보툴리눔 독소는 매질에 결합하지 않고 통과하여 흘러 나오게 되며, 불순물은 매질에 결합하게 된다. 흘러나온 액체는 추가적으로 분리에 사용된다.

<23> 상기 단계에서, 상기 접촉은 상기 보툴리눔 독소 함유 용액을 매질에 흘려주는 것, 예를 들면 칼럼 중에 충진된 상기 매질에 일정한 조건에서 흘려주는 것을 포함한다. 흘려주는 유속, 온도, 시간 등의 조건은 조절될 수 있다. 상기 접촉은 컬럼 완충액으로는 포스페이트 완충액, 시트레이트 완충액 또는 아세테이트 완충액을 사용할 수 있다. 컬럼 완충액의 농도는 5mM 내지 30 mM, 예를 들면, 7mM 내지 27 mM, 10mM 내지 25 mM, 또는 15mM 내지 20 mM로 조절할 수 있다. 이동상의 유속은 0.5㎖/분 내지 5.0㎖/분, 1.0 내지 30.0㎖/분, 10 내지 30.0㎖/분, 15 내지 25.0㎖/분일 수 있다. 이때, 완충액의 전도도는 3 내지 30mS/cm로 맞추고 컬럼의 평형화가 끝나면 시료를 주입할 수 있다.

<24> 상기 방법은 또한, 상기 매질과 결합하지 않은 상기 용액을 보툴리눔 독소의 등전점 이하의 산도에서 양이온 교환 크로마토그래피 매질과 접촉시키는 단계를 포함한다. 상기 "등전점 이하의 산도" 및 "접촉"에 대하여는 상기한 바와 같다.

<25> 상기 단계에서, 상기 양이온 교환 크로마토그래피는 매질로서 술포프로필

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(sulfopropyl: SP) 및 메틸 술포네이트(S)와 같은 강산성의 양이온 교환 기를 갖거나 카르복시메틸(carboxymethyl: CM)과 같은 약산성의 양이온 교환기를 갖는 매질일 수 있다.

<26> 상기 방법은 또한, 상기 양이온 교환 크로마토그래피 매질로부터 보툴리눔 독소를 분리하는 단계를 포함한다. 상기 독소를 분리하는 단계는 용출 용매를 상기 매질과 접촉시켜 보툴리눔 독소를 상기 용출 용매 중에서 회수하는 단계를 포함한다. 상기 용출 용매는 상기 매질로부터 상기 독소를 떼어낼 수 있는 것이면 어느 것이나 사용될 수 있다. 상기 용출 용매는 예를 들면, 상기 칼럼의 완충에 사용된 완충액 또는 염 농도 또는 전기 전도도가 적적하게 조절된 상기 완충액가 될 수 있다. 상기 염은 Na, 또는 K의 염일 수 있다. 상기 컬럼 완충액으로는 포스페이트 완충액, 시트레이트 완충액 또는 아세테이트 완충액을 사용할 수 있다. 컬럼 완충액의 농도는 5mM 내지 100 mM, 예를 들면, 10mM 내지 100 mM, 20mM 내지 100 mM, 또는 30mM 내지 70 mM로 조절할 수 있다. 이동상인 용출 용매의 유속은 0.5㎖/분 내지 5.0㎖/분, 1.0 내지 150.0㎖/분, 50.0 내지 150.0㎖/분, 75.0 내지 125.0㎖/분일 수 있다. 이때, 용출 용매액의 전도도는 3 내지 30mS/cm일 수 있다. 상기 용출 용매는 0.5 내지 2.0M 예를 들면 1.0M NaCl을 포함하는 포스페이트 버퍼 (50mM pH 5.0)일 수 있다.

<27> 상기 방법에 있어서, 상기 음이온 교환 크로마토그래피 매질과 접촉시키는

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단계 전, 양이온 교환 크로마토그래피 매질과 접촉시키는 단계 전 또는 이 두 단계의 각 단계 전에 상기 보툴리눔 독소 또는 그를 함유하는 용액의 산도를 보툴리눔 독소의 등전점 이하로 조정하는 단계를 더 포함할 수 있다. 상기 조정하는 단계는 상기 보툴리눔 독소 또는 그를 함유하는 용액을 보툴리눔 독소의 등전점 이하의 산도를 갖는 버퍼와 혼합하는 단계를 포함할 수 있다. 상기 버퍼는 포스페이트 버퍼, 시트레이트 버퍼, 아세테이트 버퍼 또는 이들의 혼합물일 수 있다. 상기 버퍼는 pH 3.5 내지 6.5, 또는 3.5 내지 5.5인 것일 수 있다. 상기 버퍼는 10mM 내지 100mM의 포스페이트, 시트레이트 또는 아세테이트를 포함할 수 있다.

<28> 상기 방법에 있어서, 상기 음이온 교환 크로마토그래피 매질과 접촉시키는 단계 전, 양이온 교환 크로마토그래피 매질과 접촉시키는 단계 전 또는 이 두 단계의 각 단계 전에 상기 보툴리눔 독소 함유 용액을 한외여과하는 단계를 더 포함할 수 있다. 상기 한외여과는 분자량 컷-오프 25 kDa 내지 125 kDa의 막을 사용하는 것일 수 있다. 상기 한외여과는 상기 음이온 교환 크로마토그래피 매질과 접촉시키는 단계 전에 수행되는 것의 분자량 컷-오프, 예를 들면 분자량 컷-오프 100 kDa에 비하여 상기 양이온 교환 크로마토그래피 매질과 접촉시키는 단계에서 수행되는 것의 분자량 컷-오프, 예를 들면 분자량 컷-오프 50 kDa가 더 작도록 조절된 것일 수 있다.

<29> 상기 방법에 있어서, 음이온 교환 크로마토그래피 매질과 접촉시키는 단계

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전에, 배지 중에서 클로스트리디움 보툴리눔 (*Clostridium botulinum*)을 배양하여, 클로스트리디움 보툴리눔 독소를 세포 외로 발현시키는 단계;를 포함하고, 상기 보툴리눔 독소 함유 용액은 얻어진 배양물로부터 세포를 제거하여 세포 외에 발현된 클로스트리디움 보툴리눔 독소가 포함된 부분일 수 있다.

<30> 상기 방법에 있어서, 음이온 교환 크로마토그래피 매질과 접촉시키는 단계 전에, 배지 중에서 클로스트리디움 보툴리눔 (*Clostridium botulinum*)을 배양하여, 클로스트리디움 보툴리눔 독소를 세포 외로 발현시키는 단계; 배양물로부터 세포를 제거하고 얻어진 배양액에 산을 첨가하여 독소를 침전시킨 다음 침전물을 분리하는 단계; 및 상기 분리된 침전물을 매질 중에 용해시키고 한외여과하는 단계를 포함하고, 상기 보툴리눔 독소 함유 용액은 상기 한외여과에 의하여 얻어진 용액인 것일 수 있다.

<31> 상기 방법에 있어서, 세포의 제거는 심층 여과 (depth filtration) 및 마이크로여과 (microfiltration)로 이루어진 군으로부터 선택되는 하나 이상의 여과에 의하여 이루어지고, 독소의 침전은 산을 첨가하여 배양액을 pH 3 내지 4로 유지하여 이루어지는 것이고, 한외여과는 분자량 컷오프 100kDa 이하의 막을 사용하여 이루어지는 것일 수 있다.

<32> 다른 방법은 또한 보툴리눔 독소 함유 용액을 등전점 이하의 산도에서 양이온 교환 크로마토그래피 매질과 접촉시키는 단계, 상기 양이온 교환 크로마토그래피 매질로부터 보툴리눔 독소를 분리하는 단계, 상기 매질에서 분리된 독소를 포함

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하는 용액을 등전점 이하의 산도에서 음이온 교환 크로마토그래피 매질과 접촉시키는 단계, 및 상기 매질과 결합하지 않은 상기 용액으로부터 보툴리눔 독소를 분리하는 단계;를 포함하는, 보툴리눔 독소 함유 용액으로부터 보툴리눔 독소를 분리하는 방법을 제공한다.

<33> 상기 보툴리눔 독소 함유 용액을 등전점 이하의 산도에서 양이온 교환 크로마토그래피 매질과 접촉시키는 단계, 및 상기 양이온 교환 크로마토그래피 매질로부터 보툴리눔 독소를 분리하는 단계는 상기에서 "상기 매질과 결합하지 않은 상기 용액" 대신에 보툴리눔 독소 함유 용액을 사용하는 것을 제외하고는 상기한 바와 같다. 또한, "상기 매질에서 분리된 독소를 포함하는 용액을 등전점 이하의 산도에서 음이온 교환 크로마토그래피 매질과 접촉시키는 단계, 및 상기 매질과 결합하지 않은 상기 용액으로부터 보툴리눔 독소를 분리하는 단계"는 "보툴리눔 독소 함유 용액"을 대신에 "상기 매질에서 분리된 독소를 포함하는 용액"을 사용하는 것을 제외하고 상기한 바와 동일하다.

【발명의 효과】

<34> 일 양상에 따른 보툴리눔 독소 함유 용액으로부터 보툴리눔 독소를 분리하는 방법에 의하면, 보툴리눔 독소 함유 용액으로부터 보툴리눔 독소 A형 거대 복합체 즉 900kDa을 효율적으로 분리할 수 있다.

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【도면의 간단한 설명】

<35> 도 1은 클로스트리디움 보툴리눔 배양액으로부터 클로스트리디움 보툴리눔 타입 A 독소의 분리 공정을 나타낸 도면이다.

도 2는 양이온교환 크로마토그래피 후에 얻어진 시료에 대하여 SEC-HPLC 한 결과를 나타낸 도면이다.

【발명을 실시하기 위한 구체적인 내용】

<36> 이하 본 발명을 실시예를 통하여 보다 상세하게 설명한다. 그러나, 이들 실시예는 본 발명을 예시적으로 설명하기 위한 것으로 본 발명의 범위가 이들 실시예에 한정되는 것은 아니다.

<37> 실시예 1: 클로스트리디움 보툴리눔 배양 및 클로스트리디움 보툴리눔 독소 분리

<38> 1. 클로스트리디움 보툴리눔의 배양

<39> 본 실시예에서는 무 동물 유래 성분 배지에서 클로스트리디움 보툴리눔을 배양하여 세포 외로 클로스트리디움 보툴리눔 타입 A 독소를 다량 발현시켰다.

<40> 사용된 배지는 배지 총 중량에 대하여 효모 추출물 4%, 및 포도당 0.5%를 포함하는 것을 사용하였다. 상기 배지 성분을 주사용수 (Water For Injection:WFI)에 녹이고, NaOH를 이용해 pH를 7.2로 조정하여 사용하였다.

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<41> 이렇게 준비된 상기 배지 200ml를 멸균된 일회용 마이크로여과 시스템 (single use microfiltration system: 0.2㎛ Sartorius 2, Sartorius 사)을 통하여 여과하여 Cellbag™ (GE healthcare사 제품, 일회용 발효 백)에 주입하고, 여기에 클로스트리디움 보툴리눔 10^6 ~ 10^7세포/ml 농도의 시드 배양액 1ml를 시료 주입 포트를 통하여 접종하였다. Cellbag™은 낮은 전단 응력에서 혼합을 일으키기 위하여 락킹 운동 (rocking motion)을 사용하는 일회용 생물반응기 (disposable bioreactor)이다. Cellbag™은 공기 입구 및 출구 및 시료 포트가 형성되어 있는 가요성 (flexible) 재질의 백으로서 구성되어 있으며, 상기 백의 재질을 통하여 공기가 통과할 수 없다 (air impermeable). 또한, 상기 백에는 결합된 공기 펌프가 구비되어 있어 백 부풀리기 (bag inflation) 및 세포 공급을 위하여 사용된다. 사용된 백은 2 L 용량을 갖는 것이었다.

<42> 배양은 200mL 종 배양액을 포함하는 2L Cellbag™을 Cellbag™에 포함되어 있는 락커 (rocker unit)를 사용하여 10rpm으로 락킹하면서, 37℃에서 12 내지 30시간 동안 종 배양하고, 이를 본 배양 배지 20L를 포함하고 있는 상기 백에 폴리카보네이트 재질의 멸균 일회용 커넥터 (Kleenpack connector PALL)를 통하여 접종한 뒤 48~72시간 동안 혐기 조건을 유지하면서 배양하였다. 상기 백의 시료 주입 포트에 제균 필터 (0.2㎛)를 설치하고 이를 통해 종 배양 배지 200mL를 상기 백으로 주입하였다. 배지의 주입은 페리스탈틱 펌프를 사용하여 수행되었다.

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<43> 상기 종 배양 배지는 상기 백에 헤드 공간 (headspace)없이 주입하여 혐기 조건이 유지되도록 주입되었다. 상기 종 배양액을 주입할 때에도 동일하게 주입하였다. 또한, 상기 본 배양은 상기 백을 Cellbag™의 락커 (Rocker unit)의 플랫폼에 올려놓고, 온도 37℃로 유지하면서 10rpm으로 락킹 (rocking)하였다. 배양 중 백 내부를 혐기 조건으로 유지하기 위하여 1800L의 질소를 6 시간 동안 주입하였다. 상기 배양 온도는 37℃ 외에 34℃, 35℃ 또는 36℃를 사용할 수도 있다.

<44> 배양이 종료된 후, 상기 백의 시료 포트에 멸균 커넥터인 QDC connector (사토리우스 제품)를 연결하고, 이를 통하여 배양물을 다른 일회용 백 (가요성 일회용 백)으로 이송하였다. 다음으로, 배양물이 포함되어 있는 일회용 용기에 멸균 일회용 심층여과 (Depthfilter, Sartoclear 또는 SupraCap, PALL) 및 마이크로여과기 (0.2㎛, Sartopore 2, Sartorius)을 연결하고, 이를 통하여 여과하여 세포를 제거하고, 이를 통해 얻어진 상등액은 또 다른 일회용 백에 담았다. 상기 상등액으로부터 클로스트리디움 보툴리눔 독소를 분리하였다.

<45> 2. 배양물로부터 클로스트리디움 보툴리눔 독소의 분리

<46> 이하에서는 세포를 제거한 배양액으로부터 타입 A 독소를 생산하는 방법에 대하여 구체적으로 설명한다.

<47> (1) 산 침전

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<48> 상기에 얻은 배양 상등액 20L가 포함되어 있는 백에 1N 황산을 첨가하여 pH를 3.0 내지 4.5로 조정하여, 독소 단백질을 침전시켰다. 이 공정 역시 외부와의 접촉 없이 일회용 용기 (disposable sample bag) 내에서 모든 작업이 이루어졌다.

<49> 다음으로, 침전이 완료되면, 멸균된 일회용 여과 시스템 (disposable slice 200 0.2㎛ 필터: Sartorius 사)을 통하여 외부와 접촉없이 여과하여 상등액을 제거하고, 침전물을 일회용 백 (sample bag, disposable)에 회수하였다. 회수된 침전물에 주사용수(WFI) 5L를 첨가하여 황산을 씻어주고, 멸균된 일회용 마이크로여과 시스템 (disposable slice 200 0.2㎛ 필터)을 상기 백에 연결하고 여과하여 상등액을 제거함으로써, 침전물을 일회용 백 내로 회수하였다.

<50> (2) 농축 및 여과

<51> 회수된 침전물에 시트레이트 버퍼 (25mM pH 5.5) 10L를 첨가하여, 침전물에 있는 독소 단백질을 재용해시켰다. 이 공정 역시 일회용 백에서 작업하였으며, 외부와의 접촉은 일어나지 않았다. 재용해된 시료가 포함되어 있는 백에 멸균된 일회용 여과 시스템 (0.2㎛, ULTA Cap HC, GE, 미국)을 연결하고 외부와 접촉없이 여과하여 상등액을 멸균된 일회용 백에 취하고, 불용성 불순물들은 제거하였다.

<52> 불용성 불순물들이 제거된 시료가 포함되어 있는 상기 백에 분자량 컷오프 100 kDa의 멸균된 일회용 한외여과 막 (disposable slice 200 100kDa: Sartorius 사)을 연결하고, 외부와의 접촉없이 여과하여 농축하였다. 이 과정 역시, 독소 단백질은 무균 용기의 내부 표면과 접촉하고 있었으며, 외부와의 접촉은 일어나지 않

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았다.

<53> (3) 음이온 교환 크로마토그래피

<54> 다음으로, 농축 및 버퍼 교환이 완료된 시료가 포함된 상기 백에 일회용 DEAE 음이온 교환 크로마토그래피 시스템 (DEAE BPG column™: GE Healthcare)을 사용하여 외부와의 접촉없이 DEAE 음이온 교환 크로마토그래피를 수행하였다. 구체적으로, 상기 시트레이트 버퍼 (25mM pH 5.5)를 상기 DEAE 음이온 교환 수지가 충진된 칼럼에 흘려주어 평형화하고, 상기 독소 함유 상기 시트레이트 버퍼 (25mM pH 5.5)를 칼럼을 통하여 흘려 주었다. 이 단계에서, 클로스트리디움 보툴리눔 타입 A 독소는 음이온 교환수지에 흡착되지 않고 대부분이 주요 불순물은 흡착되어 제거되었다. 흘려주는 동안 유속은 20ml/분이고, pH는 5.5으로 유지하였다.

<55> (4) 농축 및 여과

<56> 다음으로, 상기 음이온 교환 크로마토그래피에서 음이온 교환 수지에 결합하지 않고 흘러 나오는(flow through) 용액 중 클로스트리디움 보툴리눔 타입 A 독소를 포함하는 분획을 취하였다.

<57> 상기 분획이 포함되어 있는 백에 분자량 컷오프 50 kDa의 멸균된 일회용 한외여과 막 (disposable slice 200 100kDa: Sartorius 사)을 연결하고, 외부와의 접촉없이 여과하여 농축하고, 포스페이트 버퍼 (50mM pH 5.0)를 사용하여 버퍼 교환을 실시하였다. 이 과정 역시, 독소 단백질은 무균 용기의 내부 표면과 접촉하고

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있었으며, 외부와의 접촉은 일어나지 않았다. 버퍼 교환에 사용되는 버퍼는 포스페이트 버퍼 (50mM pH 5.0) 뿐만 아니라, 10 내지 75mM의 포스페이트 버퍼, 시트레이트 버퍼 또는 아세테이트 버퍼로서 클로스트리디움 보툴리눔 독소의 pI 값보다 낮은 pH 3.5 내지 6.5, 또는 pH 3.5 내지 5.5인 것이 사용될 수 있다.

<58> (5) 양이온 교환 크로마토그래피

<59> 다음으로, 버퍼 교환에 의하여 얻어진 시료가 포함된 상기 백에 일회용 SP 양이온 교환 크로마토그래피 시스템 (GE healthcare, SP sepharose FF)을 사용하여 외부와의 접촉없이 양이온 교환 크로마토그래피를 수행하였다. 구체적으로, 상기 포스페이트 버퍼 (75mM pH 5.0)를 상기 SP 양이온 교환 수지가 충진된 컬럼에 흘려주어 평형화하고, 상기 시료를 컬럼을 통하여 흘려 주었다. 이 단계에서 클로스트리디움 보툴리눔 타입 A 독소는 양이온 교환수지에 흡착된다. 유속은 100 mL/min으로 25분 동안 흘려주고, pH 5.0으로 유지하였다. 그 후 1M NaCl을 포함한 포스페이트 버퍼 (75mM pH 5.0)를 사용하여 용출하였다.

<60> 얻어진 시료에 대하여 크기 배제 크로마토그래피(size exclusion chromatography: SEC-HPLC)를 통하여 독소의 순도를 분석하였다. 이동상은 pH6.5의 40 mM 포스페이트 용액을 사용하였으며, SRT SEC-500 (Sepax Technologies사, P/N 215500) 컬럼을 연결하고 보툴리눔 A형 독소단백질 40 μL를 로딩(loading)하여

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0.8 mL/min으로 20분간 흘려주었다.

<61> 도 1은 클로스트리디움 보툴리눔 배양액으로부터 클로스트리디움 보툴리눔 타입 A 독소의 분리 공정을 나타낸 도면이다.

<62> 도 2는 양이온교환 크로마토그래피 후에 얻어진 시료에 대하여 SEC-HPLC 한 결과를 나타낸 도면이다.

<63> 【표 1】

이름	체류 시간(분)	면적	% 면적
12S	12.306	77163	0.19
19S	9.459	40065228	99.64
HMW1	7.000	-	-
HMW2	8.578	127860	0.32

<64> 표 1은 도 2의 피크에 대한 데이터를 나타낸 것으로, 타입 A 독소의 순도는 99.64%이었다. 특히, 체류 시간(RT)에서 나타내는 HMW1는 0%로서 존재하지 않았다. 표 1에서 HMW1 및 HMW2는 응집물1 및 응집물2를 나타내고, 12S(300kDa) 및 19S(900kDa)는 각각 BoNT 독소와 비-독성 비-HA(NTNHA)로 구성된 12S 독소 ('M 독소'라고도 함) 및 BoNT, NTNHA 및 HA 성분으로 구성된 16S(500kDa) 독소가 HA 단백질 중 하나인 HA1에 의하여 두 16S 독소가 연결된 디머(dimer)인 것으로 여겨진다. 상기 HA 성분은 3개 다른 단백질 즉, HA1, HA2 및 HA3을 갖는다. 독소 타입A는 12S, 16S 및 19S의 형태를 갖는다.

<65> 따라서, 상기한 정제 방법에 의하면, 해리되지 않은 보툴리눔 독소 타입 A의

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거대 복합체를 고순도로 분리할 수 있다.

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【특허청구범위】

【청구항 1】

보툴리눔 독소 함유 용액을 보툴리눔 독소의 등전점(isoelectric point: PI) 이하의 산도에서 음이온 교환 크로마토그래피 매질과 접촉시키는 단계;

상기 매질과 결합하지 않은 상기 용액을 보툴리눔 독소의 등전점 이하의 산도에서 양이온 교환 크로마토그래피 매질과 접촉시키는 단계; 및

상기 양이온 교환 크로마토그래피 매질로부터 보툴리눔 독소 A형 거대 복합체를 분리하는 단계; 또는

보툴리눔 독소 함유 용액을 등전점 이하의 산도에서 양이온 교환 크로마토그래피 매질과 접촉시키는 단계, 상기 양이온 교환 크로마토그래피 매질로부터 보툴리눔 독소를 분리하는 단계, 상기 매질에서 분리된 독소를 포함하는 용액을 등전점 이하의 산도에서 음이온 교환 크로마토그래피 매질과 접촉시키는 단계, 및 상기 매질과 결합하지 않은 상기 용액으로부터 보툴리눔 독소 A형 거대 복합체를 분리하는 단계;를 포함하는, 보툴리눔 독소 함유 용액으로부터 보툴리눔 독소 A형 거대 복합체를 분리하는 방법.

【청구항 2】

청구항 1에 있어서, 상기 보툴리눔 독소의 등전점 이하의 산도는 pH 3.5 내지 6.0인 것인 방법.

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【청구항 3】

청구항 1에 있어서, 상기 음이온 교환 크로마토그래피 매질과 접촉시키는 단계 전, 양이온 교환 크로마토그래피 매질과 접촉시키는 단계 전 또는 이 두 단계의 각 단계 전에 상기 보툴리눔 독소 또는 그를 함유하는 용액의 산도를 보툴리눔 독소의 등전점 이하로 조정하는 단계를 더 포함하는 것인 방법.

【청구항 4】

청구항 3에 있어서, 상기 조정하는 단계는 상기 보툴리눔 독소 또는 그를 함유하는 용액을 보툴리눔 독소의 등전점 이하의 산도를 갖는 버퍼와 혼합하는 단계를 포함하는 것인 방법.

【청구항 5】

청구항 4에 있어서, 상기 버퍼는 포스페이트 버퍼, 시트레이트 버퍼, 또는 아세테이트 버퍼인 것인 방법.

【청구항 6】

청구항 4에 있어서, 상기 버퍼는 pH 3.5 내지 6.0인 것인 방법.

【청구항 7】

청구항 4에 있어서, 상기 버퍼는 10mM 내지 100mM의 포스페이트, 시트레이트 또는 아세테이트를 포함하는 것인 방법.

【청구항 8】

청구항 1에 있어서, 상기 음이온 교환 크로마토그래피는 DEAE, 또는 Q 음이

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온 교환 크로마토그래피인 것인 방법.

【청구항 9】

청구항 1에 있어서, 상기 양이온 교환 크로마토그래피는 CM, S, 또는 SP 양이온 교환 크로마토그래피인 것인 방법.

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【요약서】

【요약】

음이온 교환 크로마토그래피 및 양이온 교환 크로마토그래피하는 과정을 포함하는 보툴리눔 독소 함유 용액으로부터 보툴리눔 독소 A형 거대 복합체를 분리하는 방법을 제공한다.

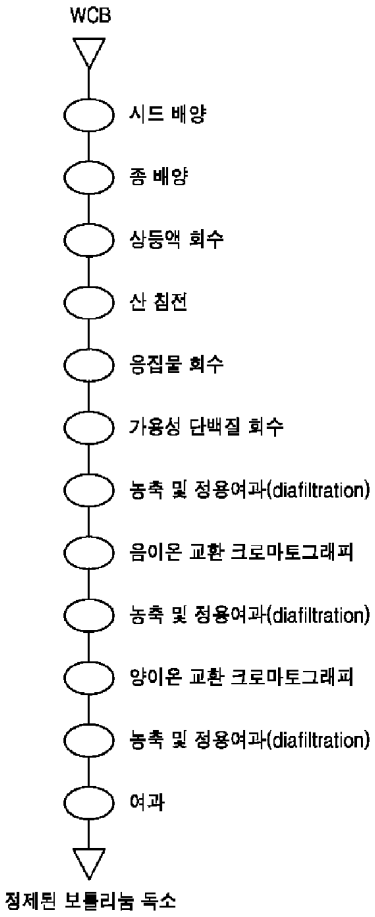
【대표도】

도 2

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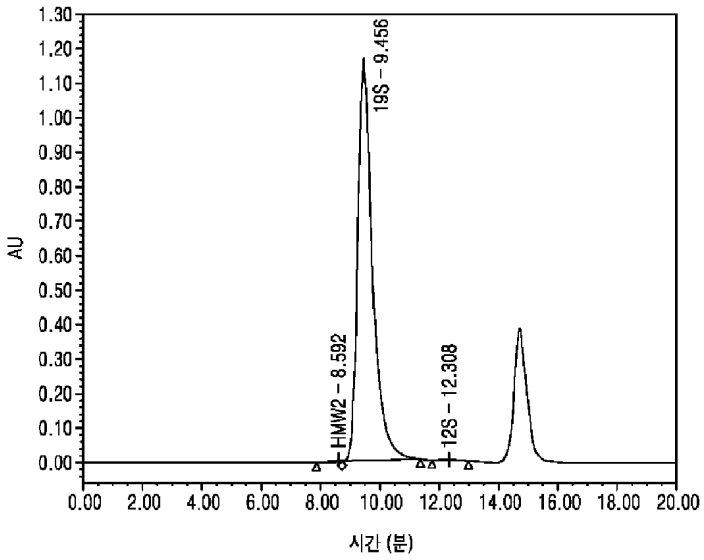
【도면】

【도 1】



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【도 2】


AU
1.30
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
HMW2 - 8.592
19S - 9.456
12S - 12.308
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
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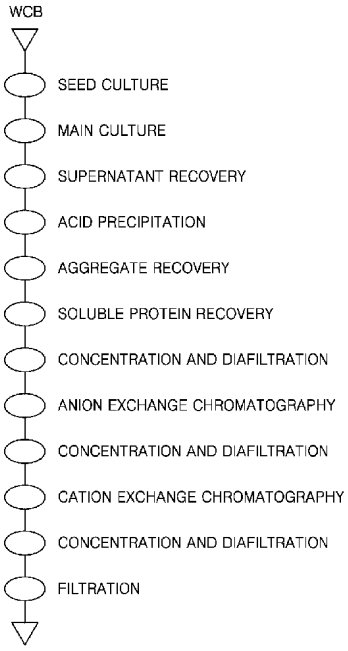
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(54) Title: METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

[Fig. 1]



(57) Abstract: Provided is a method of isolating a botulinum toxin type A macro complex from a botulinum toxin-containing solution, the method including performing anion exchange chromatography and cation exchange chromatography.

[Continued on next page]

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Description

Title of Invention: METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

Technical Field

[1] CROSS-REFERENCE TO RELATED APPLICATION

[2] This application claims the benefit of Korean Patent Application No. 10-2016-0127540, filed on October 4, 2016, in the Korean Intellectual Property Office, the disclosure of which is incorporated herein in its entirety by reference.

[3] The present disclosure relates to a method of isolating botulinum toxin from a botulinum toxin-containing solution.

Background Art

[4] *Clostridium* strains which secrete toxins having neurotoxicity have been discovered from 1890 until the present day, and research into the properties of toxins secreted by these strains has been conducted for the past 70 years (Schant, E. J. et al., Microbiol. Rev. 56; 80; 1992).

[5] The genus *Clostridium* includes more than 127 species, which are divided according to their morphology and functions. *Clostridium botulinum* is an anaerobic and Gram-positive bacterium which produces a strong polypeptide neurotoxin that causes a neuroparalytic illness, known as botulism, in humans and animals. Spores of *Clostridium botulinum* are found in soil, and a large amount of these spores may even cultured even be cultured in foods which have not been processed properly. These spores may cause many forms of botulism.

[6] Botulinum toxin is divided into seven types of A through G according to their serological characteristics. Each toxin includes about 150 kDa of toxin protein, and naturally is composed of a complex with a variety of non-toxic proteins bound thereto. An intermediate complex (300 kDa) is composed of a toxin protein and a non-toxic non-hemaglutinin (HA) protein, and a large complex (500 kDa) and a macro complex (900 kDa), each have a structure in which the intermediate complex is bound to hemaglutinin (Sugiyama, H., Microbiol. Rev., 44, 419; 1980). Such non-toxic non-hemaglutinin proteins are known to protect the toxin from low pH and various kinds of protein hydrolytic enzymes in intestines (Sugiyama, H., Microbiol. Rev., 44, 419; 1980).

[7] Botulinum toxin is synthesized in a cell as a single-chain polypeptide with a molecular weight of approximately 150 kDa, and then cleaved into two subunits of a light chain (a molecular weight of 50 kDa) and a heavy chain (a molecular weight of 100 kDa) at a position that is one-third the distance from the N-terminus by action of

intracellular protease or by artificial treatment with an enzyme such as trypsin. Such a cleaved toxin has highly increased toxicity in comparison with the single-chain polypeptide. The two subunits are bound to each other by a disulfide bond, and each subunit has a different function. The heavy chain binds with a receptor of a target cell (FEMS Microbiol. Lett. 72, 243; 1990), and reacts with a bio-membrane at a low pH (pH 4.0) to form a channel (Mantecucco, C. et al., TIBS 18, 324; 1993), and the light chain has pharmacological activities, and thus interferes with secretion of neurotransmitters, when cells are provided with permeability by a surfactant or the light chain is introduced into cells by electroporation, (Poulain, B. et al., Proc. Natl. Acad. Sci. USA. 85, 4090; 1988).

[8] Botulinum toxin type A is a natural biological agent which is known to be the most lethal to humans. On a molar basis, botulinum toxin type A is 1.8 billion times more lethal than diphtheria toxin, 600 million times more lethal than sodium cyanide, 30 million times more lethal than cobratoxin, and 12 million times more lethal than cholera toxin (Singh, Critical Aspects of Bacterial protein Toxins, page 63-84 of natural toxins II, edited by B. R. Sigh et al., Plenum Press, New York (1976)).

[9] Botulinum toxin inhibits exocytosis of acetylcholine at a cholinergic presynapse of a neuromuscular junction, thereby causing asthenia. Taking into account that toxicity is exhibited even with exposure to a very small amount of botulinum toxin, it has been considered that this toxin may have enzymatic activity (Simpson, L. L. et al., Ann. Rev. Pharmaeol. Toxicol. 26, 427; 1986). According to recent reports, botulinum toxin has metallopeptidase activity, and substrates thereof are unit proteins of an exocytosis machinery complex, such as synaptobrevin, syntaxin, synaptosomal associated protein of 25 kDa (SNAP 25), etc. Each type of botulinum toxin uses one of the three proteins as a substrate, and it is known that botulinum toxin types B, D, F, and G cleave synaptobrevin at a specific site, botulinum toxin types A and E cleave SNAP25 at a specific site, and botulinum toxin type C cleaves syntaxin at a specific site (Binz, T. et al., J. Biol. Chem. 265, 9153; 1994).

[10] Korean Patent Publication NO. 10-2012-0105417 discloses a method of isolating botulinum toxin from a culture of *Clostridium botulinum*, the method including recovering the biologically active botulinum neurotoxin from a fermentation medium by contacting the fermentation medium with an anion exchange chromatography medium and then contacting an eluent from the anion exchange chromatography medium with a cation exchange chromatography medium. In this method, elution is performed after binding the toxin to the anion exchange chromatography medium, but the method does not disclose flowing of the toxin without binding it to the medium.

[11] According to the method, there is a problem in that during purification of the botulinum toxin protein, a neurotoxic component (i.e., a neurotoxic molecule of ap-

proximately 150 kDa), an intermediate complex (300 kDa), or a large complex (500 kDa) is dissociated and removed from the botulinum toxin protein.

Disclosure of Invention

Technical Problem

[12] An aspect provides a method of isolating a botulinum toxin type A macro complex from a botulinum toxin-containing solution.

Solution to Problem

[13] An aspect provides a method of isolating a botulinum toxin type A macro complex from a botulinum toxin-containing solution, the method including contacting the botulinum toxin-containing solution with an anion exchange chromatography medium at a pH lower than an isoelectric point (PI) of botulinum toxin; contacting the solution, which is not bound to the anion exchange chromatography medium, with a cation exchange chromatography medium at a pH lower than PI of botulinum toxin; and separating the botulinum toxin type A macro complex from the cation exchange chromatography medium; or

[14] contacting the botulinum toxin-containing solution with the cation exchange chromatography medium at a pH lower than PI of botulinum toxin; separating botulinum toxin from the cation exchange chromatography medium; contacting the solution containing the toxin separated from the cation exchange chromatography medium, with the anion exchange chromatography medium at a pH lower than PI of botulinum toxin; and separating the botulinum toxin type A macro complex from the solution which is not bound to the anion exchange chromatography medium.

[15] The method includes contacting the botulinum toxin-containing solution with the anion exchange chromatography medium at a pH lower than an isoelectric point (PI) of botulinum toxin; contacting the solution, which is not bound to the anion exchange chromatography medium, with the cation exchange chromatography medium at a pH lower than PI of botulinum toxin; and separating the botulinum toxin type A macro complex from the cation exchange chromatography medium.

[16] As used herein, the "botulinum toxin type A macro complex" may be a dimer of two 16S toxins linked via HA1 which is one of HA proteins, each 16S toxin, i.e., a toxin of about 500 kDa, is composed of botulinum toxin (BoNT), non-toxic non-HA (NTNHA), and HA components.

[17] The method includes contacting the botulinum toxin-containing solution with the anion exchange chromatography medium at a pH lower than PI of botulinum toxin.

[18] In the above process, the botulinum toxin-containing solution may be a culture of *Clostridium botulinum* or a solution derived therefrom. The solution derived therefrom may be a solution obtained by partially purifying the toxin from the culture. Culturing

of *Clostridium botulinum* may be performed by a known method. The culturing may be performed in a medium containing plant-derived components. The culturing is performed by using the medium containing plant-derived components, thereby expressing the toxin outside cells, unlike the prior art. As used herein, the "expressing the toxin outside cells" indicates that, after the cells produce the toxin therein, the cells excrete the toxin spontaneously, and the cells containing the toxin expressed therein are also lysed, whereby the toxin is exposed to a solution of the culture, not inside the cells of the culture.

[19] The medium containing plant-derived components may include phytone peptone, and preferably, phytone peptone, yeast extract, and glucose. In addition, the medium containing plant-derived components may further include one or more components selected from the group consisting of vegetable tryptone, soytone, and sodium thioglycolate. Further, the medium containing plant-derived components may be a medium selected from the group consisting of a medium containing glucose, yeast extract, and phytone peptone; a medium containing glucose, yeast extract, phytone peptone, and vegetable tryptone; a medium containing glucose, yeast extract, phytone peptone, and soytone; a medium containing glucose, yeast extract, soytone, and vegetable tryptone; a medium containing glucose, yeast extract, sodium thioglycolate, phytone peptone, vegetable tryptone, and soytone; and a medium containing glucose, yeast extract, phytone peptone, vegetable tryptone, and soytone.

[20] Further, amounts of glucose, yeast extract, sodium thioglycolate, phytone peptone, vegetable tryptone, and soytone in each medium described as the medium containing plant-derived components may be 0.2 % to 2 %, 0.5 % to 5 %, 0.05 % to 2 %, 0.5 % to 2 %, 0.5 % to 2 %, and 0.5 % to 2 %, respectively, based on a total weight of the medium. The medium containing plant-derived components may be selected from the group consisting of a medium containing glucose, yeast extract, and phytone peptone; a medium containing glucose and yeast extract; a medium containing glucose, yeast extract, phytone peptone, and vegetable tryptone; a medium containing glucose, yeast extract, phytone peptone, and soytone; a medium containing glucose, yeast extract, soytone, and vegetable tryptone; a medium containing glucose, yeast extract, sodium thioglycolate, phytone peptone, vegetable tryptone, and soytone; and a medium containing glucose, yeast extract, phytone peptone, vegetable tryptone, and soytone. Amounts of glucose, yeast extract, sodium thioglycolate, phytone peptone, vegetable tryptone, and soytone in each medium described above are 0.2 % to 2 %, 0.5 % to 5 %, 0.05 % to 2 %, 0.5 % to 2 %, 0.5 % to 2.0 %, and 0.5 % to 2 %, respectively, based on a total weight of the medium, and the rest of the medium may be composed of water.

[21] The toxin may be selected from the group consisting of botulinum toxin A, B, C, D, E, F and G, and preferably, the toxin may be type A toxin. An isoelectric point of

botulinum toxin A may be about 6.06. Therefore, the "pH lower than the isoelectric point" may differ depending on the type of the toxin to be isolated. For example, in the case of type A toxin, the pH may be 6.06 or lower, 6.05 or lower, 6.00 or lower, 5.7 or lower, 5.5 or lower, 5.0 or lower, 4.5 or lower, 4.0 or lower, or 3.5 or lower. In the case of type A toxin, the pH may be 2.5 to 6.05, 3.0 to 6.00, 3.5 to 5.5, 3.5 to 5.0, 3.5 to 4.5, 3.5 to 4.0, 3.5 to 6.00, 4.0 to 6.0, 4.5 to 6.0, 5.5 to 6.0, 4.0 to 5.5, or 4.5 to 5.5.

[22] An anion exchange resin used in the anion exchange chromatography may be a resin substituted with a diethylaminoethyl (DEAE) group or a quaternary ammonium (Q) group, but is not limited thereto. For example, DEAE-Sephadex as described in US Patent NO. 5,696,077, International Patent Publication NO. WO96/05222, and US Patent NO. 5,846,929 may be used. Preferably, any one selected from anion exchange resins having a strong basic quaternary ammonium group or a weak basic diethylaminoethyl (DEAE) group may be used.

[23] For example, the strong basic anion exchange group may be Q Sepharose Fast Flow, Q Sepharose High Performance, Resource Q, Source 15Q, Source 30Q, Mono Q, Mini Q, Capto Q, Capto Q ImpRes, Q HyperCel, Q Cermic HyperD F, Nuvia Q, UNOsphere Q, Macro-Prep High Q, Macro-Prep 25 Q, Fractogel EMD TMAE(S), Fractogel EMD TMAE Hicap (M), Fractogel EMD TMAE (M), Eshmono Q, Toyopearl QAE-550C, Toyopearl SuperQ-650C, Toyopearl GigaCap Q-650M, Toyopearl Q-600C AR, Toyopearl SuperQ-650M, Toyopearl SuperQ-650S, TSKgel SuperQ-5PW (30), TSKgel SuperQ-5PW (20), TSKgel SuperQ-5PW, etc., but is not limited thereto. Any anion exchange resin known in the art may be used.

[24] By the anion exchange chromatography, *Clostridium botulinum* toxin may flow out without binding to the medium, and impurities bind to the medium. The liquid flowing out of the chromatography may be used in further separation.

[25] In the above process, the contacting may include flowing of the botulinum toxin-containing solution into the medium, for example, flowing of the botulinum toxin-containing solution into the medium packed in a column under predetermined conditions. Conditions such as a flow rate, temperature, time, etc. may be controlled. In the contacting, a phosphate buffer solution, a citrate buffer solution, or an acetate buffer solution may be used as a column buffer solution. A concentration of the column buffer solution may be controlled from 5 mM to 30 mM, for example, from 7 mM to 27 mM, from 10 mM to 25 mM, or from 15 mM to 20 mM. A flow rate of a mobile phase may be 0.5 ml/min to 5.0 ml/min, 1.0 ml/min to 30.0 ml/min, 10 ml/min to 30.0 ml/min, or 15 ml/min to 25.0 ml/min. In this regard, after conductivity of the buffer solution may be adjusted at 3 mS/cm to 30 mS/cm and column equilibration is finished, a sample may be injected.

[26] Further, the method may include contacting the solution, which is not bound to the

medium, with the cation exchange chromatography medium at a pH lower than PI of botulinum toxin. The "pH lower than PI" and "contacting" are the same as described above.

[27] In the above process, the cation exchange chromatography medium may be a medium having a strong acidic cation exchange group such as sulfopropyl (SP) and methyl sulfonate (S) or a weak acidic cation exchange group such as carboxymethyl (CM).

[28] Further, the method includes separating the botulinum toxin from the cation exchange chromatography medium. Separating of the toxin may include recovering of the botulinum toxin from an elution solvent by contacting the medium with the elution solvent. Any elution solvent may be used, as long as it may separate the toxin from the medium. The elution solvent may be, for example, a buffer solution used in the buffering of the column or the buffer solution of which a salt concentration or conductivity may be properly controlled. The salt may be a salt of Na or K. The column buffer solution may be a phosphate buffer solution, a citrate buffer solution, or an acetate buffer solution. A concentration of the column buffer solution may be controlled from 5 mM to 100 mM, for example, from 10 mM to 100 mM, from 20 mM to 100 mM, or from 30 mM to 70 mM. A flow rate of the elution solvent which is a mobile phase may be 0.5 ml/min to 5.0 ml/min, 1.0 ml/min to 150.0 ml/min, 50.0 ml/min to 150.0 ml/min, or 75.0 ml/min to 125.0 ml/min. In this regard, conductivity of the elution solvent may be 3 mS/cm to 30 mS/cm. The elution solvent may be a phosphate buffer (50 mM, pH 5.0) containing 0.5 M to 2.0 M NaCl, for example, 1.0 M NaCl.

[29] The method may further include adjusting the pH of the botulinum toxin or the botulinum toxin-containing solution to a pH lower than PI of botulinum toxin, before contacting with the anion exchange chromatography medium, before contacting with the cation exchange chromatography medium, or before each of these two processes. The adjusting may include mixing the botulinum toxin or the botulinum toxin-containing solution with a buffer having a pH lower than PI of botulinum toxin. The buffer may be a phosphate buffer, a citrate buffer, an acetate buffer, or a mixture thereof. The buffer may have pH of 3.5 to 6.5, or pH of 3.5 to 5.5. The buffer may include 10 mM to 100 mM phosphate, citrate, or acetate.

[30] The method may further include performing ultrafiltration of the botulinum toxin-containing solution, before contacting with the anion exchange chromatography medium, before contacting with the cation exchange chromatography medium, or before each of these two processes. The ultrafiltration may be performed by using a membrane with a molecular weight cut-off of 25 kDa to 125 kDa. The ultrafiltration may be adjusted such that a molecular weight cut-off before contacting with the cation

exchange chromatography medium, for example, a molecular weight cut-off of 50 kDa may be lower than a molecular weight cut-off before contacting with the anion exchange chromatography medium, for example, a molecular weight cut-off of 100 kDa.

[31] The method includes expressing *Clostridium botulinum* toxin outside cells by culturing *Clostridium botulinum* in a medium, before contacting with the anion exchange chromatography medium, and the botulinum toxin-containing solution may be a portion containing *Clostridium botulinum* toxin expressed outside cells by removing the cells from the obtained culture.

[32] The method includes expressing *Clostridium botulinum* toxin outside cells by culturing *Clostridium botulinum* in a medium, before contacting with the anion exchange chromatography medium; and precipitating the toxin by adding an acid to a culture solution which is obtained by removing the cells from the culture, and then separating precipitates; and dissolving the precipitates in a medium and performing ultrafiltration, wherein the botulinum toxin-containing solution may be a solution obtained by the ultrafiltration.

[33] In the method, removing of the cells may be performed by one or more filtrations selected from the group consisting of depth filtration and microfiltration, precipitation of the toxin may be performed by maintaining the culture at pH 3 to pH 4 with an acid, and ultrafiltration may be performed by using a membrane with a molecular weight cut-off of 100 kDa or less.

[34] Another aspect provides a method of isolating botulinum toxin from the botulinum toxin-containing solution, the method including contacting the botulinum toxin-containing solution with the cation exchange chromatography medium at a pH lower than PI, separating the botulinum toxin from the cation exchange chromatography medium, contacting the solution containing the toxin which is separated from the medium, with the anion exchange chromatography medium at a pH lower than PI, and isolating the botulinum toxin from the solution which is not bound to the medium.

[35] The contacting of the botulinum toxin-containing solution with the cation exchange chromatography medium at a pH lower than PI and the separating of the botulinum toxin from the cation exchange chromatography medium are the same as described above, except that the botulinum toxin-containing solution is used instead of "the solution which is not bound to the medium". Further, "the contacting of the solution containing the toxin which is separated from the medium, with the anion exchange chromatography medium at a pH lower than PI, and the isolating of the botulinum toxin from the solution which is not bound to the medium" are the same as described above, except that "the solution containing toxin which is separated from the medium" is used instead of "the botulinum toxin-containing solution".

[36] Reference will now be made in detail to embodiments, examples of which are illustrated in the accompanying drawings, wherein like reference numerals refer to like elements throughout. In this regard, the present embodiments may have different forms and should not be construed as being limited to the descriptions set forth herein. Accordingly, the embodiments are merely described below, by referring to the figures, to explain aspects.

[37]

Advantageous Effects of Invention

[38] According to the method of isolating botulinum toxin from the botulinum toxin-containing solution of an aspect, botulinum toxin type A macro complex, i.e., 900 kDa may be efficiently isolated from the botulinum toxin-containing solution.

Brief Description of Drawings

[39] These and/or other aspects will become apparent and more readily appreciated from the following description of the embodiments, taken in conjunction with the accompanying drawings in which:

[40] FIG. 1 is a schematic view showing a process of purifying *Clostridium botulinum* type A toxin from a culture of *Clostridium botulinum*; and

[41] FIG. 2 is a schematic view showing results of SEC-HPLC of a sample obtained by cation exchange chromatography.

Best Mode for Carrying out the Invention

[42] Hereinafter, the present invention will be described in more detail with reference to Examples. However, these Examples are for illustrative purposes only, and the scope of the invention is not intended to be limited by these Examples.

[43] **Example 1: Culture of *Clostridium botulinum* and Purification of *Clostridium botulinum* toxin**

[44] **1. Culture of *Clostridium botulinum***

[45] In this Example, a large amount of *Clostridium botulinum* type A toxin was expressed outside cells by culturing *Clostridium botulinum* in an animal product-free medium.

[46] The used medium was a medium containing 4 % yeast extract and 0.5 % glucose, based on a total weight of the medium. The components of the medium were dissolved in water for injection (WFI), and pH was adjusted with NaOH to 7.2.

[47] 200 ml of the medium thus prepared was filtered through a sterile single use microfiltration system (0.2 μm Sartorius 2, manufactured by Sartorius), and injected into Cellbag ™ (manufactured by GE healthcare, a single use fermentation bag). Then, the medium was inoculated with 1 ml of a seed culture of *Clostridium botulinum* at a density of 10^6 cells/ml ~ 10^7 cells/ml through a sample injection port. Cellbag ™ is a

disposable bioreactor that uses rocking motion in order to cause mixing under low shear stress. Cellbag ™ is a bag including an air inlet and outlet and a sample port, and is composed of a flexible material, and the bag is air impermeable. In addition, the bag is equipped with a pump combined thereto, which is used for bag inflation and cell supply. The used bag has a volume of 2 L.

[48] Culturing was performed such that 2 L Cellbag ™ containing 200 ml of the species culture was species-cultured at 37 ℃ for 12 hours to 30 hours under rocking at 10 rpm by using a rocker unit in Cellbag ™, and the resultant was inoculated in a bag containing 20 L of a main culture medium through a sterile single use connector (Kleenpack connector PALL) made of polycarbonate, and then cultured for 48 hours to 72 hours while maintaining anaerobic conditions. A sterile filter (0.2 μm) was installed at the sample injection port of the bag, and 200 ml of the species culture medium was injected into the bag through the sterile filter. The injection of the medium was performed using a peristaltic pump.

[49] The species culture medium was injected to the bag without a headspace so as to maintain anaerobic conditions. The species culture was also injected in the same manner. In addition, the main culture was performed by mounting the bag on a platform of the rocker unit of Cellbag ™ and rocking the bag at 10 rpm at a temperature of 37 ℃. During the main culture, 1800 L of nitrogen was purged for 6 hours in order to maintain anaerobic conditions inside the bag, and the culture temperature may be 34 ℃, 35 ℃, or 36 ℃, in addition to 37 ℃.

[50] After the culturing was terminated, a QDC connector (manufactured by Satorious) which is a sterile connector was connected to the sample port of the bag, and the culture was transferred to another disposable bag (flexible disposable bag) through the sterile connector. Then, a sterile disposable depthfilter (Sartoclear or SupraCap, PALL) and microfilter (0.2 μm, Sartopore 2, Sartorius) were connected to the disposable container containing the culture, and the culture was filtered therethrough to remove the cells. Then, the obtained supernatant was put in another disposable bag. From the supernatant, *Clostridium botulinum* toxin was purified.

[51] **2. Purification of *Clostridium botulinum* toxin from culture**

[52] Hereinbelow, a method of producing type A toxin from the cell-removed culture will be described in detail.

[53] **(1) Acid precipitation**

[54] 1 N sulfuric acid was added to a bag containing 20 L of the culture supernatant obtained as above to adjust pH from 3.0 to 4.5, and as a result, toxin protein was precipitated. All procedures of this process were also performed in a disposable sample bag without being in contact with the outside.

[55] Then, when the precipitation was completed, the resulting solution was filtered

through a sterile disposable filtration system (disposable slice 200 0.2 μm filter, manufactured by Sartorius) without being in contact with the outside, thereby removing the supernatant, and the precipitate was collected in a disposable sample bag. 5 L of WFI was added to the collected precipitate to wash sulfuric acid, and a sterile disposable microfiltration system (disposable slice 200 0.2 μm filter) was connected to the bag and the supernatant was removed. As a result, the precipitate was collected into a disposable bag.

[56] **(2) Concentration and Filtration**

[57] 10 L of citrate buffer (25 mM, pH 5.5) was added to the collected precipitate to re-dissolve toxin protein existing in the precipitate. This process was also performed in a disposable bag without being in contact with the outside. A sterile disposable filtration system (0.2 μm, ULTA Cap HC, GE, USA) was connected to the bag containing the re-dissolved sample, and filtration was performed without being in contact with the outside. As a result, the supernatant was collected in a sterile disposable bag and insoluble impurities were removed.

[58] A sterile disposable ultrafiltration membrane (disposable slice 200 100 kDa, manufactured by Sartorius) having a molecular weight cut-off of 100 kDa was connected to the bag containing the sample from which the insoluble impurities were removed. Then, the sample was filtered without being in contact with the outside to be concentrated. Even in this process, toxin protein was in contact with an inner surface of the sterile container, and not in contact with the outside.

[59] **(3) Anion exchange chromatography**

[60] Next, a disposable DEAE anion exchange chromatography system (DEAE BPG column ™: GE Healthcare) was connected to the bag containing the sample after the concentration and buffer exchange were completed, and DEAE anion exchange chromatography was performed without being in contact with the outside. In detail, the citrate buffer (25 mM pH 5.5) was passed through a column packed with a DEAE anion exchange resin to equilibrate the column, and the toxin-containing citrate buffer (25 mM pH 5.5) was passed through the column. In this process, *Clostridium botulinum* type A toxin was not adsorbed to the anion exchange resin, and most impurities were adsorbed thereto and removed. A flow rate was 20 ml/min, and pH was maintained at 5.5.

[61] **(4) Concentration and Filtration**

[62] Next, a fraction containing *Clostridium botulinum* type A toxin was collected from the flow-through solutions which were not bound to the anion exchange resin during the anion exchange chromatography.

[63] A sterile disposable ultrafiltration membrane (disposable slice 200 100 kDa, manufactured by Sartorius) having a molecular weight cut-off of 50 kDa was connected to

the bag containing the fraction, and concentration was performed by filtration without being in contact with outside, and buffer exchange was performed by using a phosphate buffer (50 mM pH 5.0). Even in this process, toxin protein was in contact with an inner surface of the sterile container, and not in contact with the outside. As the buffer used in the buffer exchange, 10 mM to 75 mM phosphate buffer, citrate buffer, or acetate buffer having pH of 3.5 to 6.5, or pH of 3.5 to 5.5 lower than pI of *Clostridium botulinum* toxin may be also used, in addition to the phosphate buffer (50 mM pH 5.0).

[64] **(5) cation exchange chromatography**

[65] Next, a disposable SP cation exchange chromatography system (GE healthcare, SP sepharose FF) was connected to the bag containing the sample obtained by buffer exchange, and cation exchange chromatography was performed without being in contact with outside. In detail, the phosphate buffer (75 mM pH 5.0) was passed through a column packed with a SP cation exchange resin to equilibrate the column, and the sample was passed through the column. In this process, *Clostridium botulinum* type A toxin was adsorbed to the cation exchange resin. This procedure was performed for 25 minutes at a flow rate of 100 ml/min, and pH was maintained at 5.0. Then, elution was performed by using a phosphate buffer (75 mM pH 5.0) containing 1 M NaCl.

[66] The obtained sample was subjected to size exclusion chromatography (SEC-HPLC) and purity of the toxin was analyzed. As a mobile phase, a 40 mM phosphate solution of pH 6.5 was used. SRT SEC-500 (manufactured by Sepax Technologies, P/N 215500) column was connected, and 40 μL of botulinum type A toxin protein was loaded and passed through the column for 20 minutes at 0.8 mL/min.

[67] FIG. 1 is a schematic view showing the process of purifying *Clostridium botulinum* type A toxin from the culture of *Clostridium botulinum*.

[68] FIG. 2 is a schematic view showing results of SEC-HPLC of the sample obtained by cation exchange chromatography.

[69] [Table 1]

Name	Retention time (min)	Area	%Area
12S	12.306	77163	0.19
19S	9.459	40065228	99.64
HMW1	7.000	-	-
HMW2	8.578	127860	0.32

[70] Table 1 shows data of peaks of FIG. 2, and purity of type A toxin was 99.64 %. In

particular, HMW1 appearing at a retention time (RT) was 0 %, indicating absence. In Table 1, HMW1 and HMW2 represent aggregate 1 and aggregate 2, respectively and 12S(300kDa) and 19S(900kDa) are regarded as 12S toxin (also called "M toxin" consisting of BoNT toxin and non-toxic non-HA(NTNHA) and a dimer of two 16S toxins linked via HA1 which is one of HA proteins, each 16S(500kDa) toxin consisting of BoNT, NTNHA, and HA components. The HA component has three different proteins, i.e., HA1, HA2 and HA3. Toxin type A has forms of 12S, 16S, and 19S.

[71] Therefore, according to the purification method, a macro complex of botulinum toxin type A which is not dissociated may be isolated with high purity.

[72] It should be understood that embodiments described herein should be considered in a descriptive sense only and not for purposes of limitation. Descriptions of features or aspects within each embodiment should typically be considered as available for other similar features or aspects in other embodiments.

[73] While one or more embodiments have been described with reference to the figures, it will be understood by those of ordinary skill in the art that various changes in form and details may be made therein without departing from the spirit and scope as defined by the following claims.

Industrial Applicability

[74] Therefore, according to the purification method, a macro complex of botulinum toxin type A which is not dissociated may be isolated with high purity.

[75]

[76]

Claims

[Claim 1] A method of isolating a botulinum toxin type A macro complex from a botulinum toxin-containing solution, the method comprising:
contacting the botulinum toxin-containing solution with an anion exchange chromatography medium at a pH lower than an isoelectric point (PI) of botulinum toxin;
contacting the solution, which is not bound to the anion exchange chromatography medium, with a cation exchange chromatography medium at a pH lower than the PI of botulinum toxin; and
separating the botulinum toxin type A macro complex from the cation exchange chromatography medium; or
contacting the botulinum toxin-containing solution with the cation exchange chromatography medium at a pH lower than the PI of botulinum toxin;
separating botulinum toxin from the cation exchange chromatography medium;
contacting the solution containing the toxin separated from the cation exchange chromatography medium, with the anion exchange chromatography medium at a pH lower than the PI; and
separating the botulinum toxin type A macro complex from the solution which is not bound to the anion exchange chromatography medium.

[Claim 2] The method of claim 1, wherein the pH lower than the PI of botulinum toxin is pH 3.5 to 6.0.

[Claim 3] The method of claim 1, further comprising adjusting the pH of the botulinum toxin or the botulinum toxin-containing solution to a pH lower than the PI of botulinum toxin, before the contacting of the solution with the anion exchange chromatography medium, before the contacting of the solution with the cation exchange chromatography medium, or before each of these two processes.

[Claim 4] The method of claim 3, wherein the adjusting comprises mixing the botulinum toxin or the botulinum toxin-containing solution with a buffer having a pH lower than the PI of botulinum toxin.

[Claim 5] The method of claim 4, wherein the buffer is a phosphate buffer, a citrate buffer, or an acetate buffer.

[Claim 6] The method of claim 4, wherein the buffer has pH of 3.5 to 6.0.

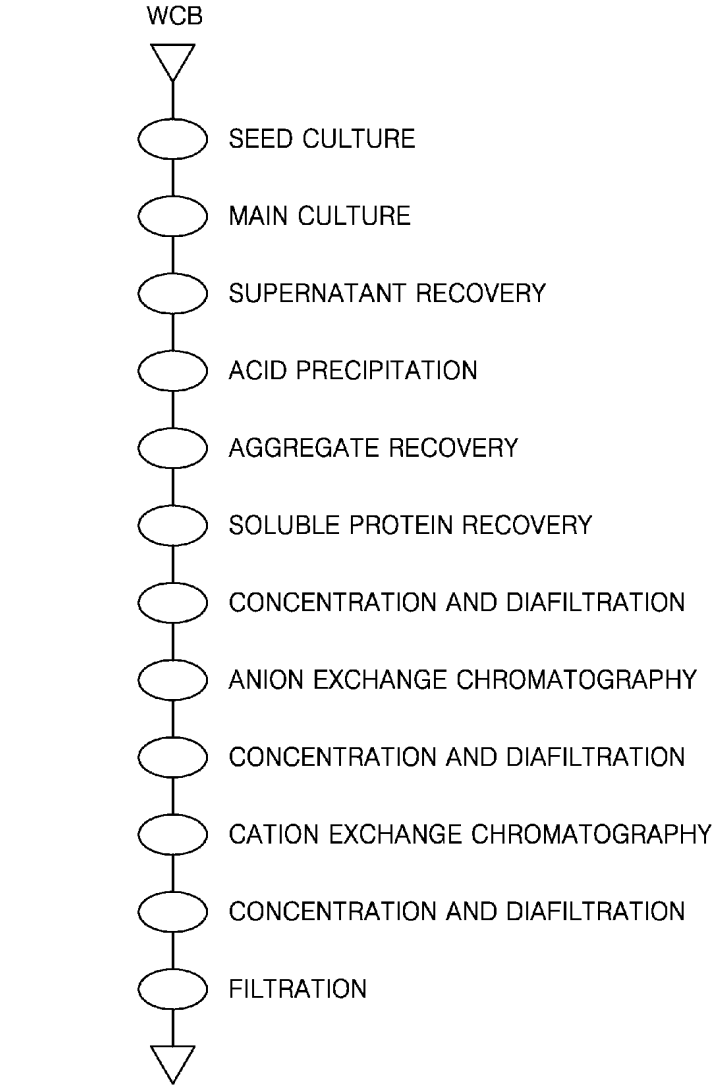
[Claim 7] The method of claim 4, wherein the buffer comprises a 10 mM to 100 mM phosphate buffer, a 10 mM to 100 mM citrate buffer, or a 10 mM

to 100 mM acetate buffer.

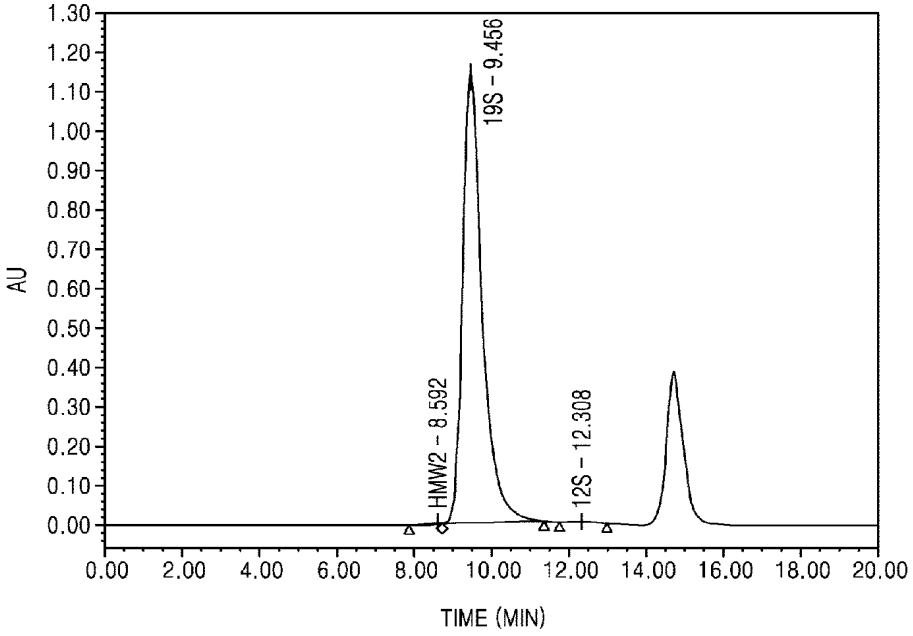
[Claim 8] The method of claim 1, wherein the anion exchange chromatography is DEAE or Q anion exchange chromatography.

[Claim 9] The method of claim 1, wherein the cation exchange chromatography is CM, S, or SP cation exchange chromatography.

[Fig. 1]


WCB
SEED CULTURE
MAIN CULTURE
SUPERNATANT RECOVERY
ACID PRECIPITATION
AGGREGATE RECOVERY
SOLUBLE PROTEIN RECOVERY
CONCENTRATION AND DIAFILTRATION
ANION EXCHANGE CHROMATOGRAPHY
CONCENTRATION AND DIAFILTRATION
CATION EXCHANGE CHROMATOGRAPHY
CONCENTRATION AND DIAFILTRATION
FILTRATION
PURIFIED BOTULINUM TOXIN

[Fig. 2]


AU
TIME (MIN)
19S - 9.456
HMW2 - 8.592
12S - 12.308
1.30
1.20
1.10
1.00
0.90
0.80
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0.60
0.50
0.40
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4.00
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16.00
18.00
20.00

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	PATENT NUMBER	GROUP ART UNIT	REQUEST ID
16/338,561			92751

PAIR Correspondence Address/Fee Address Change

The following fields have been changed to Customer Number 137713 on 07/31/2019 via Private PAIR in view of the certification copied below that authorized the change.

- Correspondence Address

The address for Customer Number 137713 is:
137713
Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

I certify, in accordance with 37 CFR 1.4(d)(4) that I am:

An attorney or Agent of Record registered to practice before the Patent and Trademark Office who has been given power of attorney in this application

Signature:	/Judith U. Kim/
Name:	Judith U. Kim
Registration Number:	40679

United States Patent and Trademark Office

Office of the Chief Financial Officer

Document Code:WFEE

User :69012

Sale Accounting Date:09/30/2019

Sale Item Reference Number	Effective Date
16338561	04/01/2019

Document Number	Fee Code	Fee Code Description	Amount Paid	Payment Method
I20199TA18514630	1617	OATH/DECL > 30 MOS FROM 371 COMMENCEMENT	$140.00	Deposit Account

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
16/338,561	04/01/2019		1720	2479-0007US01	9	1

CONFIRMATION NO. 3639

FILING RECEIPT

137713
Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182



Date Mailed: 10/01/2019

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

Inventor(s)

Hyun Ho JUNG, Seoul, KOREA, REPUBLIC OF;
Gi Hyeok YANG, Cheonan-si, KOREA, REPUBLIC OF;
Chang Hoon RHEE, Seoul, KOREA, REPUBLIC OF;
Hack Woo KIM, Cheongju-si, KOREA, REPUBLIC OF;
Gi Hyuck RYU, Daejeon, KOREA, REPUBLIC OF;
Hyurg Pyo HONG, Cheongju-si, KOREA, REPUBLIC OF;
Jung Hyun SONG, Gunsan-si, KOREA, REPUBLIC OF;
Joon Chan KONG, Cheongju-si, KOREA, REPUBLIC OF;

Applicant(s)

MEDYTOX INC., Cheongju-si, KOREA, REPUBLIC OF

Power of Attorney: The patent practitioners associated with Customer Number 141404

Domestic Priority data as claimed by applicant

This application is a 371 of PCT/IB2017/057476 11/29/2017

Foreign Applications (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.)
REPUBLIC OF KOREA 10-2016-0127540 10/04/2016

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 09/30/2019
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/338,561**
Projected Publication Date: 01/09/2020
Non-Publication Request: No
Early Publication Request: No
Title

METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

Preliminary Class

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific

countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop

technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

UNITED STATES PATENT AND TRADEMARK OFFICE

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Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

U.S. APPLICATION NO.	FIRST NAMED INVENTOR	ATTY. DOCKET NO.
16/338,561	Hyun Ho JUNG	2479-0007US01

137713
Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

INTERNATIONAL APPLICATION NO.	
PCT/IB2017/057476	
I.A. FILING DATE	PRIORITY DATE
11/29/2017	10/04/2016

CONFIRMATION NO. 3639
371 ACCEPTANCE LETTER

OC000000111541589

Date Mailed: 10/01/2019

NOTICE OF ACCEPTANCE OF APPLICATION UNDER 35 U.S.C 371 AND 37 CFR 1.495

The applicant is hereby advised that the United States Patent and Trademark Office, in its capacity as a Designated / Elected Office (37 CFR 1.495), has ACCEPTED the above identified international application for national patentability examination in the United States Patent and Trademark Office.

The United States Application Number assigned to the application is shown above. A Filing Receipt will be issued for the present application in due course. **THE DATE APPEARING ON THE FILING RECEIPT AS THE "FILING DATE or 371(c) DATE" IS THE DATE ON WHICH THE LAST OF THE 35 U.S.C. 371 (c)(1) and (c)(2) REQUIREMENTS HAS BEEN RECEIVED IN THE OFFICE. THIS DATE IS SHOWN BELOW.** The filing date of the above identified application is the international filing date of the international application (Article 11(3) and 35 U.S.C. 363)

04/01/2019
DATE OF RECEIPT OF 35 U.S.C.
371(c)(1) and (c)(2) REQUIREMENTS

The following items have been received:

- Copy of the International Application filed on 04/01/2019
- Copy of the International Search Report filed on 04/01/2019
- Request for Immediate Examination filed on 04/01/2019
- U.S. Basic National Fees filed on 04/01/2019
- Authorize Access to Search Results filed on 04/01/2019
- Priority Documents filed on 04/01/2019
- Power of Attorney filed on 04/01/2019
- Authorization to Permit Access filed on 04/01/2019
- Application Data Sheet (37 CFR 1.76) filed on 04/01/2019

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.495(c).

- Properly executed inventor's oath or declaration for the following inventor(s) has not been submitted: **Hyun Ho JUNG**, **Gi Hyeok YANG**, **Chang Hoon RHEE**, **Hack Woo KIM**, **Gi Hyuck RYU**, **Hyurg Pyo HONG**, **Jung Hyun SONG**, and **Joon Chan KONG**

FORM PCT/DO/EO/903 (371 Acceptance Notice)

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

FREDERICK SMITH

Telephone: (571) 272-8679

FORM PCT/DO/EO/903 (371 Acceptance Notice)

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 16/338,561

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	300
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	520
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	760
TOTAL CLAIMS (37 CFR 1.16(i))	9 minus 20 =	*			OR	x 100 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 460 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1580

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
16338561	

Applicant(s) Hyun Ho JUNG

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT	
	Indep	Depend	Indep	Depend	Indep	Depend
1	1					
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Total Indep	1		0		0	
Total Depend	8	↵	0	↵	0	↵
Total Claims	9		0		0	

	*		*		*	
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Docket No.: 2479-0007US01
(Patent)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
MEDYTOX INC.

Confirmation No.: 3639

Application No.: 16/338,561
Int'l Filing Date: November 29, 2017

Art Unit: 1778

For: **METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION**

Examiner: Not yet assigned

REQUEST FOR CORRECTED FILING RECEIPT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

A corrected filing receipt is hereby requested in the captioned application in view of the errors which appear in the filing receipt dated October 1, 2019, in regard to the sixth inventor's given name.

The sixth inventor's name is currently listed as "Hyurg Pyo HONG," however the correct spelling is "**Hyung Pyo HONG**" as reflected in the Application Data Sheet submitted April 1, 2019. For the convenience of the U.S. Patent and Trademark Office, attached is a photocopy of the filing receipt on which the errors have been noted in red. As the errors appear to be a data capture error on behalf of the U.S. Patent and Trademark Office, a corrected Application Data Sheet is not required.

Applicant respectfully requests that the name of the sixth inventor be updated, and that a Corrected Filing Receipt be issued to reflect the updated information.

Although Applicant believes no fee is due, the Commissioner is authorized to charge deposit account number 50-5875 for any required fees.

Date: November 1, 2019

Respectfully submitted,

Potomac Law Group, PLLC
8229 Boone Blvd.
Suite 430
Vienna, VA 22182
(443) 926-6811

By: /Judith U. Kim/
Judith U. Kim
Attorney for Applicant
Registration No.: 40,679

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
16/338,561	04/01/2019		1720	2479-0007US01	9	1

CONFIRMATION NO. 3639

137713
Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

FILING RECEIPT



Date Mailed: 10/01/2019

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

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Inventor(s)

Hyun Ho JUNG, Seoul, KOREA, REPUBLIC OF;
Gi Hyeok YANG, Cheonan-si, KOREA, REPUBLIC OF;
Chang Hoon RHEE, Seoul, KOREA, REPUBLIC OF;
Hack Woo KIM, Cheongju-si, KOREA, REPUBLIC OF;
Gi Hyuck RYU, Daejeon, KOREA, REPUBLIC OF;
~~Hyurg Pyo HONG,~~ Cheongju-si, KOREA, REPUBLIC OF; Hyung Pyo HONG
Jung Hyun SONG, Gunsan-si, KOREA, REPUBLIC OF;
Joon Chan KONG, Cheongju-si, KOREA, REPUBLIC OF;

Applicant(s)

MEDYTOX INC., Cheongju-si, KOREA, REPUBLIC OF

Power of Attorney: The patent practitioners associated with Customer Number 141404

Domestic Priority data as claimed by applicant

This application is a 371 of PCT/IB2017/057476 11/29/2017

Foreign Applications (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.)
REPUBLIC OF KOREA 10-2016-0127540 10/04/2016

Permission to Access Application via Priority Document Exchange: Yes

PTO/AIA/82A (07-13)
Approved for use through 01/31/2018. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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TRANSMITTAL FOR POWER OF ATTORNEY TO ONE OR MORE REGISTERED PRACTITIONERS

NOTE: This form is to be submitted with the Power of Attorney by Applicant form (PTO/AIA/82B) to identify the application to which the Power of Attorney is directed, in accordance with 37 CFR 1.5, unless the application number and filing date are identified in the Power of Attorney by Applicant form. If neither form PTO/AIA/82A nor form PTO/AIA82B identifies the application to which the Power of Attorney is directed, the Power of Attorney will not be recognized in the application.

Application Number	16/338,561
Filing Date	November 29, 2017
First Named Inventor	Hyun Ho JUNG
Title	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
Art Unit	1778
Examiner Name	Not yet assigned
Attorney Docket Number	2479-0007US01

SIGNATURE of Applicant or Patent Practitioner

Signature	/Judith U. Kim/	Date (Optional)	11-01-2019
Name	Judith U. Kim	Registration Number	40679
Title (if Applicant is a juristic entity)	Attorney for Applicant		
Applicant Name (if Applicant is a juristic entity)	MEDYTOX INC.		

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. If more than one applicant, use multiple forms.

☐ *Total of _____ forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82B (07-13)
Approved for use through 01/31/2018. OMB 0651-0035
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number

POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date
16/338,561	November 29, 2017

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

☒ I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 137713

OR

☐ I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

☒ The address associated with the above-mentioned Customer Number

OR

☐ The address associated with Customer Number: 137713

OR

☐ Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

Medytox Inc.

☐ Inventor or Joint Inventor (title not required below)

☐ Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

☒ Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

☐ Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature	E. M. Byun	Date (Optional)	
Name	Eun Myung		
Title	Senior Manager		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

☐ Total of forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16338561
Filing Date	2017-11-29
First Named Inventor	Hyun Ho JUNG
Art Unit	1778
Examiner Name	
Attorney Docket Number	2479-0007US01

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	5696077	A	1997-12-09	Johnson et al.	
	2	5846929	A	1998-12-08	Johnson et al.	
	3	9206409	B2	2015-12-08	Ton et al.	

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	1	20110171226	A1	2011-07-14	Johnson et al.	

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	1	1020120105417	KR	A	2012-09-25	ALLERGAN, INC.	Corresponds to WO 2011/008713 A1	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Art Unit	1778
	Examiner Name	
	Attorney Docket Number	2479-0007US01

	2	20030060150	KR	A	2003-07-16	Medy-Tox Inc.	English language abstract attached.	
	3	2011008713	WO	A1	2011-01-20	ALLERGAN, INC.	Corresponds to KR 10-2012-0105417 A	
	4	9605222	WO	A1	1996-02-22	Wisconsin Alumni Research Foundation		

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	1	BINZ et al., "The Complete Sequence of Botulinum Neurotoxin Type A and Comparison with Other Clostridial Neurotoxins," The Journal of Biological Chemistry, 265:9153-9158 (1990), American Society of Biological Chemists; Rockefeller Institute for Medical Research; American Society for Biochemistry and Molecular Biology, American Society for Biochemistry and Molecular Biology.	
	2	EISELE et al., "Studies on the dissociation of botulinum neurotoxin type A complexes," Toxicon, 47:555-565 (2011), International Society on Toxinology, Elsevier.	
	3	HASEGAWA et al., "Characterization of Toxin Complex Produced by a Unique Strain of Clostridium botulinum Serotype D 4947," The Protein Journal, 23:371-378 (2004), Kluwer Academic Publishers-Plenum Publisher.	
	4	International Search Report dated February 22, 2018 for International Application No. PCT/IB2017/057476, ISA/KR, Korean Intellectual Property Office, Republic of Korea.	
	5	MANTECUCCO et al., "Tetanus and botulism neurotoxins: a new group of zinc proteases," Trends in Biochemical Sciences, 18:324-327 (1993), Elsevier Inc.	
	6	PARK et al., "Binding of Clostridium botulinum type B toxin to rat brain synaptosome," FEMS Microbiology Letters, 72:243-247 (1990), Federation of European Microbiological Societies.	

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	Filing Date	2017-11-29
	First Named Inventor	Hyun Ho JUNG
	Art Unit	1778
	Examiner Name	
	Attorney Docket Number	2479-0007US01

	7	POULAIN et al., "Neurotransmitter release is blocked intracellularly by botulinum neurotoxin, and this requires uptake of both toxin polypeptides by a process mediated by the larger chain," Proceedings of the National Academy of Sciences of the United States of America, 85:4090-4094 (1988).	
	8	SCHANTZ et al., "Properties and Use of Botulinum Toxin and Other Microbial Nerotoxins in Medicine," Microbiological Reviews, 56:80-99 (1992), American Society for Microbiology Journals.	
	9	SIMPSON, "Molecular Pharmacology of Botulinum Toxin and Tetanus Toxin," Annual Review of Pharmacology and Toxicology, 26:427-453 (1986).	
	10	SINGH et al., "Critical Aspects of Bacterial Protein Toxins," Advances in Experimental Medicine and Biology, pp. 63-84 (1996), Plenum Press, New York.	
	11	SUGIYAMA, "Clostridium botulinum Neurotoxin," Microbiological Reviews, 44:419-448 (1980), American Society for Microbiology (ASM)	

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EXAMINER SIGNATURE

Examiner Signature		Date Considered	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16338561
	Filing Date	2017-11-29
	First Named Inventor	Hyun Ho JUNG
	Art Unit	1778
	Examiner Name	
	Attorney Docket Number	2479-0007US01

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Judith U. Kim/	Date (YYYY-MM-DD)	2019-11-01
Name/Print	Judith U. Kim	Registration Number	40679

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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(19) 대한민국특허청(KR)
(12) 공개특허공보(A)

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(뒷면에 계속)
(74) 대리인
최경준

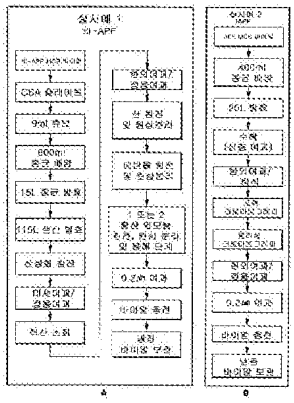
전체 청구항 수 : 총 30 항

(54) 발명의 명칭 **보툴리눔 신경독소를 수득하기 위한 공정 및 시스템**

(57) 요 약

연구, 치료 및 미용 용도를 위하여 고역가, 고수율 보툴리눔 신경독소를 수득하기 위한 신속하고 동물 단백질이 없는 크로마토그래피 공정 및 시스템.

대 표 도 - 도1



Hugel Ex. 1006 - Page 139 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

(72) 발명자

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Hugel Ex. 1006 - Page 140 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

특허청구의 범위

청구항 1

다음 단계를 포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위하여 크로마토그래피를 이용하는 실질적으로 동물 산물이 없는(APF) 공정:

(a) 실질적으로 APF 발효 배지를 제공하는 단계;

(b) 발효 배지에서 클로스트리디움 보툴리눔 박테리아를 발효시키는 단계, 및;

(c) 발효 배지를 음이온 교환 크로마토그래피 매질과 접촉시키고 이어서 음이온 교환 크로마토그래피 매질에서 나온 용리액을 양이온 교환 크로마토그래피와 접촉시켜 발효 배지로부터 생물학적으로 활성인 보툴리눔 신경독소를 회수하는 단계, 이에 의하여 실질적으로 APF 크로마토그래피 방법으로부터 생물학적으로 활성인 보툴리눔 신경독소를 수득함.

청구항 2

제1항에 있어서, 수득된 보툴리눔 신경독소는 백만의 수득된 보툴리눔 신경독소당 1 부 이하의 잔류 핵산을 포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위하여 크로마토그래피를 이용하는 실질적으로 동물 산물이 없는 (APF) 공정.

청구항 3

제1항에 있어서, 방법은 1 주일 이내에 수행되는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위하여 크로마토그래피를 이용하는 실질적으로 동물 산물이 없는(APF) 공정.

청구항 4

제1항의 공정에 의하여 수득된 생물학적으로 활성인 보툴리눔 신경독소.

청구항 5

다음의 순차적인 단계를 포함하는, 생물학적으로 활성인 보툴리눔 신경독소 A 형 복합체를 수득하기 위한 실질적으로 APF 크로마토그래피 공정:

(a) 실질적으로 APF 배양 배지에서 *클로스트리디움 보툴리눔* 박테리아를 배양하는 단계;

(b) 약 2 L 내지 약 75 L의 실질적으로 APF 발효 배지에서 배양 배지로부터의 *클로스트리디움 보툴리눔* 박테리아를 발효시키는 단계, 여기서 배양 배지 및 발효 배지 중 최소한 하나는 식물 단백질을 포함함;

(c) 발효 배지에 존재하는 세포 파편을 제거하여 발효 배지를 수확하는 단계;

(d) 여과에 의하여 수확 발효 배지를 농축하는 단계;

(e) 완충제를 첨가하여 농축된 발효 배지를 희석하는 단계;

(f) 생물학적으로 활성인 보툴리눔 신경독소가 이온 교환 매질로써 포획되도록, 희석된 수확 발효 배지가 이온 교환 매질과 접촉되는 제1 접촉 단계;

(g) 이온 교환 매질로부터 포획된 보툴리눔 신경독소를 용리하여 이에 의하여 제1 용리액을 수득하는 단계;

(h) 제1 용리액으로부터 불순물을 제거하기 위하여 제1 용리액이 양이온 교환 매질과 접촉되는 제2 접촉 단계, 이에 의하여 제2 용리액을 수득함;

(i) 정용여과에 의하여 제2 용리액을 가공하는 단계; 및

(j) 가공된 제2 용리액을 여과하는 단계, 이에 의하여 실질적으로 APF 크로마토그래피 공정을 이용하여 생물학적으로 활성인 보툴리눔 신경독소 A 형 복합체를 수득하고, 여기서 수득된 보툴리눔 신경독소 A 형 복합체는 2.0×10^7 내지 약 6.0×10^7 유닛/mg의 보툴리눔 신경독소 A 형 복합체 역가를 가짐.

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청구항 6

제5항에 있어서, 발효 배지는 약 5% w/v 이하의 식물-유래 단백질 산물, 약 2% w/v 이하의 효모 추출물 및 약 2% w/v 이하의 글루코오스를 포함하고, 발효 배지의 pH 수준은 발효 단계 시작 시 약 pH 6.5 내지 약 pH 8.0인, 생물학적으로 활성인 보툴리눔 신경독소 A 형 복합체를 수득하기 위한 실질적으로 APF 크로마토그래피 공정.

청구항 7

제5항에 있어서, 배양 단계는 약 540 nm에서 배양 배지의 광학 밀도가 약 0.8 AU 내지 약 4.5 AU일 때까지 수행되는, 생물학적으로 활성인 보툴리눔 신경독소 A 형 복합체를 수득하기 위한 실질적으로 APF 크로마토그래피 공정.

청구항 8

제5항에 있어서, 발효 단계는 약 890 nm에서 발효 배지의 광학 밀도가 약 0.05 AU 내지 약 0.7 AU로 감소될 때까지 약 60 내지 80 시간 동안 수행되는, 생물학적으로 활성인 보툴리눔 신경독소 A 형 복합체를 수득하기 위한 실질적으로 APF 크로마토그래피 공정.

청구항 9

제5항에 있어서, 배양 단계는 *클로스트리디움 보툴리눔* APF 제조용 세포 은행(working cell bank) 내용물을 배양 배지에 도입하여 개시되고, 제조용 세포 은행은 제조용 세포 은행 밀리리터당 최소한 약 1×10^4 내지 5×10^7 콜로니형성수(colony-forming unit)의 *클로스트리디움 보툴리눔*을 포함하고, 제조용 세포 은행 중의 *클로스트리디움 보툴리눔* 박테리아는 실질적으로 균일한 형태를 가지는, 생물학적으로 활성인 보툴리눔 신경독소 A 형 복합체를 수득하기 위한 실질적으로 APF 크로마토그래피 공정.

청구항 10

제5항에 있어서, 수득된 보툴리눔 신경독소는 수득된 보툴리눔 신경독소의 각 mg에 대하여 1 ng 또는 1 ng 미만의 잔류 핵산을 포함하는, 생물학적으로 활성인 보툴리눔 신경독소 A 형 복합체를 수득하기 위한 실질적으로 APF 크로마토그래피 공정.

청구항 11

제5항에 있어서, 공정은 1 주일 이내에 수행되는, 생물학적으로 활성인 보툴리눔 신경독소 A 형 복합체를 수득하기 위한 실질적으로 APF 크로마토그래피 공정.

청구항 12

다음의 순차적인 단계를 포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 APF 크로마토그래피 공정:

(a) APF 제조용 세포 은행으로부터의 *클로스트리디움 보툴리눔* 박테리아를 APF 배양 배지에 첨가하는 단계;

(b) 배양 배지에서 *클로스트리디움 보툴리눔* 박테리아를 배양하는 단계;

(c) *클로스트리디움 보툴리눔* 세포 용해가 일어날 때까지 APF 발효 배지에서 단계 (b)로부터의 *클로스트리디움 보툴리눔* 박테리아를 발효시키는 단계;

(d) 발효 배양물을 수확하여 수확 발효 배지를 제공하는 단계;

(e) 수확 발효 배지가 여과에 의한 농축을 거치는 단계;

(f) 완충제를 첨가하여 여과된 발효 배지를 희석하여 희석된 발효 배지를 수득하는 단계;

(g) 희석된 발효 배지가 포획 크로마토그래피 매질과 접촉되는 제1 접촉 단계, 여기서 포획 크로마토그래피 매질은 음이온 교환 매질임;

(h) 제1 접촉 단계의 용리액이 폴리싱(polishing) 크로마토그래피 매질과 접촉되는 제2 접촉 단계, 여기서 폴

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리성 크로마토그래피 매질은 양이온 교환 매질임, 및

(i) 제2 접촉 단계의 용리액을 여과하는 단계, 이에 의하여 개선된 APF 공정에 의하여 생물학적으로 활성인 보툴리눔 신경독소를 수득하고, 여기서 수득된 보툴리눔 신경독소는 수득된 보툴리눔 신경독소의 각 mg에 대하여 1 ng 또는 1 ng 미만의 잔류 핵산을 포함하고 공정은 1 주일 이내에 수행됨.

청구항 13

다음을 포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 동물 산물이 없는(APF) 크로마토그래피 시스템:

(a) 실질적으로 APF 발효 배지;

(b) 발효 배지에서 발효시키기 위한 *클로스트리디움 보툴리눔* 박테리아;

(c) 발효 배지로부터 생물학적으로 활성인 보툴리눔 신경독소를 회수하기 위한 음이온 교환 크로마토그래피 매질, 및;

(d) 음이온 교환 크로마토그래피 매질에서 나온 용리액으로부터 생물학적으로 활성인 보툴리눔 신경독소를 추가로 회수하기 위한 양이온 교환 크로마토그래피 매질, 이에 의하여 실질적으로 APF 크로마토그래피 공정으로부터 생물학적으로 활성인 보툴리눔 신경독소를 수득함.

청구항 14

제13항에 있어서, 실질적으로 APF 배양 배지에서 *클로스트리디움 보툴리눔* 박테리아를 혐기성으로 배양하기 위한 제1 장치를 추가로 포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 동물 산물이 없는(APF) 크로마토그래피 시스템.

청구항 15

제14항에 있어서, 실질적으로 APF 발효 배지에서 제1 장치로부터 수득된 *클로스트리디움 보툴리눔* 박테리아를 혐기성으로 발효시기키 위한 제2 장치를 추가로 포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 동물 산물이 없는(APF) 크로마토그래피 시스템.

청구항 16

제15항에 있어서, 제2 장치로부터 수득된 발효 배지로부터 세포 파편을 제거하여, 이에 의하여 수확 발효 배지를 제공하는 수확 장치를 추가로 포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 동물 산물이 없는(APF) 크로마토그래피 시스템.

청구항 17

제16항에 있어서, 수확 발효 배지를 농축시킨 다음 희석시키는 농축 및 희석 장치를 추가로 포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 동물 산물이 없는(APF) 크로마토그래피 시스템.

청구항 18

제13항에 있어서, 양이온 교환 크로마토그래피 매질에서 나온 용리액으로부터 추가로 정제된 생물학적으로 활성인 보툴리눔 신경독소를 회수하기 위한 소수성 상호작용 매질을 추가로 포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 동물 산물이 없는(APF) 크로마토그래피 시스템.

청구항 19

제13항에 있어서, 수득된 생물학적으로 활성인 보툴리눔 신경독소 중 생물부하(bioburden)를 감소시키기 위한 여과 장치를 추가로 포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 동물 산물이 없는(APF) 크로마토그래피 시스템.

청구항 20

제18항에 있어서, 수득된 생물학적으로 활성인 보툴리눔 신경독소 중 생물부하를 감소시키기 위한 여과 장치

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를 추가로 포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 동물 산물이 없는(APF) 크로마토그래피 시스템.

청구항 21

제13항에 있어서, 실질적으로 APF 배양 배지에서 *클로스트리디움 보툴리눔* 박테리아를 배양하기 위한, 작업 공간 내에 집적된 고효율 미립자 공기 필터를 가지는 혐기성 챔버를 추가로 포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 동물 산물이 없는(APF) 크로마토그래피 시스템.

청구항 22

제13항에 있어서, 수득된 보툴리눔 신경독소는 최소한 약 2.0 x 10^7 유닛/mg의 보툴리눔 신경독소 역가를 가지고, 수득된 보툴리눔 신경독소는 수득된 보툴리눔 신경독소의 각 mg에 대하여 1 ng 또는 1 ng 미만의 잔류 핵산을 포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 동물 산물이 없는(APF) 크로마토그래피 시스템.

청구항 23

제13항에 있어서, 실질적으로 APF 발효 배지는 약 2 L 내지 약 75 L의 양으로 제공되는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 동물 산물이 없는(APF) 크로마토그래피 시스템.

청구항 24

제14항에 있어서, 약 2 L 내지 약 75 L의 실질적으로 APF 발효 배지가 이용되고, 약 200 mL 내지 약 1 L의 실질적으로 APF 배양 배지가 이용되는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 동물 산물이 없는(APF) 크로마토그래피 시스템.

청구항 25

다음을 포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 APF 크로마토그래피 시스템:

클로스트리디움 보툴리눔 박테리아를 배양하기 위한 제1 장치, 제1 장치는 실질적으로 APF 배양 배지를 포함할 수 있음;

제1 장치에서 배양된 *클로스트리디움 보툴리눔* 박테리아를 발효시키기 위한 제2 장치, 제2 장치는 실질적으로 APF 발효 배지를 포함할 수 있음;

발효 배지를 수확하기 위한 제3 장치;

수확 발효 배지를 농축하고 여과된 발효 배지를 희석하기 위한 제4 장치;

수확된 배지로부터 보툴리눔 신경독소의 제1 정제를 수행하여, 이에 의하여 제1 정제된 보툴리눔 신경독소를 수득하기 위한 제5 장치, 여기서 제5 장치는 음이온 교환 크로마토그래피 매질을 포함함; 및

제1 정제된 보툴리눔 신경독소의 제2 정제를 수행하여, 이에 의하여 제2 정제된 보툴리눔 신경독소를 수득하기 위한 제6 장치 , 여기서 제6 장치는 양이온 교환 크로마토그래피 매질을 포함함, 여기서 수득된 보툴리눔 신경독소는 최소한 약 2.0 x 10^7 유닛/mg의 보툴리눔 신경독소 역가를 가지고, 수득된 보툴리눔 신경독소는 수득된 보툴리눔 신경독소 각 mg에 대하여1 ng 또는 1 ng 미만의 잔류 핵산을 포함하고, 공정은 1 주일 이내에 수행됨.

청구항 26

제25항에 있어서, 제6 장치로부터 수득된 보툴리눔 신경독소의 추가의 정제를 수행하여 제3 정제된 보툴리눔 신경독소를 수득하기 위한 제7 장치를 추가로 포함하고, 여기서 제7 장치는 소수성 상호작용 매질을 포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 APF 크로마토그래피 시스템.

청구항 27

제26항에 있어서, 제6 또는 제7 장치에서 나온 용리액을 여과하기 위한 여과막을 포함하는 제8 장치를 추가로

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포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 APF 크로마토그래피 시스템.

청구항 28

제25항의 시스템을 이용하여 수득된 생물학적으로 활성인 보툴리눔 신경독소.

청구항 29

다음을 포함하는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 APF 크로마토그래피 시스템:

클로스트리디움 보툴리눔 박테리아를 혐기성 배양하기 위한 제1 장치, 제1 장치는 약 200 mL 내지 약 1 L의 실질적으로 APF 배양 배지를 포함할 수 있음;

제1 장치에서 배양된 *클로스트리디움 보툴리눔* 박테리아의 혐기성 발효를 위한 제2 장치, 제2 장치는 약 2 L 내지 약 75 L의 실질적으로 APF 발효 배지를 포함할 수 있고 환원-산화 탐침, pH 탐침 및 탁도 탐침으로 이루어진 군에서 선택되는 최소한 하나의 일회용 탐침을 포함할 수 있음;

발효 배지를 수확하기 위한 제3 장치;

수확 발효 배지를 농축하고 여과된 발효 배지를 희석하기 위한 제4 장치;

수확 발효 배지로부터 수득된 보툴리눔 신경독소의 제1 정제를 수행하여, 이에 의하여 제1 정제된 보툴리눔 신경독소를 수득하기 위한 제5 장치, 제5 장치는 음이온 교환 크로마토그래피 매질을 포함함;

보툴리눔 신경독소의 제2 정제를 수행하여, 이에 의하여 제2 정제된 보툴리눔 신경독소를 수득하기 위한 제6 장치, 제6 장치는 양이온 교환 크로마토그래피 매질을 포함함;

보툴리눔 신경독소의 제3 정제를 수행하여, 이에 의하여 제3 정제된 보툴리눔 신경독소를 수득하기 위한 제7 장치, 제7 장치는 소수성 상호작용 매질을 포함함; 및

제3 정제된 보툴리눔 신경독소를 여과하기 위한 제8 장치, 제8 장치는 여과막을 포함함, 여기서 수득된 보툴리눔 신경독소는 약 2.0x 10^7 내지 약 6.0 x 10^7 유닛/mg의 보툴리눔 신경독소 역가를 가지고, 수득된 보툴리눔 신경독소는 수득된 보툴리눔 신경독소의 각 mg에 대하여 약 1 ng 또는 약 1 ng 미만의 잔류 핵산을 포함하고, 공정은 1 주일 이내에 수행됨.

청구항 30

제29항에 있어서, 제1 장치를 수용할 수 있는 혐기성 챔버를 포함하는 모듈식 대기 제어 시스템(modular atmosphere controlled system)을 추가로 포함하고, 여기서 혐기성 챔버는 혐기성 챔버 내에 집적된 고효율 미립자 공기 필터를 가지는, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 APF 크로마토그래피 시스템.

명 세 서

기 술 분 야

[0001] 교차 참조

[0002] 이 출원은 2009년 7월 13일자 미국 특허 출원번호 제12/502,181호의 이익을 청구하며, 상기 특허의 전체 개시는 본 명세서에 구체적인 참조로 포함된다.

[0003] 배경

[0004] 본 발명은 클로스트리디움 신경독소를 수득하기 위한 시스템 및 공정, 클로스트리디움 신경독소로부터 약제학적 조성물을 제조하는 방법 및 이렇게 제조된 약제학적 조성물의 치료 및 미용 용도에 관한 것이다. 특히, 본 발명은 고역가(high potency), 고순도, 및 고수율의 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한, 신속하고 동물 단백질이 없는 크로마토그래피 공정 및 시스템에 관한 것이다.

배 경 기 술

[0005] 치료, 진단, 연구 또는 미용 목적을 위하여 인간 또는 동물에게 투여하기 적절한 약제학적 조성물은 활성 성

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분 및 한 가지 이상의 부형제, 완충제, 담체, 안정화제, 삼투성 조정제, 보존제 및/또는 증량제를 포함한다. 약제학적 조성물 중의 활성 성분은 보툴리눔 신경독소와 같이 생물학적일 수 있다. 약제학적 조성물 중의 활성 성분으로서 유용한 보툴리눔 신경독소를 수득하기 위한 공지 방법(예컨대 Schantz 방법)은 배양 및 발효 배지에서 사용되는 고기 육즙(meat broth) 및 카제인과 같은 동물-유래 단백질, 및 동물 유래의 정제 효소를 사용하는 수주일의 배양, 발효 및 정제 공정이다. 동물 유래 산물의 사용을 통하여 제조된 약제학적 조성물을 환자에게 투여하는 것은 프리온과 같은 감염원 또는 병원체를 투여할 위험을 수반할 수 있다. 추가적으로, 보툴리눔 독소를 수득하기 위한 동물 단백질이 없는 공지된 방법은 또한 많은 상류 (배양 및 발효) 및 하류 (정제) 단계를 가지는 시간-소모적 공정(즉 완료를 위하여 일주일 이상이 걸림)이며, 여전히 검출 가능한 불순물을 가지는 보툴리눔 신경독소 수득을 초래한다.

[0006] 보툴리눔 독소

[0007] 클로스트리디움 속에는 형태 및 기능에 의하여 분류되는 127 종 이상이 속한다. 혐기성, 그람 양성 박테리아 *클로스트리디움 보툴리눔(Clostridium botulinum)*은 강력한 폴리펩타이드 신경독소인 보툴리눔 독소(동의어로 "독소")를 생성하는데, 이는 인간 및 동물에게 보툴리눔증(botulism)으로 알려진 신경마비성 병을 일으킨다. 보툴리눔 독소 중독의 증상은 보행장애, 연하장애 및 언어장애로부터 호흡기 근육의 마비 및 사망으로 진행할 수 있다.

[0008] 보툴리눔 독소의 1 유닛(unit)은 각각 약 18-20 그램으로 계량되는 암컷 스위스 웹스터 마우스에게 복강내 주사 시의 LD_{50}으로 정의된다. 보툴리눔 독소의 1 유닛은 암컷 스위스 웹스터 마우스 그룹의 50%를 죽이는 보툴리눔 독소의 양이다. 일반적으로 면역학적으로 구별되는 일곱 가지의 보툴리눔 신경독소가 특징분석되었고, 이들은 각각 보툴리눔 신경독소 혈청형(serotype) A, B, C_1, D, E, F 및 G이며, 이들 각각은 유형-특이성 항체로 중화하여 구별된다. 보툴리눔 독소의 상이한 혈청형들은 이들이 영향을 미치는 동물 종 및 이들이 일으키는 마비의 중증도 및 지속기간에 차이가 있다. 보툴리눔 독소는 높은 친화성을 가지고 콜린작동성 모터 뉴런에 명백하게 결합하고, 뉴런으로 위치를 옮기며, 아세틸콜린의 시냅스전 방출을 차단한다.

[0009] 보툴리눔 독소는, 예를 들어 활동항진성 골격근을 특징으로 하는 신경근육 장애의 치료를 위한 임상적 상황에서 사용되어 왔다. 보툴리눔 독소 A 형은 미국 식품의약국(FDA)에 의하여 12 세 이상 환자의 본태성 안검경련, 사시 및 반측안면 경련에 대하여 승인되었고, 경부 근긴장이상증, 미간 (안면) 주름의 치료 및 다한증 치료에 대하여 승인되었다. FDA는 또한 경부 근긴장이상증의 치료에 대하여 보툴리눔 독소 B 형을 승인했다.

[0010] 비록 모든 보툴리눔 독소 혈청형들이 신경근육 접합부에서 신경전달물질 아세틸콜린의 방출을 명백하게 억제하기는 하지만, 보툴리눔 독소 혈청형들은 여러 상이한 신경분비 단백질에 작용하고 및/또는 이러한 단백질들을 여러 상이한 부위에서 분해하여 이를 수행한다. 보툴리눔 독소 A 형은 세포내, 소포-결합 단백질(vesicle-associated protein, VAMP, 또한 시냅토브레빈(synaptobrevin)으로 지칭됨) 25 킬로달톤 (kDa) 시냅토솜 결합 단백질(SNAP-25)의 펩타이드 결합을 특이적으로 가수분해할 수 있는 아연 엔도펩티데이즈이다. 보툴리눔 E 형 또한 SNAP-25를 절단하지만, 보툴리눔 독소 A 형과 비교하여 이 단백질 내의 상이한 아미노산 서열을 표적으로 한다. 보툴리눔 독소 B, D, F 및 G 형은 VAMP에 작용하며, 각각의 혈청형은 상이한 부위에서 단백질을 절단한다. 마지막으로, 보툴리눔 독소 C_1 형은 신택신 및 SNAP-25 모두를 절단하는 것으로 나타났다. 이러한 작용 메커니즘의 상이함은 다양한 보툴리눔 독소 혈청형 작용의 상대적 효력 및/또는 작용기간에 영향을 미칠 수 있다.

[0011] 일곱 가지의 모든 공지 보툴리눔 독소 혈청형에 대하여, 보툴리눔 독소 복합체의 활성 보툴리눔 독소 단백질 분자("순수 독소" 또는 "신경독성 성분"으로도 공지임)의 분자량은 약 150 kDa이다. 흥미롭게도, 보툴리눔 독소는 하나 이상의 결합된 비-독소 단백질과 함께150 kDa 신경독성 성분을 포함하는 복합체로서 클로스트리디움 박테리아에 의하여 방출된다. 따라서, 보툴리눔 독소 A 형 복합체는 900 kDa, 500 kDa 및 300 kDa 형태(대략의 분자량)로서 클로스트리디움 박테리아에 의하여 생성될 수 있다. 보툴리눔 독소 B 및 C_1 형은 명백하게 500 kDa 복합체로서만 생성된다. 보툴리눔 독소 D 형은 300 kDa 및 500 kDa 복합체 두 가지로서 생성된다. 마지막으로, 보툴리눔 독소 E 및 F 형은 대략 300 kDa 복합체로서만 생성된다. 복합체(즉, 분자량이 약 150 kDa 보다 큰 것)는 헤마글루티닌(HA) 단백질 및 비-독소 비-헤마글루티닌(NTNH) 단백질을 포함한다. 따라서, 보툴리눔 독소 복합체가 보툴리눔 독소 분자(신경독성 성분) 및 하나 이상의 HA 단백질 및/또는 NTNH 단백질을 포함할 수 있다. (보툴리눔 독소 분자와 함께 관련 신경독소 복합체를 이룰 수 있는) 이러한 두 가지 유형의 비-독소 단백질은 보툴리눔 독소 분자의 변성에 대한 안정성 및 독소가 섭취될 때 소화성 산에 대한 보호를 제

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공하는 작용을 할 수 있다. 추가적으로, 더 큰 (분자량이 약 150 kDa보다 큰) 보툴리눔 독소 복합체가 보툴리눔 독소 복합체의 근육내 주사 부위로부터 더 느린 보툴리눔 독소 확산 속도를 유발할 수 있다. 다양한 임상 상태를 치료하는 보툴리눔 독소 A 형의 성공은 다른 보툴리눔 독소 혈청형에 대한 관심을 유발시켰다. 따라서, 적어도 보툴리눔 독소 A, B, E 및 F 형이 인간에게 임상적으로 사용되었다. 추가적으로, 신경독성 성분의 제제(즉 결합된 비-독소 단백질이 없음)가 상표명 XEOMIN(독일, 프랑크푸르트 소재의 Merz Pharmaceuticals)으로 유럽에서 판매된다.

[0012] 보툴리눔 독소 A 형은 pH 4-6.8의 묽은 수용액에 용해성인 것으로 공지이다. 약 7 이상의 pH에서 안정화 비-독소 단백질이 신경독소로부터 분리되어, 그 결과 독성이 점진적으로 손실되는데, 특히 pH 및 온도가 상승함에 따라 그러하다 (Schantz E.J., et al *Preparation and characterization of botulinum toxin type A for human treatment* (특히 44-45 쪽), Jankovic, J., et al, Therapy with Botulinum Toxin, Marcel Dekker, Inc, 1994의 3장).

[0013] 효소가 일반적으로 그렇듯이, 보툴리눔 독소(이는 세포내 펩티데이즈임)의 생물학적 활성은 이의 삼차원 형상에 최소한 부분적으로 의존적이다. 독소를 밀리그램 양으로부터 밀리리터당 수나노그램을 함유하는 용액으로 희석하는 것은, 예를 들어 독소가 표면에 부착되어 따라서 가용 독소의 양을 감소시키는 경향과 같은 상당한 어려움을 나타낸다. 독소는 독소 함유 약제학적 조성물이 제제화된 지 수개월 또는 수년 후에 사용될 수 있으므로, 알부민, 수크로오스, 트레할로오스 및/또는 젤라틴와 같은 안정화제로써 안정화된다.

[0014] 상용화되어 입수 가능한 보툴리눔 독소 함유 약제학적 조성물은 캘리포니아, 어바인 소재의 Allergan, Inc.로부터 상용화되어 입수 가능한 상표명 BOTOX®(보툴리눔 독소 A 형 정제된 신경독소 복합체)로 판매된다. 각각의 BOTOX®의 100 유닛 바이알은 약 5 ng의 정제된 보툴리눔 독소 A 형 복합체, 0.5 mg 인간 혈청 알부민, 및 0.9 mg 염화나트륨으로 구성되고 진공-건조 형태이며, 보존제 없이(0.9% 염화나트륨 주입) 멸균 생리식염수로써 복원되도록 의도된다. 다른 상용화되어 입수 가능한 보툴리눔 독소-함유 약제학적 조성물에는 Dysport®(보툴리눔 독소 약제학적 조성물 중에 락토오스 및 인간 혈청 알부민을 가지는 *클로스트리디움 보툴리눔* A 형 독소 헤마글루티닌 복합체, 영국, 버크셔 소재의 Ipsen Limited로부터 상용화되어 입수 가능, 사용 전에 0.9% 염화나트륨로써 복원될 분말), 및 MyoBloc™(보툴리눔 독소 B 형, 인간 혈청 알부민, 숙신산나트륨, 및 염화나트륨을 포함하는 약 pH 5.6 주사 용액, 캘리포니아, 샌 디에고 소재의 Solstice Neurosciences로부터 상용화되어 입수 가능)이 포함된다. 신경독성 성분(150 kDa 독소 분자) 및 보툴리눔 독소 복합체(300 kDa 내지 900 kDa)는, 예를 들어 캘리포니아, 캠벨 소재의 List Biological Laboratories, Inc.; 영국, 포턴 다운 소재의 the Centre for Applied Microbiology and Research; Wako (일본, 오사카), 또한 미주리, 세인트 루이스 소재의 Sigma Chemicals로부터 입수될 수 있다.

[0015] 보툴리눔 신경독소를 수득하기 위한 동물 단백질이 없는 및/또는 크로마토그래피 방법이 미국 특허 제7,445,914호; 제7,452,697호; 제7,354,740호; 제7,160,699호; 제7,148,041호 및; 제7,189,541호에 개시된다. 발명의 명칭이 "Media for Clostridium Bacterium"인 2006년 12월 12일자 미국 특허 출원 제11/609,449호; 발명의 명칭이 "Animal Product Free Media and Processes for Obtaining a Botulinum Toxin"인 2008년 4월 7일자 제12/098,896호; 발명의 명칭이 "Chromatographic Method and System for Purifying a Botulinum Toxin"인 2007년 10월 31일자 제11/932,689호; 발명의 명칭이 "Chromatographic Method and System for Purifying a Botulinum Toxin"인 2007년 10월 31일자 2007 제11/932,789호 및; 발명의 명칭이 "Animal Product Free Media And Processes For Obtaining A Botulinum Toxin"인 2008년 9월 19일 제12/234,537호가 또한 관심사이다.

[0016] 약제학적 조성물에서 사용하기 위한 보툴리눔 독소는 공지된 Schantz 공정을 이용하여 *클로스트리디움 보툴리눔*의 혐기성 발효에 의하여 수득될 수 있다 (예를 들어 Schantz E.J., et al., *Properties and use of botulinum toxin and other microbial neurotoxins in medicine,* Microbiol Rev 1992 Mar;56(1):80-99; Schantz E.J., et al., *Preparation and characterization of botulinum toxin A type for human treatment*, chapter 3 in Jankovic J, ed. Neurological Disease and Therapy. Therapy with botulinum toxin (1994), New York, Marcel Dekker;1994, pages 41-49, and; Schantz E.J., et al., *Use of crystalline type A botulinum toxin in medical research,* in: Lewis GE Jr, ed. Biomedical Aspects of Botulism (1981) New York, Academic Press, pages 143-50 참조). 보툴리눔 독소를 수득하기 위한 Schantz 공정은 동물 산물을 예를 들어 시약 및 배양 및 발효 배지의 일부로서 사용한다.

[0017] 다수의 단계가 치료, 진단, 연구 또는 미용 목적을 위하여 인간 또는 동물에게 투여하기 적절한 클로스트리디

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움 독소 약제학적 조성물 제조에 필요하다. 이러한 단계들은 정제된 클로스트리디움 독소를 수득하는 단계 그리고 이후 정제된 클로스트리디움 독소를 조합(compounding)하는 단계를 포함할 수 있다. 첫 번째 단계는 온난한 혐기성 대기와 같은 혐기성 박테리아 성장에 도움이 되는 환경에서, 전형적으로 혈액 한천 플레이트에 클로스트리디움 박테리아의 콜로니를 도말하고 성장시키는 것일 수 있다. 이 단계는 원하는 형태 및 다른 특징을 가지는 클로스트리디움 콜로니가 수득되도록 한다. 두 번째 단계에서, 선택된 클로스트리디움 콜로니가 적절한 제1 배지에서 발효될 수 있고, 추가적으로 필요한 경우, 제2 발효 배지에 보내진다. 일정 기간의 발효 후, 클로스트리디움 박테리아가 일반적으로 용해되고 클로스트리디움 독소를 배지에 방출한다. 세 번째로, 배지가 벌크(bulk) 독소를 수득하도록 정제될 수 있다. 전형적으로 벌크 독소를 수득하기 위한 배지 정제가 시약 중에서 특히 DNase 및 RNase와 같은 동물-유래의 효소를 사용하여 수행되는데, 상기 효소는 핵산의 분해 및 제거 촉진에 사용된다. 생성된 벌크 독소는 특정한 특이적 활성을 가지는 고도로 정제된 독소일 수 있다. 적절한 완충제에서 안정화시킨 후, 인간에게 투여하기 적절한 클로스트리디움 독소 약제학적 조성물을 제조하기 위하여 벌크 독소가 한 가지 이상의 부형제와 조합될 수 있다. 클로스트리디움 독소 약제학적 조성물은 활성 약제학적 성분(API)으로서 클로스트리디움 독소를 포함할 수 있다. 약제학적 조성물은 또한 한 가지 이상의 부형제, 완충제, 담체, 안정화제, 보존제 및/또는 증량제를 포함할 수 있다.

[0018] 클로스트리디움 독소 발효 단계는 완전(whole) 클로스트리디움 박테리아, 용해된 박테리아, 배양 배지 영양물 및 발효 부산물을 포함하는 발효 배지 용액을 생성할 수 있다. 완전 및 용해된 박테리아와 같은 큰 성분을 제거하도록 이 배양 용액을 여과하여 수확/정화(harvest/clarified) 배지를 제공한다. 정화 배지는 클로스트리디움 독소 및 다양한 불순물을 포함하고, 벌크 독소로 지칭되는 농축된 클로스트리디움 독소를 수득하도록 가공된다.

[0019] 한 가지 이상의 동물 유래 산물(예컨대 젖 소화 카제인, DNase 및 RNase)을 사용하여 벌크 클로스트리디움 독소를 수득하기 위한 발효 및 정제 공정이 공지이다. 보툴리눔 독소 복합체를 수득하기 위한 이러한 공지의 비-동물 산물이 없는 (non-animal product free, "NAPF") 공정의 예는 Schantz 공정 및 이의 변형이다. Schantz 공정(초기의 도말, 세포 배양으로부터 발효 및 독소 정제까지)은 예를 들어 배양 바이알 중의 동물 유래의 박토 조리 고기 배지(Bacto Cooked Meat medium), 콜로니 성장 및 선택을 위한 콜롬비아 혈액 한천 플레이트, 및 발효 배지 중의 카제인과 같은 다수의 동물 공급원 유래 산물을 사용한다. 추가적으로, Schantz 벌크 독소 정제 공정은 독소 함유 발효 배지에 존재하는 핵산을 가수분해하기 위하여 소 원천의 DNase 및 RNase를 사용한다. API를 수득하기 위한 공정에서 및/또는 이러한 API를 사용하여 약제학적 조성물을 제조(조합)하기 위한 공정에서 동물 산물이 사용될 경우 바이러스성 및 전염성 해면형 뇌증(transmissible spongiform encephalopathy, TSE), 예컨대 소 해면형 뇌증(bovine spongiform encephalopathy, BSE) 오염에 대한 잠재성에 관하여 우려가 나타났다.

[0020] 감소된 양의 동물-유래 산물을 사용하는, 파상풍 유독소를 수득하기 위한 발효 공정(동물 산물이 없는 또는 "APF" 발효 공정으로 지칭됨; APF는 동물 단백질 없는을 포괄함)이 공지이고, 예를 들어 미국 특허 제6,558,926호를 참조하라. 클로스트리디움 독소를 수득하기 위한 APF 발효 공정은, 얻어진 벌크 독소가 바이러스, 프리온 또는 다른 원하지 않는 성분으로써 오염되고, 벌크 독소가 인간에게 투여하기 위한 약제학적 조성물에 조합됨에 따라 바이러스, 프리온 또는 다른 원하지 않는 성분이 이후 활성 약제학적 성분인 클로스트리디움 독소에 동반할 수 있는 가능성을 낮추는 (이미 매우 낮음) 잠재적인 장점을 가진다.

[0021] 예를 들어 컬럼 크로마토그래피와 같은 크로마토그래피가 단백질, 핵산, 세포 파편 등의 혼합물로부터 특정 단백질(예컨대 보툴리눔 신경독소)을 분리하기 위하여 분획화(fractionation) 또는 정제로 공지인 공정에서 사용될 수 있다. 단백질 혼합물은 전형적으로, 예를 들어 흔히 다공성인 고체 매질(흔히 비드 또는 수지로 지칭됨)을 포함하는 유리 또는 플라스틱 컬럼을 통과한다. 상이한 단백질들 및 다른 화합물들은, 이들의 특이적인 화학적 특징 및 이러한 특징들이 이용되는 특정 크로마토그래피 매질과 상호작용하도록 야기하는 방식에 따라, 상이한 속도로 매트릭스를 통과한다.

[0022] 매질의 선택은 단백질의 분획화가 기초하는 화학적 특징의 유형을 결정한다. 네 가지 기본적 유형의 컬럼 크로마토그래피가 있다; 이온-교환, 겔 여과, 친화성 및 소수성 상호작용. 이온-교환 크로마토그래피는 양전하 또는 음전하를 보유하는 소형 비드로 패킹(packing)된 컬럼을 사용하여 표면 정전기적 전하에 기초한 분획화를 수행한다. 겔 여과 크로마토그래피에서, 단백질은 크기에 기초하여 분획화된다. 친화성 크로마토그래피에서, 단백질은 컬럼 매트릭스의 비드에 부착된 특이적 화학 그룹(리간드)에 결합하는 능력에 기초하여 분리된다. 리간드는 표적 단백질에 대하여 생물학적으로 특이적일 수 있다. 소수성 상호작용 크로마토그래피는 표면 소수성에 기초하여 분획화를 수행한다.

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[0023] 클로스트리디움 독소를 정제(분획화)하기 위한 컬럼 크로마토그래피가 공지이다. 예를 들어 다음 간행물을 참조하라:

[0024] 1. Ozutsumi K., et al, *Rapid, simplified method for production and purification of tetanus toxin*, App & Environ Micro, Apr. 1985, p 939-943, vol 49, no. 4.(1985)는 파상풍 독소를 정제하기 위한 고압 액체 크로마토그래피(HPLC) 겔 여과의 사용을 개시한다.

[0025] 2. Schmidt J.J., et al., *Purification of type E botulinum neurotoxin by high-performance ion exchange chromatography,* Anal Biochem 1986 Jul;156(1):213-219는 보툴리눔 독소 E형을 정제하기 위한 크기 배제 크로마토그래피 또는 이온 교환 크로마토그래피의 사용을 개시한다. 리보뉴클리에이즈(RNase) 대신 황산프로타민의 사용이 또한 개시된다.

[0026] 3. Simpson L.L., et al., *Isolation and characterization of the botulinum neurotoxins Simpson LL; Schmidt JJ; Middlebrook JL,* In: Harsman S, ed. Method in Enzymology. Vol. 165, Microbial Toxins: Tools in Enzymology San Diego, CA: Academic Press;vol 165:pages 76-85 (1988)은 중력 흐름 크로마토그래피를 사용한 보툴리눔 신경독소의 정제, HPLC, 친화성 수지를 사용한 포획 단계, 크기 배제 크로마토그래피, 및 두 가지의 상이한 이온 교환 컬럼의 사용을 포함하는 이온 (음이온 및 양이온) 교환 크로마토그래피를 개시한다. 다양한 Sephadex, Sephacel, Trisacryl, S 및 Q 컬럼이 개시된다.

[0027] 4. Zhou L., et al., *Expression and purification of the light chain of botulinum neurotoxin A: A single mutation abolishes its cleavage of SNAP-25 and neurotoxicity after reconstitution with the heavy chain,* Biochemistry 1995;34(46):15175-81 (1995)는 보툴리눔 신경독소 경사슬 융합 단백질을 정제하기 위한 아밀로오스 친화성 컬럼의 사용을 개시한다.

[0028] 5. Kannan K., et al., *Methods development for the biochemical assessment of Neurobloc (botulinum toxin type B),* Mov Disord 2000;15(Suppl 2):20 (2000)는 보툴리눔 독소 B 형을 검사하기 위한 크기 배제 크로마토그래피의 사용을 개시한다.

[0029] 6. Wang Y-c, *The preparation and quality of botulinum toxin type A for injection (BTXA) and its clinical use,* Dermatol Las Faci Cosm Surg 2002;58 (2002)는 보툴리눔 독소 A 형을 정제하기 위한 이온 교환 크로마토그래피를 개시한다. 이 참조문헌은 침전과 크로마토그래피 기법의 조합을 개시한다.

[0030] 7. Johnson S.K., et al., *Scale-up of the fermentation and purification of the recombination heavy chain fragment C of botulinum neurotoxin serotype F, expressed in Pichia pastoris* , Protein Expr and Purif 2003;32:1-9 (2003)은 보툴리눔 독소 F 형의 재조합 중사슬 단편을 정제하기 위한 이온 교환 및 소수성 상호작용 컬럼의 사용을 개시한다.

[0031] 8. 공개된 미국 특허 출원 제2003 0008367 A1호(Oguma)는 보툴리눔 독소를 정제하기 위한 이온 교환 및 락토오스 컬럼의 사용을 개시한다.

[0032] 상기 요약된 정제 방법은 전체 900 kDa 보툴리눔 독소 복합체의 정제와는 대조적인, 복합체의 보툴리눔 독소의 신경독성 성분(즉, 대략 150 kDa 신경독성 분자), 또는 신경독성 성분의 특정 성분의 소규모 정제에 관한 것이다.

[0033] 더욱이, 보툴리눔 독소 약제학적 조성물에 조합하기에 적절한 보툴리눔 독소를 수득하기 위한 상업적 규모 공정을 포함하는 현존 공정은, 발효 공정의 보툴리눔 독소에 동반하는 불순물로부터 독소 복합체를 분리하기 위한 일련의 침전 단계를 전형적으로 포함한다. 특히, 침전 기법은 보툴리눔 독소를 정제하기 위하여 생물약제학적 산업에서 널리 사용된다. 예를 들어, 냉각 알코올 분획화 (Cohn 방법) 또는 침전이 혈장 단백질 제거에 사용된다. 불행하게도, 보툴리눔 독소를 정제하기 위한 이전의 침전 기법은 낮은 분해능, 낮은 생산성, 조업의 어려움, 제어 및/또는 허가의 어려움 및/또는 규모 확대 또는 규모 축소의 어려움의 단점을 가진다. 이전에 공개된 공개일 2006년 10월 12일의 미국 특허 출원번호 제11/452,570호는 다양한 동물-단백질이 없는 및 NAPF 공정에서 이용되는 원심분리, 산 침전, 에탄올 침전, 산성화 단계, 및 황산암모늄 침전과 같은 단계를 개시한다 (상세한 논의에 대하여, 미국 특허 공개공보 제2006/0228780호 참조, 전체가 본 명세서에 참조로 포함됨). 보툴리눔 신경독소를 수득하기 위한 동물 단백질이 없는 공정과 비-동물 단백질이 없는 공정 사이의 몇 가지 차이점이 상기 문헌에 나타난다.

[0034] 그러므로 연구 목적을 위하여 및/또는 약제학적 조성물을 제조하기 위하여 사용될 수 있는 고순도의 매우 강

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력한 보툴리눔 신경독소를 수득하기 위한, 신속하고 상대적으로 더 작은 규모이지만 고수율인 시스템 및 공정이 요구된다.

발명의 내용

[0035] 요약

[0036] 본 발명은 이러한 요구를 충족시키며, 신속하고, 더 작은 규모이고, 상업적으로 유용하고, 고수율이며, 동물 단백질이 없는 크로마토그래피 시스템 및 공정에 의하여 수득 가능한 고순도의 매우 강력한 보툴리눔 신경독소를 제공한다. 생성된 보툴리눔 신경독소는 약제학적 조성물 제조에 유용하다. 본 발명의 실시에 의하여 수득된 클로스트리디움 독소는 바람직하게는 보툴리눔 신경독소이고 가장 바람직하게는 약 900 kDa의 보툴리눔 신경독소 A 형 복합체 또는 이의 150 kDa 신경독성 성분이다. 본 발명은 DNase 및 RNase와 같은 NAPF 시약을 필요로 하지 않는다.

[0037] 정의

[0038] 본 명세서에서 사용된 다음 단어 및 용어는 다음 정의를 가진다.

[0039] "약"은 한정된 항목, 파라미터 또는 용어가 명시된 항목, 파라미터 또는 용어의 값 위 및 아래의 플러스 또는 마이너스 십 퍼센트 범위를 포괄함을 의미한다.

[0040] "투여" 또는 "투여하다"는 약제학적 조성물 또는 활성 성분을 대상에게 제공(즉 투여)하는 단계를 의미한다. 본 명세서에 개시된 약제학적 조성물은 예를 들어 근육내 (i.m.), 진피내, 피하 투여, 경막내 투여, 두개내, 복강내 (i.p.) 투여, 국소 (경피) 및 이식 (즉 고분자 임플란트 또는 소형삼투압 펌프와 같은 서방 장치의 이식) 투여 경로에 의하여 "국지적으로 투여된다".

[0041] "동물 산물이 없는" 또는 "실질적으로 동물 산물이 없는"은 각각 "동물 단백질이 없는" 또는 "실질적으로 동물 단백질이 없는"을 포괄하고, 혈액 유래, 혈액 풀드(blood pooled) 및 다른 동물 유래 산물 또는 화합물의 부재 또는 실질적인 부재를 의미한다. "동물"은 포유류 (예컨대 인간), 조류, 파충류, 어류, 곤충, 거미 또는 다른 동물 종을 의미한다. "동물"은 박테리아와 같은 미생물을 배제한다. 따라서, 본 발명의 범위 내의APF 배지 또는 공정 또는 실질적으로 APF 배지 또는 공정은 보툴리눔 독소 또는 *클로스트리디움 보툴리눔* 박테리아를 포함할 수 있다. 예를 들어, APF 공정 또는 실질적으로 APF 공정은 면역글로불린, 고기 소화물(digest), 고기 부산물 및 젖 또는 유제품 또는 소화물과 같은 동물-유래 단백질이 실질적으로 없거나 본질적으로 없거나 완전히 없는 공정을 의미한다.

[0042] "보툴리눔 독소" 또는 "보툴리눔 신경독소는 *클로스트리디움 보툴리눔*에 의하여 생성된 신경독소 뿐만 아니라 변형, 재조합, 하이브리드 및 키메라 보툴리눔 독소를 의미한다. 재조합 보툴리눔 독소는 비-클로스트리디움 종에 의하여 재조합으로 제조된 경사슬 및/또는 중사슬을 가질 수 있다. 본 명세서에서 사용된 "보툴리눔 독소"는 보툴리눔 독소 혈청형 A, B, C, D, E, F 및 G를 포괄한다. 본 명세서에서 사용된 "보툴리눔 독소"는 또한 보툴리눔 독소 복합체(즉 300, 600 및 900 kDa 복합체) 뿐만 아니라 순수 보툴리눔 독소(즉 약 150 kDa 신경독성 분자) 두 가지 모두를 포괄하고, 이들 모두 본 발명의 실시에서 유용하다. "정제된 보툴리눔 독소"는 보툴리눔 독소가 배양 또는 발효 공정으로부터 수득될 때 보툴리눔 독소에 동반할 수 있는 다른 단백질 및 불순물로부터 분리되거나 실질적으로 분리된 순수 보툴리눔 독소 또는 보툴리눔 독소 복합체를 의미한다. 따라서, 정제된 보툴리눔 독소는 최소한 90%, 바람직하게는 95% 이상, 가장 바람직하게는 99% 이상의 비-보툴리눔 독소 단백질을 가질 수 있고 불순물이 제거된다. 신경독소가 아닌 보툴리눔 C_2 및 C_3 세포독소는 본 발명의 범위로부터 배제된다.

[0043] "클로스트리디움 신경독소"는 *클로스트리디움 보툴리눔, 클로스트리디움 부티리쿰 또는 클로스트리디움 베라티*와 같은 클로스트리디움 박테리아 원산이거나 이로부터 생성된 신경 독소 뿐만 아니라 비-클로스트리디움 종에 의하여 재조합으로 제조된 클로스트리디움 신경독소를 의미한다.

[0044] "완전히 없는"(용어 "구성되는")은 사용되는 장비 또는 공정의 검출 범위 내에서, 물질이 검출될 수 없거나 물질의 존재가 확인될 수 없음을 의미한다.

[0045] "본질적으로 없는"(또는 "본질적으로 구성되는"')은 단지 미량의 물질만이 검출될 수 있음을 의미한다.

[0046] "변형된 보툴리눔 독소"는 천연 보툴리눔 독소와 비교하여 아미노산 중 최소한 하나가 결손되거나, 변형되거나, 치환된 보툴리눔 독소를 의미한다. 또한, 변형된 보툴리눔 독소는 재조합으로 생성된 신경독소, 또는 재

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조합으로 제조된 신경독소의 유도체 또는 단편일 수 있다. 변형된 보툴리눔 독소는 보툴리눔 독소 수용체에 결합하는 능력, 또는 뉴런으로부터 방출된 신경전달물질을 억제하는 능력과 같은 천연 보툴리눔 독소의 생물학적 활성 중 최소한 한 가지를 보유한다. 변형된 보툴리눔 독소의 한 예는 한 보툴리눔 독소 혈청형(예컨대 혈청형 A)의 경사슬, 및 다른 상이한 보툴리눔 독소 혈청형(예컨대 혈청형 B)의 중사슬을 가지는 보툴리눔 독소이다. 변형된 보툴리눔 독소의 또 다른 예는 물질 P(substance P)와 같은 신경전달물질에 결합된 보툴리눔 독소이다.

[0047] "약제학적 조성물"은 활성 성분이 보툴리눔 독소일 수 있는 제제를 의미한다. 단어 "제제(formulation)"는 약제학적 조성물에 보툴리눔 신경독소 활성 성분 이외에 최소한 한 가지의 추가적인 성분(예컨대, 알부민 [예컨대 인간 혈청 알부민 또는 재조합 인간 알부민] 및/또는 염화나트륨, 예를 든 것이고 이에 제한되지 않음)이 존재함을 의미한다. 그러므로 약제학적 조성물은 인간 환자와 같은 대상에게 진단, 치료 또는 미용을 위하여 (예를 들어 근육내 또는 피하 주사에 의하여 또는 데포(depot) 또는 임플란트의 삽입에 의하여) 투여하기 적절한 제제이다. 약제학적 조성물은 예를 들어 동결건조되거나 진공건조된 상태, 동결건조되거나 진공건조된 약제학적 조성물을 식염수 또는 물로써 복원한 후 형성된 용액 또는; 복원을 필요로 하지 않는 용액일 수 있다. 활성 성분은 보툴리눔 독소 혈청형 A, B, C_1, D, E, F 또는 G 중 하나 또는 클로스트리디움 박테리아에 의하여 천연으로 제조될 수 있는 모든 보툴리눔 독소일 수 있다. 언급된 바와 같이, 약제학적 조성물은 액체 또는 고체일 수 있고, 예를 들어 진공-건조될 수 있다. 보툴리눔 독소 활성 성분 약제학적 조성물을 제제화하기 위한 대표적인 방법은 2002년 11월 5일에 공개된 미국 특허 공개공보 제20030118598호에 개시되고, 이는 본 명세서에 전체가 참조로 포함된다.

[0048] "실질적으로 없는"은 물질(예컨대 동물 산물, 동물 단백질 또는 동물 유래 산물 또는 단백질)의 중량 퍼센트가 평가되는 배양 배지, 발효 배지, 약제학적 조성물 또는 다른 원료의 1 중량 퍼센트 미만의 수준으로 존재함을 의미한다.

[0049] "치료 제제"는 제제가 장애 또는 질환 및/또는 이에 관련된 증상, 예컨대 말초 근육 또는 샘(예를 들어 땀샘)의 과활동(예를 들어 강직)을 특징으로 하는 장애 또는 질환을 치료하고 이에 의하여 경감시키기 위하여 사용될 수 있음을 의미한다.

[0050] "치료적 유효량"은 현저한 부정적 또는 역의 부작용을 야기하지 않고 질환, 장애 또는 용태를 치료하기 위하여 필요한 (예를 들어 보툴리눔 독소 또는 보툴리눔 독소 포함 약제학적 조성물과 같은) 약제의 수준, 양 또는 농도를 의미한다.

[0051] "치료하다", "치료하는", 또는 "치료"는 예컨대 상처입거나 손상된 조직의 회복에 의하여, 또는 현존하거나 인지된 질환, 장애 또는 용태의 변경, 변화, 향상, 개선, 호전 및/또는 미화에 의하여, 원하는 치료적 또는 미용적 결과를 성취하도록 하는, 둔부 변형과 같은 질환, 장애 또는 용태의 경감 또는 감소(이는 약간의 감소, 상당한 감소, 거의 완전한 감소, 및 완전한 감소를 포함함), 소산 또는 예방(일시적 또는 영구적)을 의미한다. 보툴리눔 신경독소의 투여의 경감 효과와 같은 치료 효과가 예를 들어 환자에게 보툴리눔 신경독소를 투여한 후 1 내지 7 일 사이에 임상적으로 나타나지 않을 수 있고, 예를 들어 치료되는 용태 또는 특정 사례에 따라 약 1 개월 내지 약 1 년 또는 그 사이의 임의의 시간 범위의 유효 기간을 가질 수 있다.

[0052] 퍼센티지는 달리 명시되지 않으면 부피당 중량 기준이다.

[0053] APF는 동물 산물/단백질이 없는(animal product/protein free)을 의미하고

[0054] CV는 컬럼 부피(column volume)를 의미하고

[0055] DF는 정용여과(diafiltration)를 의미하고

[0056] ELISA는 효소-결합 면역흡수 검사(enzyme-linked immunosorbent assay)를 의미한다.

[0057] "IAPF 시스템" 또는 "IAPF 공정"에서와 같은 IAPF는 "개선된 동물 단백질이 없는(improved animal protein free)" 시스템 또는 공정을 의미한다. IAPF 시스템 또는 공정은 본 명세서에 구체적으로 기재된 바와 같이 보툴리눔 독소 또는 신경독소 성분을 정제하기 위한 두 가지의 크로마토그래피 매질 또는 세 가지의 크로마토그래피 매질의 사용을 포함한다. 크로마토그래피 매질은 당해 분야에 공지된 크로마토그래피 수지를 포함한다. 두 가지의 크로마토그래피 매질의 사용에 의하여 수득된 보툴리눔 신경독소 배치(batch)는 본 명세서에서 IAPF로 명시된다.

[0058] "FAPF 시스템" 또는 "FAPF 공정"에서와 같은 FAPF는 "더욱 개선된 동물 단백질이 없는(further improved

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animal protein free)" 시스템 또는 공정을 의미한다. 따라서, FAPF는 IAPF 공정이며, FAPF 시스템 또는 공정은 보툴리눔 독소 또는 신경독소 성분을 정제하기 위하여 세 가지의 크로마토그래피 매질이 사용됨을 의미한다. 세 가지의 크로마토그래피 매질의 사용에 의하여 수득된 보툴리눔 신경독소의 배치는 본 명세서에서 FAPF로 명시된다.

[0059] NAPF는 비-동물 단백질이 없는(non-animal protein free)을 의미하고

[0060] SDS-PAGE는 소듐 도데실설페이트 폴리아크릴아미드 겔 전기영동(sodium dodecylsulfate polyacrylamide gel electrophoresis)을 의미하고

[0061] SEC-HPLC는 크기 배제 고성능 액체 크로마토그래피(size exclusion high performance liquid chromatography)를 의미하고

[0062] UF는 한외여과(ultrafiltration)를 의미한다.

[0063] 본 발명의 한 구체예에서, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 APF 크로마토그래피 공정이 제공되고, 상기 공정은 다음의 (a) 실질적으로 APF 발효 배지를 제공하는 단계; (b) 발효 배지에서 *클로스트리디움 보툴리눔* 박테리아를 발효시키는 단계, 및; (c) 발효 배지를 음이온 교환 크로마토그래피 매질과 접촉시키고 이어서 음이온 교환 크로마토그래피 매질에서 나온 용리액을 양이온 교환 크로마토그래피 매질과 접촉시켜 발효 배지로부터 생물학적으로 활성인 보툴리눔 신경독소를 회수하는 단계를 포함하고, 이에 의하여 실질적으로 APF 크로마토그래피 공정으로부터 생물학적으로 활성인 보툴리눔 신경독소를 수득한다. 특정 구체예에서, 공정은 수득된 보툴리눔 신경독소의 각 밀리그램에 대하여 1 나노그램 이하의 잔류 핵산인, 1 백만분율 (ppm) 미만의 잔류 핵산을 포함하는 보툴리눔 신경독소를 제공할 수 있다. 또 다른 양태에서, 공정은 1 주일 이내에 수행된다.

[0064] 한 예에서, 3:1:1의 비율을 가지는 배지는 3% HySoy, 1% HyYeast, 및 1% 글루코오스를 포함하는 보툴리눔 독소 배양/발효 배지를 의미한다. HySoy(Quest 제품번호 5X59022)는 대두의 효소적 가수분해에 의하여 제조된 펩타이드 공급원이다. HyYeast(HyYest, Quest 제품번호 5Z10102 또는 5Z10313)는 제빵 효모 추출물이다. 또 다른 예에서, 5:1:1의 비율을 가지는 배지는 5% HySoy, 1% HyYeast, 및 1% 글루코오스를 포함하는 보툴리눔 독소 배양/발효 배지를 의미한다.

[0065] 또 다른 구체예가 생물학적으로 활성인 보툴리눔 신경독소 A 형 복합체를 수득하기 위한 실질적으로 APF 크로마토그래피 공정을 제공하고, 상기 공정은 다음의 순차적인 실질적으로 APF 배양 배지에서 *클로스트리디움 보툴리눔* 박테리아를 배양하는 단계; 약 2 L 내지 약 75 L의 실질적으로 APF 발효 배지에서, 더욱 바람직하게는 약 2 L 내지 약 50 L의 실질적으로 APF 발효 배지에서, 더욱더 바람직하게는 약 2 L 내지 약 30 L의 실질적으로 APF 발효 배지에서 배양 배지로부터의 *클로스트리디움 보툴리눔* 박테리아를 발효시키는 단계 (특정 구체예가 식물 단백질 및/또는 식물 단백질 유도체, 예를 들어 가수분해된 식물 단백질을 포함하는 배양 배지 및 발효 배지 중 최소한 하나를 가짐); 여과 또는 원심분리를 이용하여 발효 배지에 존재하는 세포 파편을 제거하여 발효 배지를 수확하는 단계; 예를 들어 한외여과(UF)와 같은 여과에 의하여 수확 발효 배지를 농축하는 단계; 완충제를 첨가하여 농축된 발효 배지를 희석하는 단계를 포함한다. 완충제로 희석한 후, 생물학적으로 활성인 보툴리눔 신경독소가 음이온 교환 매질에 의하여 포획되도록, 희석된 수확 발효 배지가 음이온 교환 매질과 접촉되는 제1 접촉 단계가 착수되고; 이어서 포획된 보툴리눔 신경독소를 음이온 교환 매질로부터 용리하여 이에 의하여 보툴리눔 독소를 포함하는 제1 용리액을 수득하고; 제1 용리액으로부터 불순물을 제거하기 위하여, 제1 용리액이 양이온 교환 매질과 접촉되는 제2 접촉 단계를 수행하고, 이에 의하여 보툴리눔 독소를 포함하는 제2 용리액을 수득하고; 이어서 정용여과(DF)에 의하여 제2 용리액을 가공하고; 가공된 제2 용리액을 여과하여, 이에 의하여 실질적으로 APF 크로마토그래피 공정을 이용하여 생물학적으로 활성인 보툴리눔 신경독소 A 형 복합체를 수득한다. 수득된 보툴리눔 신경독소 A 형 복합체는 약 2.0×10^7 유닛/mg 내지 약 6.0×10^7 유닛/mg의 보툴리눔 신경독소 A 형 복합체 역가를 가질 수 있다. 특정 예에서, 예를 들어 약 2.4×10^7 유닛/mg 내지 약 5.9×10^7 유닛/mg의 역가를 가지는 보툴리눔 신경독소 A 형 복합체가 수득될 수 있다.

[0066] 특정 구체예에서, 상기 공정은 약 5% w/v 이하의 식물-유래 단백질 산물, 약 2% w/v 이하의 효모 추출물 및 약 2% 이하의 w/v 글루코오스를 포함하는 발효 배지를 이용하고, 여기서 발효 배지의 pH 수준은 발효 단계 시작 시 약 6.5 내지 약 pH 8.0, 더욱 바람직하게는 약 pH 6.8 내지 약 pH 7.6이다. 특정 구체예에서, 배양 단계는 약 540 나노미터(nm)에서 배양 배지의 광학 밀도가 약 0.8 흡광 단위 (AU) 내지 약 4.5 AU일 때까지 수행된다. 배양 단계는 바람직하게는 *클로스트리디움 보툴리눔* APF 제조용 세포 은행(working cell bank) 내용

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물을 배양 배지에 도입하여 개시되고, 여기서 제조용 세포 은행 내용물은 제조용 세포 은행 밀리리터당 최소한 약 1 x 10^4 콜로니형성수(colony-forming unit), 바람직하게는 약 1 x 10^4 내지 약 5 x 10^7 콜로니형성수의 *클로스트리디움 보툴리눔*을 포함하고, 제조용 세포 은행 중의 *클로스트리디움 보툴리눔* 박테리아는 실질적으로 균일한 형태를 가진다. 또 다른 구체예에서, 발효 단계는 약 890 nm에서 발효 배지의 광학 밀도가 약 0.05 AU 내지 약 0.7 AU로 감소될 때까지, 약 60 내지 80 시간 동안 수행된다. 한 양태에서, 실질적으로 APF 크로마토그래피 공정에 의하여 수득된 보툴리눔 신경독소는 1 ppm 미만의 잔류 핵산을 포함하고, 공정은 1 주일 이내에 수행된다.

[0067] 또 다른 구체예에서, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위하여 크로마토그래피를 이용하는 APF 공정이 제공되고, 상기 공정은 다음의 순차적인: (a) APF 제조용 세포 은행으로부터의 *클로스트리디움 보툴리눔* 박테리아를 APF 배양 배지에 첨가하는 단계; (b) 배양 배지에서 *클로스트리디움 보툴리눔* 박테리아를 배양하는 단계; (c) *클로스트리디움 보툴리눔* 세포 용해가 일어날 때까지 APF 발효 배지에서 단계 (b)로부터의 *클로스트리디움 보툴리눔* 박테리아를 발효시키는 단계; (d) 발효 배양물을 수확하여 수확 발효 배지를 제공하는 단계; (e) 수확 발효 배지가 여과에 의한 농축을 거치는 단계; (f) 완충제를 첨가하여 여과된 발효 배지를 희석하여 희석된 발효 배지를 수득하는 단계; (g) 희석된 발효 배지가 포획 크로마토그래피 매질과 접촉되는 제1 접촉 단계, 여기서 포획 크로마토그래피 매질은 음이온 교환 매질임; (h) 제1 접촉 단계의 용리액이 폴리싱(polishing) 크로마토그래피 매질과 접촉되는 제2 접촉 단계, 여기서 폴리싱 크로마토그래피 매질은 양이온 교환 매질임, 및 (i) 제2 접촉 단계의 용리액을 여과하는 단계를 포함하고, 이에 의하여 개선된 APF 공정에 의하여 생물학적으로 활성인 보툴리눔 신경독소를 수득하고, 여기서 수득된 보툴리눔 신경독소는 1 ppm의 잔류 핵산 또는 1 ppm 미만의 잔류 핵산을 포함하고, 공정은 1 주일 이내에 수행된다.

[0068] 한 양태에서, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 동물 산물이 없는(APF) 크로마토그래피 시스템이 제공되고, 이는 실질적으로 APF 발효 배지, 발효 배지에서 발효시키기 위한 *클로스트리디움 보툴리눔* 박테리아, 발효 배지로부터 생물학적으로 활성인 보툴리눔 신경독소를 회수하기 위한 음이온 교환 크로마토그래피 매질, 및 음이온 교환 크로마토그래피 매질에서 나온 용리액으로부터 생물학적으로 활성인 보툴리눔 신경독소를 추가로 회수하기 위한 양이온 교환 크로마토그래피 매질을 포함하고, 이에 의하여 실질적으로 APF 크로마토그래피 공정으로부터 생물학적으로 활성인 보툴리눔 신경독소를 수득한다. 특정 구성에서, 시스템은 실질적으로 APF 배양 배지에서 *클로스트리디움 보툴리눔* 박테리아를 혐기성으로 배양하기 위한 제1 장치를 추가로 포함할 수 있고, 실질적으로 APF 발효 배지에서 제1 장치로부터 수득된 *클로스트리디움 보툴리눔* 박테리아를 혐기성으로 발효시키기 위한 제2 장치로 추가로 이루어질 수 있다. 정화를 위하여, 시스템은 제2 장치로부터 수득된 발효 배지로부터 세포 파편을 제거하여, 이에 의하여 수확 발효 배지를 제공하는 수확 장치를 포함할 수 있다. 수확 발효 배지는 수확 발효 배지를 농축시킨 다음 희석시키는 농축 및 희석 장치를 통과할 수 있다. 특정 예에서, 시스템은 또한 양이온 교환 크로마토그래피 매질에서 나온 용리액으로부터 추가로 정제된 생물학적으로 활성인 보툴리눔 신경독소를 회수하기 위한 소수성 상호작용 매질을 포함할 수 있다. 추가적으로, 수득된 생물학적으로 활성인 보툴리눔 신경독소 중 생물부하(bioburden)를 감소시키기 위한 여과 장치가 또한 2 또는 3 크로마토그래피 매질을 이용하여 수득된 생물학적으로 활성인 보툴리눔 신경독소의 생물부하를 감소시키기 위한 시스템을 이룰 수 있다. 특정 예에서, 실질적으로 APF 배양 배지에서 *클로스트리디움 보툴리눔* 박테리아를 배양하기 위한, 작업 공간 내에 집적된 고효율 미립자 공기 필터를 가지는 혐기성 챔버가 사용될 수 있다. 대표적인 시스템은 최소한 약 2.0 x 10^7 유닛/mg의 보툴리눔 신경독소 역가를 가지는 보툴리눔 신경독소를 제공할 수 있고, 수득된 보툴리눔 신경독소는 수득된 보툴리눔 신경독소의 각 mg에 대하여 1 ng 또는 1 ng 미만의 잔류 핵산을 포함한다. 특정 구체예에서, 실질적으로 APF 발효 배지가 약 2 L 내지 약 75 L의 양으로 제공되고; 약 200 mL 내지 약 1 L의 실질적으로 APF 배양 배지가 이용된다.

[0069] 본 발명의 또 다른 양태에서, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위하여 크로마토그래피를 이용하는 실질적으로 APF 시스템이 제공되고, 시스템은 *클로스트리디움 보툴리눔* 박테리아를 배양하기 위한 제1 장치(제1 장치는 실질적으로 APF 배양 배지를 포함할 수 있음); 제1 장치에서 배양된 *클로스트리디움 보툴리눔* 박테리아를 발효시키기 위한 제2 장치(제2 장치는 실질적으로 APF 발효 배지를 포함할 수 있음); 발효 배지를 수확하기 위한 제3 장치; 수확 발효 배지를 농축하고 여과된 발효 배지를 희석하기 위한 제4 장치; 수확된 배지로부터 보툴리눔 신경독소의 제1 정제를 수행하여, 이에 의하여 제1 정제된 보툴리눔 신경독소를 수득하기 위한 제5 장치(여기서 제5 장치는 음이온 교환 크로마토그래피 매질을 포함함); 및 보툴리눔 신경독소의 제2 정제를 수행하여, 이에 의하여 제2 정제된 보툴리눔 신경독소를 수득하기 위한 제6 장치(여기서 제6 장치는 양이온 교환 크로마토그래피 매질을 포함함)을 포함하고, 여기서 수득된 보툴리눔 신경독소는 최소한

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약 2.0 x 10^7 유닛/mg의 보툴리눔 신경독소 내지 약 5.9 x 10^7 유닛/mg의 보툴리눔 신경독소 역가를 가지고, 수득된 보툴리눔 신경독소는 수득된 보툴리눔 신경독소 각 mg에 대하여 1 ng 또는 1 ng 미만의 잔류 핵산을 포함하고, 공정은 1 주일 이내에 수행된다. 특정 구체예에서, 수득된 보툴리눔 신경독소는 최소한 4.4 x 10^7 유닛/mg의 보툴리눔 신경독소 역가를 가질 수 있다. 시스템의 특정 구체예에서, 시스템은 제6 장치로부터 수득된 보툴리눔 신경독소의 추가의 정제를 수행하여, 이에 의하여 제3 정제된 보툴리눔 신경독소를 수득하기 위한 제7 장치를 추가로 포함할 수 있고, 여기서 제7 장치는 소수성 상호작용 매질을 포함한다. 추가적인 구체예에서, 시스템은 제7 장치에서 나온 용리액을 여과하기 위한 막을 포함하는 제8 장치를 추가로 포함할 수 있다.

[0070] 본 발명의 또 다른 양태는 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 APF 크로마토그래피 시스템을 포함하고, 상기 시스템은 *클로스트리디움 보툴리눔* 박테리아를 혐기성 배양하기 위한 제1 장치(제1 장치는 약 200 mL 내지 약 1 L의 실질적으로 APF 배양 배지를 포함할 수 있음); 제1 장치를 수용할 수 있는 챔버 내에 집적된 고효율 미립자 공기 필터를 가지는 혐기성 챔버를 포함하는 제2 장치; 제1 장치에서 배양된 *클로스트리디움 보툴리눔* 박테리아의 혐기성 발효를 위한 제3 장치(제3 장치는 약 2 L 내지 약 75 L의 실질적으로 APF 발효 배지, 바람직하게는 약 2 L 내지 약 30 L의 실질적으로 APF 발효 배지를 포함할 수 있고, 환원-산화 탐침, pH 탐침 및 탁도 탐침으로 이루어진 군에서 선택되는 최소한 하나의 일회용 탐침을 포함할 수 있음); 발효 배지를 수확하기 위한 제4 장치; 수확 발효 배지를 농축하고 여과된 발효 배지를 희석하기 위한 제5 장치; 수확 발효 배지로부터 수득된 보툴리눔 신경독소의 제1 정제를 수행하여, 이에 의하여 제1 정제된 보툴리눔 신경독소를 수득하기 위한 제6 장치(제6 장치는 음이온 교환 크로마토그래피 매질을 포함함); 보툴리눔 신경독소의 제2 정제를 수행하여, 이에 의하여 제2 정제된 보툴리눔 신경독소를 수득하기 위한 제7 장치(제7 장치는 양이온 교환 크로마토그래피 매질을 포함함); 제2 정제된 보툴리눔 신경독소의 제3 정제를 수행하여 제3 정제된 보툴리눔 신경독소를 수득하기 위한 제8 장치(제8 장치는 소수성 상호작용 매질을 포함함); 및 제3 정제된 보툴리눔 신경독소를 여과하기 위한 제9 장치(제9 장치는 여과막을 포함함)를 포함하고, 여기서 수득된 보툴리눔 신경독소는 약 2.4 x 10^7 유닛/mg의 보툴리눔 신경독소 내지 약 5.9 x 10^7 유닛/mg의 보툴리눔 신경독소 역가를 가지고, 수득된 보툴리눔 신경독소는 수득된 보툴리눔 신경독소의 각 mg에 대하여 1 ng 또는 1 ng 미만의 잔류 핵산을 포함하고, 공정은 1 주일 이내에 수행된다. 이러한 공정에 따르면, 이에 의하여 생물학적으로 활성인 보툴리눔 신경독소가 수득되고, 특정 예에서, 수득된 보툴리눔 신경독소는 최소한 약 4.4 x 10^7 유닛/mg의 보툴리눔 신경독소의 역가를 가진다.

[0071] 본 명세서에 개시된 공정 및 시스템에 따르면, 이에 의하여 생물학적으로 활성인 보툴리눔 신경독소가 수득된다. 특정 구체예에서, 본 명세서에 개시된 공정 및 시스템에 의하여 수득된 생물학적으로 활성인 보툴리눔 신경독소는 약 900 kDa의 분자량을 가진다.

[0072] 본 발명은 실질적으로 APF 약제학적 조성물을 제조하는 방법을 추가로 포함하며, 여기서 활성 성분은 생물학적으로 활성인 보툴리눔 신경독소이고, 상기 방법은 다음의: (a) (i) 실질적으로 동물 산물이 없는 발효 배지를 제공하고; (ii) 발효 배지에서 *클로스트리디움 보툴리눔* 박테리아를 발효시키고; (iii) 음이온 교환 크로마토그래피 매질을 사용하고 이어서 양이온 교환 크로마토그래피 매질을 사용하여 발효 배지로부터 생물학적으로 활성인 보툴리눔 신경독소를 회수하여, 생물학적으로 활성인 보툴리눔 신경독소를 수득하는 단계, 여기서 회수된 보툴리눔 신경독소는 최소한 약 2.0 x 10^7 유닛/mg의 보툴리눔 신경독소, 바람직하게는 약 2.4 x 10^7 유닛/mg의 보툴리눔 신경독소 내지 약 5.9 x 10^7 유닛/mg의 보툴리눔 신경독소, 일부 구체예에서 최소한 약 4.4 x 10^7 유닛/mg의 보툴리눔 신경독소 역가를 가지고, 보툴리눔 신경독소는 보툴리눔 신경독소의 각 mg에 대하여 1 ng 또는 1 ng 미만의 잔류 핵산을 포함하고, 단계 (i) 내지 (iii)는 1 주일 이내에 완료됨; (b) 보툴리눔 신경독소를 최소한 한 가지의 적절한 부형제와 조합하는 단계를 포함하고, 이에 의하여 실질적으로 APF 약제학적 조성물을 제조한다. 특정 구체예에서, 조합 단계는 냉동건조(freeze drying), 동결건조(lyophilization) 및 진공건조(vacuum drying)로 이루어진 공정의 군에서 선택된 공정에 의하여 보툴리눔 신경독소를 건조하는 단계를 포함하고, 여기서 적절한 부형제가 알부민, 인간 혈청 알부민, 재조합 인간 혈청 알부민, 젤라틴, 수크로오스, 트레할로오스, 하이드록시에틸 녹말, 콜라겐, 락토오스, 수크로오스 염화나트륨, 폴리사카라이드, 카프릴레이트, 폴리비닐피롤리돈 및 나트륨으로 이루어진 군에서 선택된다. 따라서, 한 양태에서 본 발명은 본 명세서에 개시된 공정 및 시스템에 의하여 수득된 생물학적으로 활성인 보툴리눔 신경독소를 조합하여 제조된 실질적으로 APF 약제학적 조성물을 또한 제공한다.

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[0073] 추가적으로, 본 발명은 환자의 용태를 치료하기 위한 방법을 또한 포함하고, 상기 방법은 활성 성분이 본 명세서에 개시된 APF 공정(즉 IAPF 및 FAPF 공정)에 의하여 수득된 생물학적으로 활성인 보툴리눔 신경독소인 실질적으로 APF 약제학적 조성물을 제조하기 위한 방법에 의하여 제조된 약제학적 조성물의 치료적 유효량을 환자에게 투여하는 단계를 포함한다. 치료될 용태의 예는 예를 들어 두통, 편두통, 긴장성 두통, 부비동 두통, 경추성 두통, 발한 장애, 겨드랑이 다한증, 손 다한증, 발 다한증, 프레이 증후군, 과운동성 피부 주름(hyperkinetic skin line), 안면 주름, 미간 주름, 눈가 주름, 입가 주름, 코입술 주름, 피부 장애, 이완불능증, 사시, 만성 치열, 안검경련, 근골격 통증, 섬유근통, 췌장염, 빈맥, 전립선 비대, 전립선염, 요폐, 요실금, 과민성 방광, 반측안면 경련, 떨림, 근경련, 위장관 장애, 당뇨병, 타액과다증, 배뇨근-조임근 협동장애, 뇌졸중 후 강직, 상처 회복, 소아 뇌성마비, 평활근 경련, 재협착, 국소 근긴장이상, 간질, 경부 근긴장이상증, 갑상선 장애, 고칼슘혈증, 강박 장애, 관절염 통증, 레이노 증후군, 튼살, 복막 유착, 혈관경련, 콧물, 근육 경축, 부상 근유, 후두 근긴장이상, 필기 경련 및 수근관 증후군으로 이루어진 군에서 선택된다.

[0074] 한 구체예에서, 환자의 용태를 치료하기 위한 방법에 있어서, 상기 방법은 다음 단계를 포함하는 방법에 의하여 제조된 유효량의 실질적으로 APF 약제학적 조성물을 환자에게 국지적으로 투여하는 단계를 포함한다: (a) (i) 동물 산물이 실질적으로 없는 발효 배지를 제공하고; (ii) 발효 배지에서 *클로스트리디움 보툴리눔* 박테리아를 발효시키고; (iii) 음이온 교환 크로마토그래피 매질을 사용하고 이어서 양이온 교환 크로마토그래피 매질을 사용하여 발효 배지로부터 생물학적으로 활성인 보툴리눔 신경독소를 회수하여, 생물학적으로 활성인 보툴리눔 신경독소를 수득하는 단계, 여기서 회수된 보툴리눔 신경독소는 최소한 약 2.0×10^7 유닛/mg의 보툴리눔 신경독소 역가를 가지고, 보툴리눔 신경독소는 보툴리눔 신경독소 각 mg에 대하여 1 ng 또는 1 ng 미만의 잔류 핵산을 포함하고, 단계 (i) 내지 (iii)는 1 주일 이내에 완료됨; (b) 보툴리눔 신경독소를 최소한 한 가지의 적절한 부형제와 조합하는 단계, 이에 의하여 실질적으로 APF 약제학적 조성물을 제조하고, 상기 방법에 의하여 실질적으로 APF 약제학적 조성물의 국지 투여가 용태를 치료한다.

[0075] 본 명세서에 개시된 IAPF 공정/시스템/방법에 의하여 제공된 생물학적으로 활성인 보툴리눔 신경독소를 포함하는 치료적 유효량의 약제학적 조성물의 국지 투여가, 예를 들어 약 2 개월 내지 약 6 개월의 간격 또는 약 2 개월 내지 약 3 개월의 간격으로 반복될 수 있다. 본 개시에 따라 제조된 치료적 유효량의 실질적으로 APF 약제학적 조성물의 환자에게 국지적으로 투여되는 대표적인 유용한 투약량은 약 0.01 유닛 내지 약 10,000 유닛의 보툴리눔 신경독소 유닛 양을 가질 수 있다. 특정 예에서, 보툴리눔 신경독소가 약 0.01 유닛 내지 약 3000 유닛의 양으로 투여된다. 특정 예에서, 약제학적 조성물 중의 활성 약제학적 성분인 생물학적으로 활성인 보툴리눔 신경독소는 예를 들어 보툴리눔 신경독소 A 형 또는 B 형이다.

[0076] 본 발명은 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위하여 크로마토그래피를 이용하는 실질적으로 APF 공정을 포함한다. 공정은 실질적으로 APF 발효 배지를 제공하는 단계, 이어서 발효 배지에서 *클로스트리디움 보툴리눔* 박테리아를 발효시키는 단계 및 음이온 교환 크로마토그래피 매질의 사용에 이어서 양이온 교환 크로마토그래피 매질을 사용하여 발효 배지로부터 생물학적으로 활성인 보툴리눔 신경독소를 회수하는 단계의 순차적인 단계를 포함할 수 있고, 이에 의하여 실질적으로 APF 크로마토그래피 공정으로부터 생물학적으로 활성인 보툴리눔 신경독소를 수득한다. 회수 단계는 또한 양이온 교환 크로마토그래피 매질의 사용 후 소수성 상호작용 매질의 사용을 포함할 수 있다. 수득된 생물학적으로 활성인 보툴리눔 신경독소는 보툴리눔 신경독소 복합체 또는 이로부터 분리된 약 150 kDa의 분자량을 가지고 보툴리눔 독소 복합체의 조합 단백질이 없는 보툴리눔 독소 신경독성 성분일 수 있다. APF 공정(2-컬럼 사용 (IAPF), 예를 들어 음이온 크로마토그래피에 이어 양이온 크로마토그래피; 또는 3-컬럼 사용 (FAPF), 예를 들어 음이온 크로마토그래피에 이어 양이온 크로마토그래피에 이어 소수성 상호작용 크로마토그래피)이 보툴리눔 신경독소 A, B, C_1, D, E, F 및 G 형과 같은 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위하여 이용될 수 있다. 수득된 보툴리눔 신경독소는 바람직하게는 보툴리눔 신경독소 A 형 복합체이다.

[0077] 본 발명의 한 양태에서, 사용된 발효 배지의 양은 약 2 L 내지 약 75 L의 실질적으로 APF 발효 배지, 바람직하게는 약 2 L 내지 약 30 L의 실질적으로 APF 발효 배지를 포함할 수 있다. 예로서, 약 100 mg 내지 약 5 그램, 바람직하게는 약 100 mg 내지 약 3 그램, 더욱 바람직하게는 약 100 mg 내지 약 1 그램의 생물학적으로 활성인 보툴리눔 신경독소가 공정으로부터 수득된다. 예로서, 사용된 발효 배지 리터당 약 20 mg 내지 약 100 mg 또는 약 20 mg 내지 약 80 mg의 생물학적으로 활성인 신경독소가 수득될 수 있다. 발효 배지는 예를 들어 식물 유래 단백질 산물, 효모 추출물 및 글루코오스를 포함할 수 있다. 예로서, 발효 배지는 약 5% w/v 이하의 식물 유래 단백질 산물을 포함한다. 또 다른 예에서, 발효 배지는 약 2% w/v 이하의 효모 추출물을 포함한다. 또 다른 구체예에서, 발효 배지는 약 2% w/v 이하의 글루코오스를 포함한다. 특정 예에서, 발효 배지는

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약 5% w/v 이하의 식물-유래 단백질 산물, 약 2% w/v 이하의 효모 추출물 및 약 2% w/v 이하의 글루코오스를 포함하고, 식물-유래 단백질 산물, 효모 추출물 및 글루코오스는 언급된 w/v 퍼센티지 양에 따른 임의의 비율이다. 일부 구체예에서, 발효 단계는 약 60 시간 내지 약 80 시간 동안 진행된다.

[0078] 한 구체예에서, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위하여 크로마토그래피를 이용하는 실질적으로 APF 공정이 제공되고, 다음의 순차적인 실질적으로 APF 배양 배지에서 *클로스트리디움 보툴리눔* 박테리아를 배양하는 단계, 이후 약 2 L 내지 약 75 L의 실질적으로 APF 발효 배지에서, 더욱 바람직하게는 약 2 L 내지 약 30 L의 실질적으로 APF 발효 배지에서 배양 배지로부터의 *클로스트리디움 보툴리눔* 박테리아를 발효시키는 단계, (여기서 실질적으로 APF 배양 배지 및 실질적으로 APF 발효 배지 중 최소한 하나는 식물 단백질을 포함함), 이어서 발효 배지에 존재하는 세포 파편을 제거하여 발효 배지를 수확하는 단계 및 여과에 의하여 수확 발효 배지를 농축하고 완충제를 첨가하여 농축된 발효 배지를 희석하는 단계를 포함한다. 일단 완충되면, 제1 접촉 단계가 실행되고, 여기서 생물학적으로 활성인 보툴리눔 신경독소가 이온 교환 매질과 결합되도록, 희석된 수확 발효 배지가 음이온 교환 매질과 접촉되고, 이후 음이온 교환 매질로부터의 포획된 보툴리눔 신경독소 용리가 진행되어, 이에 의하여 제1 용리액을 수득하고, 이어서 제2 접촉 단계가 실행되고, 여기서 제1 용리액으로부터 불순물을 제거하기 위하여 제1 용리액이 양이온 교환 매질과 접촉되고, 이에 의하여 제2 용리액을 수득하고; 이후 제2 용리액은 예컨대 UF 및/또는 DF에 의하여 가공되고; 이후 가공된 제2 용리액을 여과하고, 이에 의하여 크로마토그래피를 이용하는 실질적으로 APF 공정을 이용하여 생물학적으로 활성인 보툴리눔 신경독소를 수득한다.

[0079] 예로서, 박테리아를 배양하는 것으로부터 생물학적으로 활성인 보툴리눔 신경독소를 수득하기까지의 공정을 완료하기 위한 시간은 예를 들어 약 50 시간 내지 약 150 시간, 더욱 바람직하게는 약 80 시간 내지 약 120 시간일 수 있다. 특정 구체예에서, 배양 배지는 약 4% w/v 이하의 식물-유래 단백질 산물을 포함하고, 또 다른 예에서, 배양 배지는 약 2% w/v 이하의 효모 추출물을 포함하고, 또 다른 예에서 배양 배지는 약 2% w/v 이하의 글루코오스를 포함한다. 배양 배지는 언급된 w/v 퍼센티지 양에 따른 임의의 비율로 식물-유래 단백질 산물, 효모 추출물 및 글루코오스를 포함할 수 있다. 특정 예에서, 배양 배지의 pH 수준은 배양 단계의 시작 시 약 pH 6.5 내지 약 pH 8.0, 바람직하게는 약 pH 6.8 내지 약 pH 7.6, 더욱 바람직하게는 7.3일 수 있다. 배양 단계는 혐기성 챔버에서 약 8 시간 내지 약 14 시간, 약 10 시간 내지 12 시간, 바람직하게는 약 11 시간 동안, 약 33 ℃ 내지 약 37 ℃, 바람직하게는 약 34.5 ℃의 온도에서 수행될 수 있다. 특정 예에서, 혐기성 챔버는 작업 공간 내에 집적된 고효율 미립자 필터를 포함할 수 있고, 여기서 배양이 수행된다. 발효 단계는 약 60 시간 내지 약 80 시간, 바람직하게는 약 72 시간 동안 약 33 ℃ 내지 약 37 ℃, 바람직하게는 35 ℃의 온도에서 수행될 수 있다. 본 발명의 한 양태에 따르면, 수확 단계는 발효 배지에 포함된 최소한 약 80%의 RNA 및 DNA를 제거할 수 있고, 음이온 교환 매질은 수확 발효 배지 중의 모든 측정 가능한 잔여 DNA 및 RNA (검출 한계 아래)를 제거할 수 있다. 또 다른 양태에서, 수확 단계는 약 1 시간 내지 약 3 시간, 바람직하게는 약 2.5 시간 동안 수행될 수 있다. 특정 예에서, 수확 단계는 원래 발효 배지 부피의75%가 수집될 때까지 수행될 수 있다. 구체예의 한 양태에서, 농축 단계는 약 30 분 내지 약 2 시간, 바람직하게는 약 0.75 시간 동안 수행될 수 있다. 또 다른 양태에서, 희석 단계는 수확 발효 배지를 수확 단계의 시작 시 발효 배지의 초기 중량까지 희석한다. 제1 접촉 단계는 예를 들어 약 4 시간 내지 약 5 시간 동안 수행될 수 있다. 한 예에서, 음이온 교환 수지로부터의 제1 용리액은 280 nm에서의 분광광도계 눈금이 정점으로부터 약 150 mAU로 감소할 때까지, 약 150 mAU 이상의 분광광도계 눈금에서 수집된다. 제2 접촉 단계는 1 시간 내지 약 3 시간, 바람직하게는 약 2 시간 동안 수행될 수 있다. 이 제2 용리액은 분광광도계 눈금이 정점으로부터 예를 들어 약 100 mAU로 감소할 때까지, 약 100 mAU 이상의 분광광도계 눈금에서 양이온 교환 수지로부터 수집될 수 있다. 농축 및 정용여과에 의한 이 제2 용리액 가공 단계는 약 1 시간 내지 약 2 시간, 바람직하게는 약 1.5 시간 동안 수행될 수 있다. 특정 구체예에서, 여과 단계는 제2 용리액을 생물부하 감소 필터를 통과시키는 것에 의한 생물부하 감소를 포함한다. 생물부하 감소 필터는 약 0.1 ㎛ 내지 약 0.3 ㎛, 바람직하게는 0.2 ㎛의 공극 크기를 가질 수 있다. 특정 구체예에서, 공정은 제2 용리액으로부터 불순물을 추가로 제거하기 위하여 제2 용리액을 소수성 상호작용 매질에 접촉시키는 것에 의한 제2 접촉 단계 후의 제3 접촉 단계를 추가로 포함할 수 있고, 이에 의하여 제3 용리액을 수득한다. 이러한 제3 접촉 단계는 약 1 시간 내지 3 시간, 바람직하게는 약 2 시간 동안 수행될 수 있다. 제3 용리액은 분광광도계 눈금이 정점으로부터 예를 들어 약 50 mAU로 감소할 때까지, 약 50 mAU 이상의 분광광도계 눈금에서 소수성 상호작용 매질로부터 수집될 수 있다. 제3 접촉 단계가 존재하는 경우, 농축 및 정용여과에 의한 가공 단계가 제3 용리액에 적용되고 약 2 시간 내지 약 4 시간 동안 수행된다. 따라서 (이용된 2 또는 3-컬럼 공정으로부터) 농축되고 정용여과된 용리액을 생물부하 감소 필터에 통과시켜 생물부하 감소가 수행될 수 있다. 특정 구체예에서, 공정은 수득된 생물학적으로 활성인 보

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툴리눔 신경독소를 냉동하는 단계를 추가로 포함한다.

[0080] 특정 구체예에서, 실질적으로 APF 배양 배지는 약 100 mL 내지 약 500 mL의 부피를 포함한다. 특정 배양 단계가 약 100 ㎕ 내지 약 500 ㎕의 *클로스트리디움 보툴리눔*-포함 APF 제조용 세포 은행 배지를 실질적으로 APF 배양 배지에 도입하여 개시된다. 이후 배양 단계가 혐기성 챔버에서 예를 들어 최소한 약 8 시간, 바람직하게는 약 11 시간 동안, 약 34.5 ℃ ± 1 ℃의 온도에서 일어날 수 있다. 한 예에서, 제조용 세포 은행 배지는 최소한 약 1×10^4 콜로니형성수/mL의 제조용 세포 은행 배지, 예를 들어 약 1×10^5 내지 약 5×10^7 콜로니형성수/mL의 제조용 세포 은행 배지의 생존 세포 계수 검사치를 가질 수 있고, 제조용 세포 은행 중의 *클로스트리디움 보툴리눔* 박테리아는 실질적으로 균일한 형태를 가지도록 선택될 수 있다.

[0081] 한 구체예에서, 제조용 세포 은행 배지는 예를 들어 약 20부피% 글리세롤, 예컨대 멸균 글리세롤을 포함한다. 제조용 세포 은행 배지는 (a) 약 540 nm의 파장에서 측정된 배지 분취물의 광학 밀도가 약 2.5 ± 1.0 AU가 될 때까지, 혐기성 챔버에서 *클로스트리디움 보툴리눔* 박테리아를 약 2% w/v 대두 펩톤, 약 1% w/v 효모 추출물, 및 약 1% w/v 글루코오스를 포함하는 APF 배지에서 약 34.5 ℃ ± 1 ℃의 온도에서 성장시키고; 글리세롤을 첨가하여 약 20%의 배지 중 글리세롤 농도를 달성하여 제조될 수 있고, 이에 의하여 제조용 세포 은행을 수득한다. 제조용 세포 은행의 보관 형태는 예를 들어 약 -135 ℃ 아래에서 제조용 세포 은행을 냉동하여 제조될 수 있다. 본 개시에 따른 대표적인 공정에서 사용하기 위한 제조용 세포 은행의 보관 형태는 주위 온도에서 해동되어 배양 단계를 개시하기 위하여 사용될 수 있다.

[0082] 배양 단계는 약 8 시간 내지 약 14 시간, 바람직하게는 약 11 시간 동안 약 33 ℃ 내지 약 37 ℃, 바람직하게는 약 34.5 ℃의 온도에서, 혐기성 챔버에서, 예를 들어 바람직하게는 작업 공간 내에 집적된 고효율 미립자 공기 (high efficiency particulate air, HEPA) 필터를 가지는 혐기성 챔버/캐비닛에서 수행될 수 있다. 발효 단계는 약 20 시간 내지 약 80 시간, 바람직하게는 약 60 시간 내지 약 80 시간, 더욱 바람직하게는 약 72 시간 동안 약 33 ℃ 내지 약 37 ℃, 바람직하게는 35 ℃의 온도에서 수행될 수 있다. 공정은, 예를 들어 배양 단계 전에, 혐기성 챔버의 대기에 배지를 노출시켜 실질적으로 APF 배양 배지의 산화적 환원(oxidative reduction)을 허용하는 단계를 추가로 포함할 수 있다. 공정은 발효 단계 전에, 혐기성 챔버의 대기에 역시 발효 배지를 노출시켜 실질적으로 APF 발효 배지의 산화적 환원을 허용하는 단계를 또한 포함할 수 있다. 한 예로서, 실질적으로 APF 배양 배지의 산화적 환원을 허용하는 단계는 혐기성 챔버에서 약 10 시간 내지 약 14 시간 동안 수행될 수 있다. 유사하게, 발효기에서 실질적으로 APF 발효 배지의 산화적 환원을 허용하는 단계는 발효 단계의 시작 전에 약 10 시간 내지 약 14 시간 동안 수행될 수 있다.

[0083] 한 구체예에서, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 크로마토그래피를 포함하는 APF 공정이 개시되고, 다음의 순차적인, APF 제조용 세포 은행으로부터의 *클로스트리디움 보툴리눔* 박테리아를 APF 배양 배지에 첨가하는 단계; 배양 배지에서 *클로스트리디움 보툴리눔* 박테리아를 배양하는 단계; *클로스트리디움 보툴리눔* 세포 용해가 일어날 때까지 APF 발효 배지에서 배양 단계로부터의 *클로스트리디움 보툴리눔* 박테리아를 발효시키는 단계; APF 발효 배양물을 수확하여 수확 발효 배지를 제공하는 단계; 수확 발효 배지가 여과에 의한 농축을 거치는 단계; 완충제를 첨가하여 여과된 발효 배지를 희석하여 희석된 발효 배지를 수득하는 단계; 희석된 발효 배지가 포획 크로마토그래피 매질과 접촉되는 제1 접촉 단계, 여기서 포획 크로마토그래피 매질은 음이온 교환 매질임; 제1 접촉 단계으로부터의 용리액이 폴리싱 크로마토그래피 매질과 접촉되는 제2 접촉 단계, 여기서 폴리싱 크로마토그래피 매질은 양이온 교환 매질임, 및 제2 접촉 단계로부터의 용리액을 여과하는 단계를 포함하고, 이에 의하여 개선된 APF 공정에 의하여 생물학적으로 활성인 보툴리눔 신경독소를 수득한다. 특정 구체예에서, 공정은 제2 접촉 단계 후 및 여과 단계 전에, 제2 접촉 단계에서 나온 용리액을 소수성 상호작용 매질과 접촉시켜 제3 접촉 단계를 수행하는 단계를 추가로 포함할 수 있다. *클로스트리디움 보툴리눔* 용해 단계는 예를 들어 발효 단계의 시작 후 약 35 시간 내지 약 70 시간에 일어날 수 있다. 발효 배지는 예를 들어 약 2 L 내지 약 75 L, 약 2 L 내지 약 30 L, 또는 약 2 L 내지 20 L의 발효 배지의 부피를 가질 수 있다. 이 공정 전체는 약 50 시간 내지 약 150 시간, 더욱 바람직하게는 약 80 시간 내지 약 120 시간 동안 수행될 수 있다. 이 공정에 의하여 이렇게 수득된 생물학적으로 활성인 보툴리눔 신경독소는 예를 들어 약 2.4×10^7 내지 약 5.9×10^7 유닛/mg의 생물학적으로 활성인 보툴리눔 신경독소 역가를 가질 수 있다.

[0084] 한 양태에 따르면, 발효 종결 시, 발효 배지 리터당 약 40 mg 내지 약 85 mg의 보툴리눔 신경독소가 수득될 수 있다. 다양한 가공(여과/크로마토그래피/여과 런) 단계 다음에, 발효 배지 리터당 약 30 mg 내지 약 60 mg의 보툴리눔 신경독소; 발효 배지 리터당 약 5 mg 내지 약 25 mg의 보툴리눔 신경독소; 발효 배지 리터당 약

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6 mg 내지 약 20 mg의 보툴리눔 신경독소가 수득될 수 있다.

[0085] 한 구체예로서, 발효 배지의 pH는 발효 단계 시작 시 약 pH 6.0 내지 약 pH 8, 바람직하게는 약 pH 6.8 내지 약 pH 7.6, 더욱 바람직하게는 약 pH 7.3으로 조정될 수 있다. 또 다른 예로서, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 APF 크로마토그래피 공정이 또한 제공되고, 상기 공정은 보툴리눔 신경독소를 포함하는 실질적으로 APF 발효 배지를 수득하는 단계; 배지를 음이온 교환 크로마토그래피 수지와 접촉시켜 보툴리눔 신경독소를 포함하는 정제된 용리액을 제공하는 단계; 용리액을 양이온 교환 크로마토그래피 수지와 접촉시켜 이에 의하여 추가로 정제된 용리액을 수득하는 단계, 및 정제된 용리액을 추가로 여과하는 단계를 포함하고, 이에 의하여 실질적으로 APF 크로마토그래피 공정으로부터 정제된 생물학적으로 활성인 보툴리눔 신경독소를 수득한다. 특정 구성에서, 약 600 mL 내지 약 800 mL의 음이온 교환 크로마토그래피 수지를 포함하는 음이온 크로마토그래피 컬럼이 사용될 수 있다. 음이온 크로마토그래피 컬럼은 예를 들어 약 8 cm 내지 약 10 cm의 지름 및 약 9 cm 내지 약 16 cm의 컬럼 내 음이온 교환 크로마토그래피 수지층 높이를 가질 수 있다. 음이온 교환 크로마토그래피 수지를 통한 발효 배지의 유속은 예를 들어 약 140 cm/시간 내지 약 250 cm/시간, 또는 약 150 cm/시간 내지 약 160 cm/시간일 수 있다. 또 다른 양태에서, 크로마토그래피 컬럼 중의 약 150 mL 내지 약 300 mL의 양이온 교환 크로마토그래피 수지가 공정에서 이용될 수 있고, 여기서 양이온 크로마토그래피 컬럼은 예를 들어 약 5 cm 내지 약 8 cm의 지름 및 약 5 cm 내지 약 11 cm의 양이온 교환 크로마토그래피 수지층 높이를 가진다. 공정은 정용여과 단계 및/또는 생물부하 감소 단계 중 최소한 하나를 포함할 수 있다. 생물부하 감소 단계는 캡슐 필터를 이용할 수 있다. 정제된 용리액의 정용여과는 바람직하게는 생물부하 감소 단계 전에 수행된다. 한 예에서, 추가로 정제된 용리액의 정용여과 단계는 정용여과된 추가로 정제된 용리액의 농도 조정, 및 농도-조정된 정용여과된 더욱-정제된 용리액의 생물부하 감소 필터 통과에 선행하거나 후행한다. 공정은 마우스 LD50 생물검사에 의하여 결정된 최소한 약 2.0 $x10^7$ 유닛/mg의 보툴리눔 독소, 예컨대 약 2.4 x 10^7 내지 약 6.0 x 10^7 유닛/mg의 보툴리눔 신경독소 역가를 가지는 수득된 보툴리눔 신경독소를 제공할 수 있다. 예를 들어, 발효 배지 리터당 약 4 mg 내지 약 25 mg의 보툴리눔 독소의 예시적인 공정 종결 시 회수물이 회수될 수 있다.

[0086] 또 다른 구체예에서, 생물학적으로 활성인 보툴리눔 신경독소를 정제하기 위한 본질적으로 APF 공정은 보툴리눔 신경독소를 포함하는 약 2 L 내지 약 30 L APF 발효 배지를 수득하는 단계; APF 발효 배지 단계를 수확하여 수확된 APF 발효 배지를 제공하는 단계; 수확된 APF 발효 배지에 대하여 음이온 교환 크로마토그래피를 수행하여, 이에 의하여 제1 용리액을 제공하는 단계; 음이온 교환 크로마토그래피에서 나온 용리액을 양이온 교환 크로마토그래피 매질과 접촉시켜 양이온 교환 크로마토그래피를 수행하고, 이에 의하여 제2 용리액을 제공하는 단계; 및 양이온 교환 크로마토그래피 매질에서 나온 제2 용리액을 여과하여, 이에 의하여 정제된 보툴리눔 신경독소를 수득하는 단계를 포함할 수 있고, 여기서 수득된 정제된 보툴리눔 신경독소는 약 2.4 x 10^7 내지 약 5.9 x 10^7 유닛/mg의 생물학적으로 활성인 보툴리눔 신경독소 역가를 가지고, 사용된 APF 발효 배지 리터당 약 4 mg 내지 약 25 mg의 양으로 수득될 수 있다.

[0087] 본 발명은 또한 활성 성분이 생물학적으로 활성인 보툴리눔 신경독소인 실질적으로 APF 약제학적 조성물을 제조하는 조합 방법을 포함하고, 상기 방법은 (i) 실질적으로 동물 산물이 없는 발효 배지를 제공하고; (ii) 발효 배지에서 *클로스트리디움 보툴리눔* 박테리아를 발효시키고, (iii) 음이온 교환 크로마토그래피 매질을 사용하고 이어서 양이온 교환 크로마토그래피 매질을 사용하여 발효 배지로부터 생물학적으로 활성인 보툴리눔 신경독소를 회수하여 생물학적으로 활성인 보툴리눔 신경독소를 수득하는 단계; 이후 보툴리눔 신경독소를 최소한 한 가지의 적절한 부형제와 조합하는 단계를 포함하고, 이에 의하여 실질적으로 APF 약제학적 조성물을 제조한다. 한 예에서, 상기 방법은 냉동건조 또는 동결건조 또는 진공건조에 의하여 조합된 보툴리눔 신경독소 및 최소한 한 가지의 적절한 부형제를 건조하여 출하 또는 보관에 안정한 형태를 수득하는 단계를 포함하고, 여기서 활성 성분은 생물학적으로 활성인 보툴리눔 신경독소이고, 발효 배지는 식물로부터 수득된 단백질 산물을 포함한다. 단백질 산물을 수득할 수 있는 식물은 대두, 옥수수 또는 맥아, 쓴 맛이 제거된 (debittered) *루피누스 캄페스트리스*의 종자, 또는 이들의 가수분해된 산물일 수 있다. 수득된 보툴리눔 신경독소는 약 2.0 x 10^7 유닛/mg의 보툴리눔 신경독소 내지 약 6.0 x 10^7 유닛/mg의 보툴리눔 신경독소 역가를 가질 수 있다. 보툴리눔 신경독소는 보툴리눔 신경독소 A, B, C_1, D, E, F 및 G 형으로 이루어진 군에서 선택되고, 바람직하게는 보툴리눔 신경독소 A 형이다. 특정 예에서 보툴리눔 신경독소는 약 150 kDa의 분자량을 가지고 보툴리눔 독소 복합체의 복합 단백질이 없는 보툴리눔 독소 신경독성 성분으로서 수득된다. 특정 구체예에서, 적절한 부형제는 알부민, 인간 혈청 알부민, 재조합 인간 혈청 알부민, 젤라틴, 수크로오스, 트레할로

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오스, 하이드록시에틸 녹말, 콜라겐, 락토오스, 수크로오스, 아미노산, 염화나트륨, 염화칼륨, 폴리사카라이드, 카프릴레이트, 폴리비닐피롤리돈 및 시트르산칼륨으로 이루어진 군에서 선택된다. 생물학적으로 활성인 보툴리눔 신경독소 수득은 양이온 교환 매질의 사용 후 소수성 상호작용 매질을 사용하는 단계를 추가로 포함할 수 있다. 특정 예에서, 진공건조는 약 20 ℃ 내지 약 25 ℃의 온도에서 일어난다. 일부 구체예에서, 진공건조는 예를 들어 약 70 mmHg 내지 약 90 mmHg의 압력에서 일어난다. 진공건조를 위한 시간은 예를 들어 약 4 시간 내지 약 5 시간일 수 있다.

[0088] 본 개시의 특정 양태는 예를 들어 생물학적으로 활성인 보툴리눔 신경독소 복합체를 포함할 수 있는 약제학적 조성물을 제공하는 것에 관한 것이고, 부형제는 알부민, 인간 혈청 알부민, 재조합 인간 혈청 알부민, 젤라틴, 수크로오스, 트레할로오스, 하이드록시에틸 녹말, 콜라겐, 락토오스 및 수크로오스로 이루어진 군에서 선택되며, 여기서 약제학적 조성물에 본질적으로 핵산이 없다.

[0089] 특정 예에서, 수득된 보툴리눔 신경독소가 약 2.0×10^7 내지 약 6.0×10^7 유닛/mg의 생물학적으로 활성인 보툴리눔 신경독소 역가를 가지는 생물학적으로 활성인 보툴리눔 신경독소 및 최소한 한 가지의 부형제를 포함하는 약제학적 조성물이 제공되고, 조성물은 보툴리눔 신경독소 복합체 mg당 약 12 ppm 미만의 핵산, 바람직하게는 1 ppm 미만의 핵산을 포함한다.

[0090] 특정 구체예에서, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 APF 크로마토그래피 공정은 다음의 순차적인: 약 10 시간 내지 약 12 시간 동안 또는 배양 배지의 바이오매스 측정치가 약 540 나노미터(nm)의 파장에서 약 0.8 AU 내지 약 4.5 AU의 광학 밀도를 가질 때까지, 실질적으로 APF 배양 배지에서 *클로스트리디움 보툴리눔* 박테리아를 배양하는 단계; 약 65 시간 내지 약 75 시간 동안 또는 약 890 nm의 파장에서 온라인 바이오매스 탐침을 사용하여 발효 배지를 광학 밀도 측정하여 발효 종료 시 구한 바이오매스 측정치가 약 0.05 AU 내지 약 0.7 AU일 때까지, 배양 배지로부터의 *클로스트리디움 보툴리눔* 박테리아를 실질적으로 APF 발효 배지에서 발효시키는 단계; 발효 배지를 약 2.5 시간 동안 수확하는 단계, 이에 의하여 발효 배지의 세포 파편이 제거되고 발효 배지의 중량이 수확 단계의 시작 시 출발 중량의 약 4분의 3으로 감소됨; 수확 발효 배지를 접선 흐름 여과에 의하여 수확 단계의 시작 시 출발 부피의 약 4분의 1로 농축하는 단계; 완충제를 첨가하여 농축된 발효 배지를 희석하는 단계, 여기서 농축 및 희석 단계는 약 0.5 시간 내지 약 2 시간 동안 일어나고, 이에 의하여 농축 동안 발효 배지가 수확 단계 시작 시 출발 중량의 약 4분의 1로 감소되고, 이후 완충제를 첨가하여 희석되어, 수확 단계의 시작 시 원래의 출발 중량으로 되돌아감; 생물학적으로 활성인 보툴리눔 신경독소를 포획하기 위하여 희석된 발효 배지를 포획 크로마토그래피 매질과 약 4 시간 내지 약 5 시간 동안 접촉시키는 단계; 약 1.5 시간 내지 약 2.5 시간 동안 포획 크로마토그래피 매질에서 나온 용리액을 제1 폴리싱 크로마토그래피 매질과 접촉시켜 제1 폴리싱 런을 수행하여 불순물을 제거하는 단계; 약 1.5 시간 내지 약 2.5 시간 동안 폴리싱 크로마토그래피 매질에서 나온 용리액을 소수성 상호작용 매질에 통과시켜 제2 폴리싱 런을 수행하는 단계; 약 1 시간 내지 약 4 시간 동안 정용여과에 의하여 소수성 상호작용 매질에서 나온 용리액을 가공하는 단계; 및 약 0.5 시간 동안 가공된 용리액을 생물부하 감소 필터를 통하여 여과시키는 단계를 포함하고, 이에 의하여 생물학적으로 활성인 보툴리눔 신경독소를 수득한다.

[0091] 또 다른 양태에서, 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 APF 크로마토그래피 시스템이 개시되고, 상기 시스템은: *클로스트리디움 보툴리눔* 박테리아를 배양하기 위한 제1 장치(제1 장치는 실질적으로 APF 배양 배지를 포함할 수 있음); 제1 장치에서 배양된 *클로스트리디움 보툴리눔* 박테리아를 발효시키기 위한 제2 장치(제2 장치는 실질적으로 APF 발효 배지를 포함할 수 있음); 발효 배지를 수확하기 위한 제3 장치; 제3 장치로부터 수확된 배지 농축 및 희석을 수행하기 위한 제4 장치(제4 장치는 접선 흐름 여과 (tangential flow filtration, TFF)를 포함함); 농축 및 희석된 배지로부터의 보툴리눔 신경독소의 제1 정제를 수행하여, 이에 의하여 제1 정제된 보툴리눔 신경독소를 수득하기 위한 제5 장치(제5 장치는 음이온 교환 크로마토그래피 매질을 포함함); 및 제1 정제된 보툴리눔 신경독소의 제2 정제를 수행하여, 이에 의하여 제2 정제된 보툴리눔 신경독소를 수행하기 위한 제6 장치(제6 장치는 양이온 교환 크로마토그래피 매질을 포함함)를 포함한다.

[0092] 특정 구체예에서, 시스템은 제6 장치로부터 수득된 제2 정제된 보툴리눔 신경독소를 정제하여 추가의 정제를 수행하여, 이에 의하여 제3 정제된 보툴리눔 신경독소를 수득하기 위한 제7 장치를 추가로 포함할 수 있고, 여기서 제7 장치는 소수성 상호작용 매질을 포함한다. 시스템은 또한 제6 또는 제7 장치에서 나온 용리액을 여과하기 위한 여과막을 가지는 제8 장치로 추가로 이루어질 수 있다.

[0093] 또 다른 구체예에서, 예를 들어 약 8 cm 내지 약 15 cm의 지름을 가지는 크로마토그래피 컬럼은 음이온 교환

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크로마토그래피 매질을 포함하고, 음이온 교환 크로마토그래피 매질은 약 8 cm 내지 약 15 cm의 컬럼 내 층 높이를 가질 수 있다. 또 다른 예에서, 시스템의 제4 장치는 약 125 cm/시간 내지 약 200 cm/시간의 유속으로 작동되는 크로마토그래피 컬럼을 포함하고, 컬럼은 약 500 mL 내지 약 1 L의 컬럼 부피를 가질 수 있다. 한 양태에서, 예를 들어 제5 장치는 약 50 mL 내지 약 500 mL의 컬럼 부피, 약 8 cm 내지 약 15 cm의 층 높이를 가질 수 있다. 일부 예에서, 예를 들어 제5 장치의 크로마토그래피 컬럼은 약 2 cm 내지 약 10 cm의 컬럼 지름을 가진다. 제5 장치의 크로마토그래피 컬럼은 약 100 cm 내지 약 200 cm/시간의 예시적인 유속을 가질 수 있다. 시스템의 제7 장치는 여과막을 포함할 수 있다.

[0094] 또 다른 구체예에서, 시스템은 제9 장치를 추가로 포함할 수 있고, 제9 장치는 *클로스트리디움 보툴리눔* 박테리아를 배양하기 위한 제1 장치가 포함되는 혐기성 대기를 제공하기 위한 혐기성 챔버를 포함한다. 이러한 제9 장치는 바람직하게는 챔버/작업장소에 위치되는 집적된 고효율 미립자 공기 (HEPA) 필터를 포함한다. (발효를 위한) 시스템의 제2 장치는 산화-환원 전위 또는 pH 또는 광학 밀도를 검출하기 위한 최소한 하나의 탐침을 포함할 수 있다. 특정 예에서, 최소한 하나의 일회용 탐침이 환원-산화 탐침, pH 탐침 및 탁도 탐침으로 이루어진 군에서 선택된다. 시스템의 제8 장치는 농축 및 완충제 교환을 위하여 접선 흐름 여과 장치를 포함할 수 있다. 다른 구체예에서, 시스템은 생물부하 감소를 위한 생물부하 감소 장치를 포함하는 제10 장치를 포함할 수 있다. 한 예에서, 생물부하 감소 장치는 약 0.1 ㎛ 내지 0.3 ㎛, 바람직하게는 0.2 ㎛의 공극 크기를 가지는 필터를 포함한다. 시스템은 또한 제2 정제된 보툴리눔 신경독소를 수득한 후, 정제된 보툴리눔 신경독소 보관에 사용하기 위한 제11 장치를 포함할 수 있다. 한 예에서, 이러한 보관 장치는 약 -25 ℃ 내지 약 -80 ℃의 보관 온도를 제공한다.

[0095] 또 다른 양태에서, 생물학적으로 활성인 보툴리눔 독소가 다음 단계를 가지는 APF 공정에 의하여 제공된다: 실질적으로 APF 발효 배지를 제공하는 단계; 발효 배지에서 *클로스트리디움 보툴리눔* 박테리아를 발효시키는 단계; 음이온 교환 크로마토그래피 매질의 사용에 이어서 양이온 교환 크로마토그래피 매질을 사용하여 발효 배지로부터 생물학적으로 활성인 보툴리눔 신경독소를 회수하는 단계, 수득된 생물학적으로 활성인 보툴리눔 독소는 약 2.0×10^7 유닛/mg의 보툴리눔 신경독소 내지 약 6.0×10^7 유닛/mg의 보툴리눔 신경독소 역가를 가진다. 한 구체예에서, 공정은 소수성 상호작용 매질의 사용에 이어서 양이온 교환 매질을 사용하여 보툴리눔 신경독소를 추가로 정제하는 단계를 추가로 포함한다.

[0096] 또 다른 양태에 따르면, 본 명세서에 개시된 방법에 따라 수득된 보툴리눔 신경독소를 포함하는 약제학적 조성물을 이용하여 환자의 용태를 치료하는 방법이 제공된다.용태는 질환, 불쾌, 병, 또는 미용적 변형 또는 외관을 포함할 수 있다. 한 예에서, 환자의 용태를 치료하는 방법은 보툴리눔 신경독소 및 최소한 한 가지의 적절한 부형제를 포함하는 치료적 유효량의 약제학적 조성물을 환자에게 투여하는 단계를 포함하고, 이에 의하여 환자의 용태를 치료하고, 여기서 보툴리눔 독소는 약 1 유닛 = 약 .02 피코그램의 역가를 가진다.

[0097] 특정 예에서, 이러한 용태를 치료하기 위한 보툴리눔 신경독소는 실질적으로 APF 배양 배지에서 *클로스트리디움 보툴리눔* 박테리아를 배양하고; 보툴리눔 신경독소를 포함하는 실질적으로 APF 발효 배지를 수득하고; 배지를 음이온 교환 크로마토그래피 매질과 접촉시켜 보툴리눔 신경독소를 포함하는 정제된 용리액을 제공하고; 용리액을 양이온 교환 크로마토그래피 매질과 접촉시켜 이에 의하여 추가로 정제된 용리액을 수득하고, 추가로 정제된 용리액을 여과하는 공정에 의하여 수득될 수 있고, 이에 의하여 실질적으로 APF 크로마토그래피 공정으로부터 정제된 생물학적으로 활성인 보툴리눔 신경독소를 수득한다.

[0098] 한 구체예에서 생물학적으로 활성인 보툴리눔 신경독소를 수득하기 위한 실질적으로 APF 크로마토그래피 시스템이 포함되고, 상기 시스템은 *클로스트리디움 보툴리눔* 박테리아를 혐기성 배양하기 위한 제1 장치(제1 장치는 약 200 mL 내지 약 1 L의 실질적으로 APF 배양 배지를 포함할 수 있음); 제1 장치에서 배양된 *클로스트리디움 보툴리눔* 박테리아의 혐기성 발효를 위한 제2 장치(제2 장치는 약 5 L 내지 약 75 L, 또는 약 2 L 내지 약 75 L, 또는 약 2 L 내지 약 30 L의 실질적으로 APF 발효 배지를 포함할 수 있고 환원-산화 탐침, pH 탐침 및 탁도 탐침으로 이루어진 군에서 선택되는 최소한 하나의 일회용 탐침을 포함할 수 있음); 혐기성 대기를 제공하고 제1 장치를 수용할 수 있는 제9 장치(제9 장치는 챔버 내에 집적된 고효율 미립자 공기 필터를 가지는 혐기성 챔버를 포함하고, 상기 챔버는 *클로스트리디움 보툴리눔* 박테리아를 혐기성 배양하기 위한 제1 장치를 수용할 수 있음); 발효 배지를 수확하기 위한 제3 장치; 수확된 배지의 농축 및 희석을 수행하기 위한 제4 장치; 제4 장치로부터 수득된 보툴리눔 신경독소의 제1 정제를 수행하여, 이에 의하여 제1 정제된 보툴리눔 신경독소를 수득하기 위한 제5 장치(제5 장치는 음이온 교환 크로마토그래피 매질을 포함함); 제1 정제된 보툴리눔 신경독소의 제2 정제를 수행하여, 이에 의하여 제2 정제된 보툴리눔 신경독소를 수득하기 위한 제6 장치(제6 장치는 양이온 교환 크로마토그래피 매질을 포함함); 제2 정제된 보툴리눔 신경독소의 제3 정제를

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수행하여, 이에 의하여 제3 정제된 보툴리눔 신경독소를 수득하기 위한 제7 장치(제7 장치는 소수성 상호작용 매질을 포함함); 및 제3 정제된 보툴리눔 신경독소의 농축 및 완충제 교환을 위한 제8 장치(제8 장치는 TFF 막을 포함함)를 포함한다.

[0099] 특정 예에서, 예를 들어 발효 배지는 약 5% w/v 이하의 식물-유래 단백질 산물, 약 2% w/v 이하의 효모 추출물 및 약 2% w/v 이하의 글루코오스를 포함하고, 여기서 발효 배지의 pH 수준은 약 72 시간 발효 단계의 시작 시 약 pH 6.8 내지 약 7.6, 바람직하게는 약 pH 7.3이다. 한 구체예에서, 상기 방법은 추가로 정제된 용리액을 소수성 상호작용 매질과 접촉시켜 보툴리눔 신경독소를 포함하는 추가로 더욱 정제된 용리액을 수득하는 단계를 추가로 포함할 수 있다. 특정 예에서, 용태를 치료하는 방법은 약 150 kDa의 분자량을 가지고 보툴리눔 독소 복합체의 복합 단백질이 없는 보툴리눔 독소 신경독성 성분으로서 수득되는 보툴리눔 신경독소 사용에 의할 수 있다. 예시적인 투여 단계는 근육내, 진피내, 피하, 선내, 경막내, 직장, 경구 및 경피 투여로 이루어진 투여 경로의 군에서 선택될 수 있고, 보툴리눔 신경독소는 보툴리눔 독소 A, B, C_1, D, E, F 또는 G 형으로 이루어진 군에서 선택된다. 바람직하게는, 보툴리눔 신경독소는 보툴리눔 신경독소 A 형이다.

[0100] 일부 예에서, 시스템은 생물학적으로 활성인 보툴리눔 신경독소 복합체가 약제학적 조성물의 일부로서 사용하기 위하여 수득될 수 있는 공정을 용이하게 할 수 있고, 상기 약제학적 조성물은 보툴리눔 신경독소 복합체 mg당 약 12 ng 미만의 핵산, 바람직하게는 보툴리눔 신경독소 복합체 mg당 1 ng 아래의 핵산을 포함하고, 더욱 바람직하게는 측정 가능한 핵산을 가지지 않는다 (예를 들어 검출 한계 아래).

도면의 간단한 설명

[0101] 도면

도 1A는 실시예 1 NAPF 공정의 주요 단계를 나타내는 흐름도이다. 도 1B는 실시예 2 IAPF 공정의 주요 단계를 나타내는 흐름도이고, 여기서 포획 및 폴리싱 크로마토그래피 단계가 2-컬럼(음이온 교환에 이어 양이온 교환) 또는 3-컬럼(FAPF)(음이온 교환에 이어 양이온 교환에 이어 소수성 상호작용 컬럼)을 이용할 수 있다.

발명을 실시하기 위한 구체적인 내용

[0102] 상세한 설명

[0103] 본 발명은 고역가, 고순도의 생물학적으로 활성인 클로스트리디움 신경독소, 예컨대 보툴리눔 신경독소가 단순하고 빠르며 경제적인 APF 크로마토그래피 시스템 및 공정의 이용에 의하여 수득될 수 있다는 발견에 기초한다. 중요하게도, 본 시스템 및 공정의 이용은, 발효된 보툴리눔 신경독소를 정제하기 위하여 RNase 및 DNase와 같은 동물 유래의 효소가 사용되지 않음에도 불구하고, 수득된 정제된 보툴리눔 신경독소 1 mg당 1 ng (또는 1 ng 미만)의 핵산 (RNA 및 DNA) 불순물을 포함하는 정제된 보툴리눔 신경독소를 생성할 수 있다. 예를 들어, 본 시스템 및 공정의 이용은 수득된 정제된 보툴리눔 신경독소 밀리그램당 약 0.6 ng 미만의 핵산 (RNA 및 DNA) 불순물을 포함하는 정제된 보툴리눔 신경독소를 생성할 수 있다. 수득된 보툴리눔 신경독소는 보툴리눔 독소 A 형 복합체, 예컨대 300 kDa, 500 kDa 또는 900 kDa (대략의 분자량) 복합체 또는 이들의 혼합일 수 있다. 수득된 보툴리눔 신경독소는 또한 약 150 kDa의 분자량을 가지는 보툴리눔 독소 유형 신경독성 성분(즉 복합 단백질이 없음)일 수 있다. 보툴리눔 신경독소는 혈청형 A, B, C, D, E, F 또는 G 또는 이들의 혼합 중 임의의 하나일 수 있다. 추가적으로, 개선된 시스템 및 공정이 재조합, 하이브리드, 키메라 또는 변형된 보툴리눔 독소(경사슬, 중사슬, 또는 두 사슬 함께)로써 실시될 수 있다.

[0104] 본 발명의 중요한 양태는 *클로스트리디움 보툴리눔* 박테리아가 발효된 APF 발효 배지로부터 보툴리눔 신경독소를 정제하기 위하여 음이온 교환 (포획) 매질 크로마토그래피의 이용에 이어서 양이온 교환 (폴리싱) 매질 크로마토그래피를 이용하는 것이다. 본 발명자들은 음이온 교환의 사용에 이어 양이온 교환 크로마토그래피 매질을 사용하는 것이 고순도, 고수율의 보툴리눔 신경독소를 수득하기 위한 효과적이고 신속한 방법을 제공함을 발견했다. 종전에는, 음이온 교환 크로마토그래피의 사용은 보툴리눔 신경독소의 겔 밴딩 패턴에 유해한 영향을 미치는 것으로 생각되었고, 이에 따라 보툴리눔 신경독소 정제를 위한 음이온 교환 크로마토그래피 사용이 단념되었다. 예를 들어 미국 특허 제7,452,697호의 컬럼 55, 라인 53-57을 참조하라.

[0105] 본 발명의 또 다른 중요한 양태는 앞에서 제시한 바와 같이 고순도 보툴리눔 신경독소(즉 ≤ 1 ng 핵산/mg 수득된 보툴리눔 신경독소)를 생성하는 것이다. 본 발명의 또 다른 중요한 양태는 공지의 Schantz 공정이 보툴리눔 신경독소를 배양, 발효 및 정제하기 위하여 수주일(즉 전형적으로 약 18 내지 약 22 일)를 필요로 하는 것과 대조적으로, 본 발명의 범위 내의 시스템 및 공정은 모든 배양, 발효 및 정제 단계가 1 주일 이내에 완

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료되도록 하는 것이다. 본 발명의 바람직한 구체예에서 모든 배양, 발효 및 정제 단계는 6일 이내에 완료될 수 있다. 본 발명의 더욱 바람직한 구체예에서 모든 배양, 발효 및 정제 단계는 약 4일 이내(예를 들어 약 80 내지 약 144 시간 이내 또는 그 사이의 시간/범위 이내)에 완료될 수 있다. 본 발명자들은 8 또는 9 단계 공정(및 공정을 수행하기 위한 시스템)을 개발하고 특정 구체예에서 8 또는 9 단계 각각이 아래에 제시된 시간 이내에 완료될 수 있음을 발견하여 이러한 본 발명의 신속하고 더욱 바람직한 구체예를 발명했다:

[0106] 배양을 위하여 약 8 시간 내지 약 14 시간;

[0107] 발효를 위하여 약 60 시간 내지 약 80 시간;

[0108] 수확을 위하여 약 2.5 시간;

[0109] 농축 및 희석을 위하여 약 2 시간 내지 약 4 시간;

[0110] 음이온 교환 크로마토그래피를 위하여 약 4 시간 내지 약 6 시간 (이는 포획된 보툴리눔 독소 용리를 위한 시간을 포함한다)

[0111] 양이온 교환 크로마토그래피를 위하여 약 2 시간;

[0112] 선택적인 제3 크로마토그래피 단계(즉 소수성 상호작용 크로마토그래피)를 위하여 약 2 시간;

[0113] 농축 및 정용여과를 위하여 약 2 시간 내지 약 4 시간, 및;

[0114] 추가의 여과를 위하여 약 1/2 시간. 따라서, 본 발명의 8 또는 9 단계의 신속하고 더욱 바람직한 구체예를 완료하기 위하여 필요한 총 시간은 약 75 시간 내지 약 150 시간이다.

[0115] 본 발명은 더욱 효율적이고 시간 절약적이다. 한 양태에서, 본 발명의 새로운 공정은 사전-선택되고 검증된 세포주를 사용하고, 따라서 (세포 배양 및 발효 단계 전에) 배양한 다음 발효 배지에 접종하기 위하여 필요한, 세포 도말 및 성장, 콜로니 선택 및 수확, 및 수확된 콜로니의 단계적 세포주 확대의 종래기술 Schantz 공정 단계를 폐기한다. 한 양태에서, 본 발명은 APF 배양 배지의 접종을 위한 사전-선택된 세포 배양 없이 바로 시작하고, 따라서 시간 및 공정 단계를 단축한다.

[0116] 실험을 통하여 본 발명자들은 발효 배지에 존재하는 보툴리눔 신경독소를 정제하기 위한 2크로마토그래피 컬럼 ("IAPF") 및 3 컬럼 ("FAPF" / "FIAPF") 크로마토그래피 시스템 및 공정을 개발했고, 발효 배지는 *클로스트리디움 보툴리눔* 박테리아의 APF 발효로부터 유래했다. 중요하게도, APF 발효 공정이 클로스트리디움 박테리아를 배양하고 발효시키기 위하여 사용된 영양물로서 동물 유래 산물(예컨대 카제인 및 고기 육즙)을 배지로부터 줄이거나 제거할 수 있기는 하지만, 공지된 APF 발효 공정에는 전형적으로 동물 유래 산물, 예컨대 효소 DNase 및 RNase를 사용하는 하나 이상의 정제 단계가 이어진다. 본 발명의 APF 발효 배지에 존재하는 보툴리눔 신경독소를 정제하기 위한 시스템 및 공정은 동물 유래의 효소를 사용하지 않는다.

[0117] 본 발명은 수확 발효 배지(예를 들어 여과에 의하여 투명해짐)를 Applied Biosystems의 POROS® 50HQ 음이온 교환 크로마토그래피 수지와 같은 음이온 교환 컬럼에 로딩(loading)하는 것을 포함할 수 있다. 한 양태에서, 폴리스타이렌/디비닐벤젠의 베이스 매트릭스 및 약 50 ㎛의 입자 지름 및 약 60-70의 동적 능력(BSA mg/ml)을 가지는 강한 음이온 교환 매질이 사용될 수 있다. 음이온 교환 컬럼은 클로스트리디움 신경독소(예컨대 보툴리눔 독소 복합체)를 포획하고 불순물 수준을 감소시킨다. 음이온 교환 컬럼이 발효 배지에 보툴리눔 독소와 함께 존재하는 많은 불순물을 또한 분리하면서 보툴리눔 독소 복합체의 생물학적 활성을 보존하며 수확 발효 배지로부터의 보툴리눔 독소 복합체의 효과적인 포획을 제공함이 발견되었다. 음이온 교환 컬럼으로부터 포획된 (결합된) 클로스트리디움 신경독소를 용리시키기 위하여 적절한 완충제가 사용된다.

[0118] 본 발명의 2-컬럼 구체예에서, 음이온 교환 컬럼에서 나온 용리액(보툴리눔 신경독소 포함)이 양이온 교환 컬럼에 로딩되어 불순물로부터 보툴리눔 신경독소를 추가로 정제한다. 양이온 교환 컬럼은 Applied Biosystems의 POROS® 20HS 양이온 교환 수지일 수 있다. 한 양태에서, 폴리스타이렌/디비닐벤젠의 베이스 매트릭스 및 약 20 ㎛의 입자 크기 및 약 75 초과(>75)의 동적 결합 능력(라이소자임 mg/ml)을 가지는 강한 양이온 교환 매질이 사용될 수 있다. 본 발명의 3-컬럼 구체예(FAPF)에서, 양이온 교환 컬럼에서 나온 용리액이 GE Healthcare의 Phenyl Sepharose HP 수지와 같은 소수성 상호작용 컬럼에 로딩되어 보툴리눔 신경독소를 추가로 정제한다. 한 양태에서, 페닐기로써 유도체화되고 약 45의 동적 결합 능력(키모트립시노겐 mg/ml)을 가지는, 고도로 가교된 약 34 ㎛의 입자 크기를 가지는 아가로오스 비드의 매트릭스가 사용될 수 있다.

[0119] 2컬럼 또는 3 컬럼 공정 후, 최종 사용된 컬럼에서 나온 용리액이 더욱 가공되어 고도로 정제된 벌크 보툴리

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늄 독소 복합체를 수득할 수 있다. 크로마토그래피 후 가공 단계는 한외여과 및 정용여과에 의한 농축 및 완충제 교환, 멸균 여과, 현탁액(종래기술) 대신 정제된 보툴리눔 독소 복합체의 용액 제조를 포함할 수 있고, 용액은 바람직하게는 시트르산칼륨에 섞인 용액이고, 한 예에서 약 6.5의 pH에서 10mM 시트르산칼륨의 농도이다.

[0120] 특정한 바람직한 구체예에서, *클로스트리디움 보툴리눔*의 성장(혐기성 배양 및 혐기성 발효)을 위한 배지 및 보툴리눔 독소의 생성은 사용된 매질에 실질적으로 또는 완전히 동물-유래 산물이 없도록 동물 유래 산물을 대체하는 대두 기초 산물을 포함할 수 있다. 배양 단계는 추후의 발효를 위하여 미생물의 양을 증가시킨다. 배양은 이전에 냉동된 휴면 중인 박테리아를 활발하게 성장하는 배양물로 회복시킨다. 또한, 발효 배지에 접종하기 위하여 사용된 생존 가능한 미생물의 부피 및 양은, 보관된 비-증식 *클로스트리디움 보툴리눔* 세포 은행보다는 활발하게 성장하는 배양물로부터 더욱 정확하게 제어될 수 있다. 따라서, APF 배지에서 제조용 세포 은행의 샘플이 해동되고 선택된 APF 배양 배지에 넣어진다. 적절한 박테리아 성장 수준을 달성하면, 배양 배지가 발효 배지에 접종시키기 위하여 사용된다. 한 예로서, 성장기의 *클로스트리디움 보툴리눔*을 가지는 약 1% 내지 약 5%, 또는 그 사이의 양의 배양 배지가 발효 배지에 접종시키기 위하여 사용된다. 발효는 대규모 혐기성 환경에서 최대량의 미생물 세포 생산을 위하여 수행된다 (Ljungdahl et al., *Manual of industrial microbiology and biotechnology* (1986), edited by Demain et al, American Society for Microbiology, Washington, D.C. page. 84). 대안으로, 발효 배지에서의 *클로스트리디움 보툴리눔* 성장이 제조용 세포 은행의 샘플을 발효 배지에 직접 첨가하여 진행될 수 있다.

[0121] 종래 기술에서, 배양 배지에서 *클로스트리디움 보툴리눔*의 성장은 전형적으로, 세포 도말, 세포 콜로니 성장, 선택 및 성장의 제1 단계에 이어서 배양 배지의 접종 (전형적으로 두 단계 증대(step-up) 배양) 및 발효 배지의 접종 및 보툴리눔 독소 생성의 제2 단계의 두 단계로 진행한다. 바람직하게는, 어떤 단계의 배양 배지 중의 성장물도 배양 배지 중의 최종 성장물로 발효 배지를 접종하기 전에 세포 용해를 야기하지 않는다. 따라서, 본 발명 이전에는 발효 단계가 시작되기 전에 *클로스트리디움 보툴리눔* 박테리아를 배양하기 위하여 약 4 일이 걸렸다. 본 발명에 따르면, 종전에 이용된 세포 도말 단계, 혈액 한천 플레이트 상의 콜로니 성장을 위한 추후의 대기 시간, 배양 배지를 위한 접종물을 제공하는 작은 부피의 배양물(예를 들어 8-9 mL) 성장을 위한 플레이트로부터의 콜로니 선택이 필요하지 않으므로, 본 발명자들은 모든 배양을 단지 8 내지 14 시간 내에 완료할 수 있다. 본 발명의 한 양태에 따르면, 사전-선택된 세포가 배양 배지 접종에 직접 이용되고, 이후 배양 배지는 완전-규모 발효 배지를 접종하기 위하여 이용되며, 완전-규모 발효 배지로부터 보툴리눔 독소가 결국 정제되므로, 이후 그 자체가 발효 배지를 접종하기 위하여 이용되는 배양 배지를 접종할 세포 성장을 위하여 종전에 이용된 도말, 콜로니 형성, 선택 및 증대 단계를 생략한다.

[0122] 동물-기초의 (비-APF 또는 "NAPF") 배양 배지는 일반적으로 뇌 심장 침출 배지 (BHI), 박토-펩톤, NaCl, 및 글루코오스를 포함한다. 본 발명의 범위 내의 배양 배지는 APF 배양 배지이다. 예를 들어, 대두-기초 산물이 배양 및 발효 배지에서 BHI 및 박토-펩톤 대신에 사용될 수 있다. 바람직하게는, 비록 *클로스트리디움 보툴리눔*이 불용성 대두를 포함하는 배지에서 성장할 수 있기는 하지만, 대두-기초 산물은 물에 용해성이고 가수분해된 대두를 포함한다. 대두-기초 산물의 어떠한 원천이라도 본 발명에 따라 사용될 수 있다. 바람직하게는, 대두는 가수분해된 대두이고 가수분해는 비-동물 효소를 사용하여 수행되었다. 가수분해되거나 용해성인 대두의 원천은 Hy-Soy (Quest International), Soy peptone (Gibco) Bac-soytone (Difco), AMISOY (Quest), NZ soy (Quest), NZ soy BL4, NZ soy BL7, SE50M (DMV International Nutritionals), 및 SE50MK (DMV)를 포함한다.

[0123] 실시예

[0124] 다음 실시예는 본 발명의 특정 구체예를 제시하고, 본 발명의 범위를 제한하도록 의도되지 않는다. 실시예에서 달리 제시되지 않으면 "독소" 또는 "보툴리눔 독소"는 약 900 kDa의 분자량을 가지는 보툴리눔 독소 A 형 복합체를 의미한다. 약 900 kDa의 분자량을 가지는 보툴리눔 독소 A 형 복합체를 정제하기 위한 본 명세서에 개시된 시스템 및 방법은 약 150 kDa, 약 300 kDa, 약 500 kDa 뿐만 아니라 다른 분자량의 독소, 복합체, 보툴리눔 독소 혈청형 및 보툴리눔 독소 신경독성 성분의 정제에 준비된 적용성을 가진다.

[0125] 실시예 1

[0126] 보툴리눔 독소를 수득하기 위한 비-APF (Schantz) 공정

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[0127] 이 실시예는 보툴리눔 신경독소를 수득하기 위한 종래 기술의 Schantz 공정을 제시한다. 상기 공정은 동물 유래의 배지 및 시약을 사용하는 비-APF 공정이다 (즉 배양을 위한 소 혈액 한천 플레이트, 발효 배지 중의 카제인 및 보툴리눔 신경독소 정제를 위한 RNase 및 DNase 효소 사용). 도 1A는 Schantz 공정의 주요 단계를 나타내는 흐름도이다. Schantz 공정은 약 16 내지 20 개의 주요 단계를 가지고, 생산 규모를 위하여 115 L 발효기를 사용하며 완료에 약 3 주가 걸린다. Schantz 공정은 비-APF *클로스트리디움 보툴리눔* 마스터 세포 은행(master cell bank, MCB) 바이알을 실온으로 해동하는 것으로 시작되고 넷의 배양 단계가 이어진다. 첫 번째로, 적절한 형태를 가지는 콜로니를 선택하기 위하여, 해동된 MCB 바이알의 분취물이 사전-환원된 콜롬비아 혈액 한천(Columbia blood agar, CBA) 플레이트에 획선도말되었고 30-48 시간 동안 34 ℃ ± 1 ℃에서 혐기성으로 인큐베이션(incubation)되었다. 두 번째로, 선택된 콜로니가 6-12 시간 동안 34 ℃에서 카제인 성장 배지를 포함하는 9 mL 테스트 튜브에 접종되었다. 가장 빠른 성장 및 가장 높은 밀도(성장 선택 단계)를 가지는 9 mL 튜브 내용물이 두 증대 혐기성 인큐베이션(제3 및 제4 배양 단계)을 통하여 추가로 배양되었는데, 상기 인큐베이션은 600 mL 내지 1 L 종균 배양 병에서 34 ℃의 12-30 시간 인큐베이션에 이어서 카제인 성장 배지를 포함하는 15 L 내지 25 L 종균 발효기에서 6-16 시간 동안 35 ℃의 배양이다. 이러한 두 증대 배양이 물에 2% 카제인 가수분해물(카제인 [우유 단백질] 소화물), 1% 효모 추출물 및 1% 글루코오스(덱스트로오스)를 포함하는 pH 7.3의 영양 배지에서 수행되었다.

[0128] 증대 배양에 이어서, 제어된 혐기성 대기하에 카제인 포함 배지에서 상업적 규모(즉 115 L) 생산 발효기에서 60-96 시간 동안 35 ℃에서 추가로 인큐베이션되었다. 박테리아의 성장은 보통 24 내지 36 시간 후 완료되고, 발효 단계 동안 약 65 내지 약 72 시간 동안 수행되며 여기서 대부분의 세포가 용해 및 보툴리눔 신경독소 방출을 겪는다. 독소가 세포 용해에 의하여 유리되고 매질에 존재하는 프로테이즈에 의하여 활성화되는 것으로 생각된다. 배양 배지의 여과액이 단일층 심층 필터를 사용하여 제조되어, 큰 불순물(즉 전체 및 파쇄된 세포)이 제거되고, 이에 의하여 정화 배양물로 지칭되는 투명한 용액을 수득할 수 있다. 정화 배양물로부터의 보툴리눔 신경독소 수집은 정화 배양물의 pH를 3M 황산으로써 pH 3.5로 낮추고 20 ℃에서 생(raw) 독소를 침전시켜 이루어졌다 (산성화 침전). 이후 생 보툴리눔 신경독소가 (부피 감소를 달성하기 위하여) 초미세여과(미세여과)(MF 또는 UF로 지칭됨)에 이어 정용여과(DF)에 의하여 농축되었다. 0.1 ㎛ 필터가 미세여과 단계에 사용되었다.

[0129] 이후 수확된 미정제 또는 생 독소가 소화 용기에 옮겨지고 프로테이즈 억제제 벤즈아미딘 하이드로클로라이드의 첨가에 의하여 안정화되었다. DNase 및 RNase가 핵산 소화(가수분해)를 위하여 첨가되었다. 이후 가수분해된 핵산 및 저분자량 불순물이 추가의 UF 및 DF 단계에 의하여 제거되었다. 이후 독소가 pH 6.0 포스페이트 완충제로써 추출되었고 정화에 의하여 세포 파편이 제거되었다. 그 다음 셋의 연속 침전 단계(차가운 에탄올, 염산 및 황산암모늄 침전)가 수행되었다. 정제된 보툴리눔 신경독소 복합체(벌크 독소)가 인산나트륨/황산암모늄 완충제 중의 현탁액으로서 2 ℃ 내지 8 ℃에서 보관되었다.

[0130] 수확 및 정제 단계를 포함하는 이러한 실시예 1 Schantz (비-APF) 공정의 완료에는 약 2 내지 3 주가 걸린다. 생성된 벌크 보툴리눔 신경독소는 2×10^7 U/mg 이상($\geq 2 \times 10^7$ U/mg)의 특이역가, 0.6 미만의 A_{260}/A_{278} 및 겔 전기영동에서의 뚜렷한 밴딩 패턴을 가지는 *클로스트리디움 보툴리눔*의 Hall A 균주로부터 제조된 900 kDa 보툴리눔 독소 A 형 복합체의 고품질 현탁액이었고, 보툴리눔 독소 약제학적 조성물 조합에 사용하기에 적절했다.

[0131] 보툴리눔 신경독소는 또한 미국 특허 제7,452,697호의 실시예 7에 제시된 바와 같이 APF, 비-크로마토그래피 공정으로부터 수득될 수 있고, 완전한 APF, 비-크로마토그래피 공정(배양의 시작으로부터 모든 정제 및 가공 단계의 종료까지)은 완료에 약 2 내지 3주가 걸린다. 대안으로, 보툴리눔 신경독소가 또한 미국 특허 제7,452,697호의 실시예 16에 제시된 바와 같이 APF, 크로마토그래피 공정으로부터 수득될 수 있고, APF, 크로마토그래피 공정(배양의 시작으로부터 모든 정제 및 가공 단계의 종료까지)은 완료에 1주 이상이 걸린다.

[0132] 실시예 2

[0133] 보툴리눔 신경독소를 수득하기 위한 APF, 2 및 3 컬럼 크로마토그래피 시스템 및 공정

[0134] 본 발명자들은 고수율, 고순도의 보툴리눔 신경독소를 수득하기 위한 신속한 APF, 음이온-양이온 크로마토그래피 기초의 시스템 및 공정을 개발했다. 이 실시예 2의 공정은 (그램 양의 최종 보툴리눔 신경독소를 수득하기 위한) 생산 목적으로 단지 8-10 개의 주요 단계를 가졌고, 20 L 발효 용기를 사용했으며, 배양의 개시로부

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터 최종 정제 및 독소 보관의 완료에 이르는 공정의 모든 단계 완료에 단지 4-7 일, 바람직하게는 약 4 내지 약 6 일이 걸린다. 본 명세서에 개시된 시스템에서 사용된 장치는 아래에 논의된다. 2 크로마토그래피 매질 공정 및 3 크로마토그래피 매질 공정 두 가지가 개발되었고 본 명세서에 제시된다. 2 매질 공정은 음이온 교환 크로마토그래피에 이어 양이온 교환 크로마토그래피를 사용했다. 3 매질 공정은 음이온 교환 크로마토그래피에 이어 양이온 교환 크로마토그래피에 이어 소수성 상호작용 크로마토그래피(HIC)를 사용했다. HIC는 49 kDa 불순물(이는 아래에 논의되는 바와 같이 숙주 세포 글루코오스 포스페이트 아이소머레이즈로 밝혀졌다)과 같은 추가의 불순물을 제거했다.

[0135] *제조용 세포 은행의 준비*

[0136] 본 발명자들은 (배양 단계 개시에 사용하기 위한) 콜롬비아 혈액 한천 플레이트를 사용하지 않는 새로운 *클로스트리디움 보툴리눔* 세포 은행을 개발했고, 이는 배양 전에 콜로니를 선택해야 할 필요성을 제거하고 또한 Schantz 공정 증대 튜브 배양 및 다중 종균 (배양) 단계를 수행할 필요성을 없앴다.

[0137] 이 목적을 위하여, 종전에 확립된 Schantz 마스터 세포 은행(MCB)이 APF 연구용 세포 은행(research cell bank, RCB) 생성에 사용되었고, 이로부터 새로운 APF 마스터 세포 은행(MCB) 및 다음의 제조용 세포 은행(WCB)이 발생되었다. 연구용 세포 은행(RCB)이 Schantz(NAPF) MCB의 콜로니로부터 제조되었다. MCB 바이알로부터 동물-유래 단백질을 제거하기 위하여, 세포가 2% w/v SPTII(대두 펩톤 타입 II), 1% w/v 효모 추출물, 및 1% w/v 글루코오스를 포함하는 APF 배지에서 두 번 세척되었다. 세포는 모듈식 대기 제어 시스템(Modular Atmosphere Controlled System, MACS) 혐기성 챔버를 사용하여 엄격한 혐기성 조건하에 APF 배지에 도말되었다. 단리된 콜로니는 더욱 팽창되었고 -135 ℃ 아래에서 약 20% 글리세롤을 포함하는 APF 배지에 보관되었다.

[0138] APF-MCB는 RCB를 무산소 APF 배지(200 mL, 혐기성 챔버에서 최소 12 시간 동안 환원됨)에 팽창시켜 GMP 조건하에 제조되었고, 배양물의 OD_{540}가 2.5 ± 1.0 AU에 도달할 때까지 34.5 ℃ ± 1 ℃ MACS 혐기성 챔버(60 rpm에서 교반됨)에서 배양되었다. 생성된 배양물에 멸균 글리세롤이 약 20%의 최종 농도로 첨가되었고, 그 후 혼합물이 1 mL/바이알로 냉동바이알(APF-MCB 바이알)에 옮겨졌다. 바이알이 액체 질소에서 급속 냉동된 다음, -135 ℃ 아래에서 보관되었다. APF-WCB는 상기와 같이 팽창시켜 GMP 조건하에 제조되었다. 생성된 APF 세포 은행은 동일성, 순도, 생존력 및 유전적 안정성에 대하여 특징분석되었다.

[0139] *상류 단계 (배양 및 발효)*

[0140] 본 발명의 실시예 2 공정은 일반적인 두 단계; 상류 단계 및 하류 단계를 가졌다. 상류 단계는 출발 세포주의 팽창(실질적으로 APF 배양 배지에서 *클로스트리디움 보툴리눔* 박테리아의 성장 및 재생), 발효, 수확(세포 파편의 제거)을 포함하여, 정화된 수확 배양물을 제공하고 이는 이후 농축되고 희석된다. 따라서, 이 실시예에서 본 발명의 2 컬럼 공정의 9 단계는 배양, 발효, 수확 여과, 농축, 포획 (음이온) 크로마토그래피, 폴리싱 (양이온) 크로마토그래피, 완충제 교환, 생물부하 감소 및 바이알 충전이다.

[0141] 상류 단계는 400 mL의 (혐기성 챔버에서) 환원된 종균 APF 배양 배지(2% w/v SPTII, 1% w/v 효모 추출물, (오토클레이빙(autoclaving) 전에) 1 N 수산화나트륨 및/또는 1 N 염산으로써 pH 7.3으로 조정된) 배양 배지의 오토클레이빙 후 첨가된 1% w/v 멸균 글루코오스)를 수용하는 1 L 병에서 배양 배지의 사용을 포함했다. 배양 (종균) 배지가 400 ㎕의 해동된 *클로스트리디움 보툴리눔* WCB로 접종되었다. 인큐베이션/배양이 혐기성 챔버에서 150 rpm 교반되며 34.5 ℃ ± 1.0 ℃에서 일어났다.

[0142] 540 nm에서 배양 배지의 광학 밀도가 1.8 ± 1.0 AU였을 때, 1 L 병의 전체 내용물(대략 400 mL)이 증기 멸균 후 1 N 수산화나트륨 및/또는 1 N 염산으로써 pH 7.3으로 조정된 APF 발효 배지를 포함하는 20 L 생산 발효기에 옮겨졌고, 발효 배지는 3.25% w/v SPTII, 1.2% w/v 효모 추출물, 1.5% w/v 멸균 글루코오스(멸균 후 첨가됨; 멸균, 예를 들어 약 122 ℃에서 0.5 시간 동안)로 구성되었다. 온도 및 교반은 각각 35 ℃ ± 1 ℃ 및 70 rpm으로 제어되었다. 세포 성장을 위한 혐기성 환경을 유지하기 위하여 질소 오버레이(overlay)가 12 slpm으로 설정되었고 헤드스페이스 압력이 5 psig로 설정되었다. 발효 pH 및 세포 밀도가 pH 탐침 및 온라인 탁도 탐침 각각에 의하여 모니터링되었다. 생산 발효를 위한 세 단계는 지수성장기, 정체기, 및 자가용해기를 포함한다. 활성 BoNT/A 복합체를 배양 배지에 방출하는 세포 자가용해가 35 시간 및 발효의 종료 사이에 일정하게 일어나는 것으로 관찰되었다. 발효의 종료 시, 배양물이 수확을 위하여 25 ℃로 냉각되었다.

[0143] 발효 배지가 25 ℃로 냉각되면, 세포 파편이 심층 여과에 의하여 용해물을 함유하는 보툴리눔 신경독소 A 형 복합체로부터 분리되는데, 먼저 5 - 0.9 ㎛ 명목 체류율 구배(nominal retention rating gradient) 사전-필터를 통과하여 세포 파편이 제거된 다음, 양으로 하전된 0.8 - 0.2 ㎛ 명목 체류율 구배를 통과하여 DNA가 제거

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된다 (최대 약 80% 제거). 두 필터는 사용 전에 20 L의 주사용수(water for injuction, WFI)로 함께 헹구어졌다. 최소 15 L의 여과액이 추가의 가공을 위하여 필요했고, 공정 중 샘플채취(in-process sampling)가 완료된 후 임의의 과잉 물질이 오염제거되었다. 여과액은 한외여과에 의하여 즉시 가공되지 않을 경우 4 ℃에서 보관되었다.

[0144] 생물안전 캐비닛(biosafety cabinet, BSC) 내에서 수확 단계에서 나온 여과액이 GE Healthcare의 중공 섬유, 접선 흐름 여과(TFF) 막을 사용하여 15 L로부터 5 ± 0.5 L로 농축되었다. 이후 한외여과된 물질이 10 mM 인산나트륨 pH 6.5 완충제로써 20 L의 최종 부피로 희석되었다. 이 물질은 2 컬럼(음이온 이후 양이온) 또는 3 크로마토그래피 컬럼(음이온, 양이온, 이후 소수성 상호작용)의 사용에 의하여 정제되었다. 희석되고 한외여과된 수확 물질이 정제에 의하여 즉시 가공되지 않을 경우 4 ℃에서 보관되었다.

[0145] Schantz 공정에서 시간 및 배양물 성장의 시각적 관찰에 기초하여 배양 단계가 종료되고 발효 단계가 시작된다. 반면에, 본 발명의 실시예 2 공정에서 배양 단계를 언제 종료시킬지의 결정이 배양물 유체 광학 밀도의 분석에 기초하는데, 이는 배양물이 발효 단계의 시작 시간에 대수증식기에 있음을 보증하고, 배양 단계 기간을 약 8 시간 내지 약 14 시간으로 단축시킨다. 본 발명의 OD 파라미터 배양된 세포의 건강이 최대화된 배양 단계를 끝내고, 발효 단계에서 생성된 확고하고 풍부한 보툴리눔 독소를 고무시켰다. 배양 종결 시 (540 nm에서) 배양 배지의 평균 광학 밀도는 1.8 AU였다. 발효 단계의 평균 기간은 72 시간이었고, 발효 단계의 종결 시 발효 배지의 평균 최종 탁도(A_{890})는 0.15 AU였다. 발효 단계의 종결 시 20 L 발효 배지(전체 육즙)에 존재하는 보툴리눔 독소 A 형 복합체의 평균 양(ELISA에 의하여 결정됨)은 약 64 ㎍ 보툴리눔 독소 A 형 복합체/mL 발효 배지였다.

[0146] 수확 단계는 세포 파편 및 핵산을 제거하기 위하여 심층 여과를 이용했고, 이어서 공정의 다음 단계를 위한 발효 배지를 제조하기 위하여 한외여과 및 희석을 이용했다. 이 수확/세포 파편 세정은 Schantz 수확 공정과 근본적으로 상이한데, Schantz 수확 공정은 산성화에 의한 침전에 이어서 미세여과 및 정용여과를 이용하여 추가의 가공을 위한 제조에서 농축하고 완충제를 교환한다.

[0147] *하류 단계 (정제)*

[0148] 하류 단계는 음이온 교환 컬럼 상의 보툴리눔 신경독소 포획, 컬럼으로부터의 용리 및 양이온 교환 컬럼 상의 폴리싱에 의한 불순물로부터의 추가 분리, 및 바람직하게는 (3 컬럼 공정에서), 원하는 보툴리눔 신경독소를 함유하는 용리액의 제3 컬럼, 바람직하게는 소수성 상호작용 컬럼 통과 (예를 들어 크로마토그래피), 이어서 접선 흐름 여과(TFF)를 이용한 농축 및 완충제 교환, 및 냉장 보관, 바람직하게는 냉동에 최적화된 최종 보툴리눔 신경독소 A 형 복합체로의 생물부하 감소 (예를 들어 0.2 ㎛ 필터를 사용한 추가의 여과에 의하여), 및 보툴리눔 신경독소 A 형 복합체 약제학적 조성물로의 궁극적인 조합을 포함했다. 크로마토그래피 및 여과 단계의 순서는 더욱 안정한 약물 물질을 제공하기 위하여, 생성물 및 공정-관련 불순물을 제거하고, 잠재적인 부수 물질을 제거하고, 보툴리눔 신경독소 A 형 복합체 농도 및 최종 보툴리눔 신경독소 A 형의 완충제 매트릭스를 제어하도록 의도되었다.

[0149] 수행된 3 컬럼 하류 공정의 더욱 상세한 구체예는 다음과 같다. 정화된(희석된) 한외여과된 물질(앞에서 개시된 바와 같이 20 L)이 POROS® 50HQ 음이온 교환 크로마토그래피 수지를 통과했고, 포획된 보툴리눔 신경독소가 음이온 교환 컬럼으로부터 용리된 다음 POROS® 20HS 양이온 교환 크로마토그래피 수지를 빠져나가며, 이로부터의 용리액이 Phenyl Sepharose HP 크로마토그래피 수지를 빠져나갔다. HIC 컬럼에서 나온 용리액이 100 kDa 접선 흐름 여과에 이어 0.2 ㎛ 여과를 거쳤다. 생성된 보툴리눔 신경독소 A 형 복합체는 보관을 위하여 냉동되었다.

[0150] 이 실시예에서, 본 발명자들은 하류 공정의 제1 크로마토그래피 단계에서 약 8 cm의 내부 지름 및 약 15 cm의 컬럼 높이를 가지는 컬럼에 패킹된 POROS® 50HQ 음이온 교환 크로마토그래피 수지를 사용했다. 완전한 POROS® 50HQ 컬럼 조업은 주위 온도에서 완료되었고, 흐름은 하향 방향이었다. 보툴리눔 신경독소 A 형 복합체가 pH 단계 변화를 이용하여 음이온 컬럼으로부터 용리되었고, 여기서 핵산(예를 들어 DNA 및 RNA)과 같은 더욱 음으로 하전된 성분 및 다른 숙주 세포 단백질이 음이온 교환 컬럼에 결합된 채로 있었다.

[0151] 음이온 교환 단계의 상세사항은 다음과 같았다: 30 분의 최소 접촉 시간 동안 0.1 N 수산화나트륨을 사용하는 POROS® 50HQ 컬럼의 사용 (최소한 약 3 컬럼 부피, 230 cm/시간). 이후 컬럼이 50 mM 인산나트륨, pH 6.5 완충제(최소한 5 컬럼 부피)로써 평형화되었다. 그 다음 정화되고 한외여과되고 희석된 물질(즉 가공된 용해물

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APF 발효 물질)이 230 cm/시간으로 POROS® 50HQ 음이온 교환 컬럼에 로딩되었고, 이어서 컬럼 유출액의 280 nm에서의 흡광도가 0.10 AU로 감소될 때까지 최소한 약 20 컬럼 부피의 50 mM 인산나트륨, pH 6.5로써 230 cm/시간으로 세척하고, 이어서 50 mM 아세트산나트륨, pH 4.8으로써 230 cm/시간으로 용리했다. 280 nm에서의 흡광도(A_{280})가 피크 최대치를 통하여 최소한 약 0.15 AU로 증가하고, 트레일링 에지(trailing edge)에서 약 0.2 AU 이하일 때, 생성물 풀(pool)이 1 컬럼 부피의 50 mM 아세트산나트륨, pH 4.8을 수용하는 용기에 수집되었다. 이 용리 풀은 약 2 ℃ 내지 약 8 ℃에서 최대 48 시간 동안 보관되었다.

[0152] 이 실시예 2의 하류 공정에서 제2 크로마토그래피 단계는 8 cm의 내부 지름 및 5 cm의 컬럼 높이를 가지는 컬럼에 패킹된 POROS® 20HS 양이온 교환 크로마토그래피 수지를 사용했다. 전체 POROS® 20HS 컬럼 조업은 주위 온도에서 완료되었고, 흐름은 하향 방향이었다. 보툴리눔 신경독소 A 형 복합체는 POROS® 20HS 컬럼 수지와 결합한다. 이후 보툴리눔 신경독소 A 형 복합체가 염 단계 변화를 이용하여 컬럼으로부터 용리되었다. 생성물-관련 불순물이 세척 완충제 및 오염제거 용액으로써 용리되었다.

[0153] 양이온 교환 단계의 상세사항은 다음과 같았다: 30 분의 최소 접촉 시간 동안 0.1 N 수산화나트륨 용액을 사용하는 POROS® 20HS 컬럼의 사용 (최소한 약 3 컬럼 부피, 230 cm/시간). 이후 컬럼이 50 mM 아세트산나트륨, pH 4.8 완충제(최소한 약 5 컬럼 부피)로써 평형화되었다. 그 다음 POROS® 50HQ 생성물 풀(앞에서 기재된 바와 같이 수집됨, 새롭거나 냉장상태로부터)이 POROS® 20HS 컬럼에 로딩되었다. 이후 컬럼이 50 mM 아세트산나트륨, pH 4.8 완충제(최소한 약 3 컬럼 부피)로 세척된 다음 50 mM 아세트산나트륨, 150 mM 염화나트륨, pH 4.8 완충제로 다시 세척되었다. 보툴리눔 신경독소 A 형 복합체가 POROS® 20HS 컬럼으로부터 50 mM 아세트산나트륨, 250 mM 염화나트륨, pH 4.8 완충제로써 200 mL/min으로 용리되었고, A_{280}이 피크 최대치를 통하여 약 0.1 AU 이상(≥ 0.1 AU)으로 증가될 때, 용리 피크의 트레일링 에지의 A_{280}이 0.1 AU 이하(≤ 0.1 AU)의 트레일링 에지 값으로 감소될 때까지, 용리액이 생물공정 수집 백(1 컬럼 부피의 50 mM $NaH_3C_2O_2$, pH 4.8 수용)으로 우회되었다. POROS® 20HS 생성물 풀이 수집 백에 주위 온도에서 최대 약 6 시간 동안 보관되었다.

[0154] 이 실시예 2의 3-컬럼 크로마토그래피 매질 공정에서, 제2(양이온 교환) 컬럼에서 나온 용리액이 HIC 컬럼을 통과했다. 사용된 HIC 컬럼은 Phenyl Sepharose HP 소수성 상호작용 크로마토그래피 수지가 패킹된, 약 8 cm의 내부 지름 및 약 5 cm의 컬럼 높이를 가지는 컬럼이었다. 전체 Phenyl Sepharose HP 컬럼 조업은 주위 온도에서 완료되었고, 흐름은 하향 방향이었다. 보툴리눔 신경독소 A 형 복합체가 감소되는 염 단계 변화를 이용하여 컬럼으로부터 용리되었다. 불순물이 로딩 동안 세척 완충제 및 오염제거 용액으로써 용리되었다.

[0155] 소수성 상호작용 크로마토그래피 단계의 상세사항은 다음과 같았다: Phenyl Sepharose HP 컬럼이 30 분의 최소 접촉 시간 동안 0.1 N 수산화나트륨 용액으로써 초기에 위생처리되었다 (최소한 약 3 컬럼 부피의 0.1 N 수산화나트륨 용액으로써 200 cm/시간으로). 이후 컬럼이 최소한 약 5 컬럼 부피의 50 mM 아세트산나트륨, 0.4 M 황산암모늄, pH 4.8 완충제로써 평형화되었다. 그 다음 POROS® 20HS (양이온 교환 컬럼) 생성물 풀(상기)이 50 mM 아세트산나트륨, 0.8 M 황산암모늄, pH 4.8 완충제와 1:1로 조합되고 Phenyl Sepharose HP 컬럼에 로딩되었다. 컬럼은 먼저 최소한 약 3 컬럼 부피의 50 mM 아세트산나트륨, 0.4 M 황산암모늄, pH 4.8 완충제로 세척된 다음, 50 mM 인산나트륨, 0.4 M 황산암모늄, pH 6.5 완충제로 세척되었다. 보툴리눔 신경독소 A 형 복합체는 10 mM 인산나트륨, 0.14 M 황산암모늄, pH 6.5 완충제로써 컬럼으로부터 용리되었다. A_{280}이 0.05 AU 이상(≥ 0.05 AU)으로 증가했을 때, 용리액이 생물공정 수집 백으로 우회되었다. 용리 피크의 트레일링 에지의 A_{280}이 0.05 AU 이하(≤ 0.05 AU)의 값으로 감소할 때까지 용리액이 수집되었다. Phenyl Sepharose HP 생성물 풀이 수집 백에 주위 온도에서 최대 6 시간 동안 보관되었다.

[0156] Phenyl Sepharose HP 크로마토그래피 단계 생성물 풀을 약물 물질 제제화 완충제로써 농축하고 정용여과하기 위하여 접선 흐름 여과 시스템이 사용되었다. 100 kDa 분자량 차단 막을 가지는 Pall® Filtron Minimate 카세트가 농축 및 정용여과 단계를 위하여 사용되었다. 이후 제제화된 물질이 Pall Mini Kleenpak ® 0.2 ㎛ 필터를 통과하여 잠재적인 생물부하를 감소시켰다. 앞에서 언급된 바와 같이, UF/DF 단계는 Phenyl Sepharose HP 생성물 풀(HIC 컬럼의 용리액)을 0.7 g/L의 BoNT/A 복합체 농도까지 농축했고 10 mM 시트르산칼륨, pH 6.5 완충제로써 농축된 물질을 정용여과했다.

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[0157] 사용된 한외여과/정용여과 공정의 상세사항은 다음과 같았다. UF/DF 유닛 및 Pall 100 kDa 폴리에테르 설폰 막이 패킹 용액 제거를 위한 최소 5 L의 주사용수(WFI)로 처음에 플러싱되었고, 최소 10 분, 바람직하게는 최소한 30 분 동안의 재순환 조건하에 최소 200 mL의 1 N 수산화나트륨 용액으로써 위생처리(sanitization)되어 UF/DF 유닛을 위생처리했다. 그 다음 투과액 및 체류액 pH가 pH 6.5일 때까지 막 및 UF/DF 시스템이 충분한 부피의 10 mM 시트르산칼륨, pH 6.5 제제화 완충제로써 평형화되었다. 그 후 Phenyl Sepharose HP 생성물 풀이 Minimate® 접선 흐름 여과 카세트에 로딩되었고, HIC 용리액이 0.7 g/L로 농축되었다. 농축 단계 이후에, 체류액 풀이 7.5 psig(제곱인치당 파운드 게이지)의 막간차압(transmembrane pressure)으로 최소 5 정용여과 부피의 약물 물질 제제화 완충제(10 mM 시트르산칼륨, pH 6.5)에 대하여 정용여과되었다. 이후 투과액 유출구가 폐쇄되었고, UF/DF 시스템이 최소한 2 분 동안 가동되었으며, 시스템이 50 mL의 10 mM 시트르산칼륨, pH 6.5 제제화 완충제로써 헹구어졌다. 헹굼 후, 체류액 풀 중의 BoNT/A 복합체의 농도가 오프라인 A_{278}를 측정하고 A_{278} 눈금에 기초하여 결정되었고, 체류액 풀의 농도가 10 mM 시트르산칼륨, pH 6.5 완충제로써 0.5 g/L로 조정되었다. 이후 농도-조정된 체류액 풀이 Pall Mini Kleenpak 0.2 ㎛ 필터를 통하여 여과되어 잠재적인 생물부하가 감소되었다. 여과된 농도-조정된 체류액 풀은 수집 백에 2 ℃ - 8 ℃에서 최대 2 일 동안 보관되었다.

[0158] 수득된 최종 정제된 보툴리눔 신경독소 A 형 복합체가 바이알당 700 ㎕로 1 mL Nunc® 냉동바이알에 채워지고 냉동 보관되었다. 충전 조업이 주위 온도의 클래스 100 생물안전 캐비닛에서 수행되었다.

[0159] 하류 공정(2 또는 3 크로마토그래피 컬럼의 사용을 포함함)은 단지 1 내지 3 일 후 완료되었고, 수득된 보툴리눔 신경독소 A 형 복합체는 시트르산칼륨, pH 6.5 완충제 중의 0.5 g/L의 농도의 용액으로서 냉동 보관되었다. 비교하면, 종래기술의 Schantz 하류 (독소 정제) 공정은 보툴리눔 신경독소 A 형 복합체를 정제하기 위하여 다중 여과, 침전, 추출 및 원심분리 단계를 사용하고, 단지 하류 단계를 완료하기 위하여 1-2 주를 필요로 하며, 결과적인 약물 물질(회수된 보툴리눔 신경독소)이 대략 2.7 g/L 농도의 황산암모늄 현탁액으로서 냉장 보관된다. 침전 대신 크로마토그래피의 사용 및 단축된 가공 시간은 본 명세서에 개시된 것과 같은 현저하게 개선되고 일관된 하류 공정을 야기했다.

[0160] 한 양태에 따르면, 식물-기초 산물, 예컨대 대두-기초 산물의 농축물은 배양 및 발효 배지에서 대두 펩톤 타입 II Hy-Soy® 또는 SE50MK(Kosher 대두 펩톤)일 수 있다. 종균 배양 배지에서 Hy-Soy®는 10-200 g/L 범위일 수 있다. 바람직하게는, 종균 배지에서 Hy-Soy®의 농도는 15-150 g/L 범위이다. 가장 바람직하게는, 종균 배지에서 Hy-Soy®의 농도는 대략 약 20-30 g/L 또는 그 사이의 양이다. 종균 배지에서 글루코오스의 농도는 0.1 g/L 내지 20 g/L 범위일 수 있다. 바람직하게는, 글루코오스의 농도는 0.5-15 g/L 범위이다. 가장 바람직하게는, 배양 배지에서 글루코오스의 농도는 대략 10 g/L이다. 효모 추출물 양은 약 5-20 g/L, 더욱 바람직하게는 약 10-15 g/L 또는 그 사이의 양일 수 있다. 예를 들어, *클로스트리디움 보툴리눔* 성장 전의 배양 배지의 pH는 대략 pH 7.0-7.5, 또는 그 사이, 바람직하게는 pH 7.3일 수 있다.

[0161] 예로서, 생산 발효 배지 중의 Hy-Soy® 양은 10-200 g/L 범위일 수 있다. 바람직하게는, 발효 배지 중의 Hy-Soy® 농도는 15-150 g/L 범위이다. 가장 바람직하게는, 발효 배지 중의 Hy-Soy® 농도는 대략 약 20-40 g/L 또는 그 사이의 양이다. 발효 배지 중의 글루코오스 농도는 0.1 g/L 내지 20 g/L 범위일 수 있다. 바람직하게는, 글루코오스 농도는 0.5-15 g/L 또는 그 사이의 양 범위이다. 필수적이지는 않지만, 상기와 같이, 글루코오스는 발효 배지의 다른 성분과 함께 오토클레이빙하여 멸균될 수 있다. 성장 전 발효 배지의 pH 수준은 pH 7.0-7.8, 바람직하게는 약 7.0-7.5 또는 그 사이, 더욱 바람직하게는 pH 7.3일 수 있다.

[0162] 도 1의 우측에 나타나는 바와 같이, 생물학적으로 활성인 보툴리눔 신경독소 복합체를 수득하기 위하여 이 실시예 2에서 사용된 2 컬럼 APF 공정은 다음 단계로 이루어졌다: (a) 종균/배양 병에서 APF WCB 바이알로부터의 *클로스트리디움 보툴리눔* 박테리아와 같은 박테리아를 배양하는 단계, (b) 이후 APF 발효 배지를 가지는 발효기(독소 생산 발효기)에서 *클로스트리디움 보툴리눔* 박테리아를 발효시켜 세포주를 팽창시키고, 원하는 세포 용해 상에 도달할 때까지 발효 및 보툴리눔 독소 생산을 진행하는 단계. 그 다음, (c) APF 발효 배지를 수확(예를 들어 여과에 의한 정화)하여 수확 발효 배지를 수득하는 단계, (d) 농축 및 희석을 진행하여 희석된 수확 발효 배지를 생성하는 단계, 희석된 수확 발효 배지는 (e) 포획 컬럼을 통과하여 불순물이 제거됨, (f) 포획 컬럼에서 나온 용리액을 폴리싱 컬럼과 접촉시켜 불순물을 추가로 제거하고, 선택적으로 제2 폴리싱 컬럼과 접촉시키는 단계 (g) 폴리싱 컬럼 용리액의 농축 및 완충제 교환 단계, (h) 이어서 생물부하 감소 여

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과 단계 및 (i) 바이알의 충전 단계.

[0163] 한 예에서, 발효 부피는 20 L이고, 모든 단계에 대한 총 공정 시간이 단지 4 내지 6 일이었으며, 높은 보툴리눔 신경독소 수율이 달성되었다.

[0164] 다음은 본 발명의 범위 내의 특정 구체예의 더욱 상세한 설명을 제공한다. 발효 단계가 30 L 스테인리스 스틸 발효기를 사용하여 APF 배지에서 수행되었다.

[0165] 하기 이 실시예에서, 여전히 고수율의 고역가 보툴리눔 신경독소 A 형 복합체를 제공하면서 훨씬 감소된 부피의 발효 배지가 사용되었다. 다음의 프로토콜을 사용하여, 예를 들어 보툴리눔 신경독소를 수득하기 위하여 상업적으로 유용한 양 생산에 필요한 전형적으로 더 큰 종전 부피(예를 들어 115 L)의 발효 배지 대신 단지 20 L 이하의 APF 발효 배지가 필요했다.

[0166] 기밀실(airlock)을 가지는MACS 혐기성 워크스테이션(Don Whitley)이 혐기성 생물을 처리하기 위한 산소-결핍 환경을 제공했다. 챔버로의 접근 및 챔버로부터의 퇴거는 내부 및 외부 문으로 구성된 포트홀(porthole) 시스템을 통했다. 유닛이 온도 제어되어 챔버 내의 사용자 설정을 유지했다. 습도조절장치-제어된 응축 플레이트가 챔버 내 과잉 수분의 효과적인 제거를 보장했다. 챔버는 작업자 사용을 위하여 조사되었고, 낮은 기체 압력, 연속적인 기체 흐름, 및 전력 조절(power condition) 손실에 대하여 경보했다. 챔버는 혐기성 챔버 내의 생육성 및 비생육성 미립자 수준을 감소시키기 위한 HEPA 필터를 구비했다. 혐기성 조건은 "Anotox" 및 Palladium Deoxo "D" Catalyst 대기 스크러빙(scrubbing) 시스템을 이용하여 유지되었다. 응축 플레이트로부터의 응축물 물이 수집되고, 물이 제거되는 외부 저장고에 파이프로 물이 운반되었다.

[0167] 앞에서 개시한 바와 같이, -135 ℃ 아래에서 보관된 세포 은행 바이알을 가지고서 APF 공정이 APF WCB의 제조에 이용되었다. APF WCB 세포 은행 바이알이 배양 배지 접종 전에 실온에서 약 15 min 동안 해동되었고, "종균" 배양을 확립하기 위하여 앞에서 개시한 바와 같은 단일 배양 단계가 이어졌다. 이는 전반에 걸쳐 무균 기법을 이용하는 모듈식 대기 제어 시스템에서 수행되어 생물부하를 최소화했다. 모듈식 대기 제어 시스템은 완료된 종균 배양 바이알에 WCB 바이알 내용물로써 접종을 착수하기 전에 APF 세정되었다. 배양 배지가 (pH 조정을 위한) 1 N 염산 및 1 N 수산화나트륨, D(+) 글루코오스, Anhydrous (Mallinckrodt Baker, Cat# 7730, 4.00 g), 대두 펩톤 타입 II(Soy Peptone Type II, SPTII)(Marcor, Cat #1130, 8.00 g), 주사용수(WFI) 400.0 mL 및 효모 추출물(YE)(BD Cat #212730, 4.00 g)을 사용하여 제조되었다. 대두 펩톤 타입 II 및 효모 추출물 용액이 500 mL 메스실린더로 300 mL의 WFI를 측정하여 제조되고 종균 배양 병에 부어졌다. 종균 배양 병을 교반기에 넣었고 교반기가 가동되었다. 8.00 g의 SPTII 및 4.00 g의 효모 추출물이 종균 배양 병에 첨가되고 용해될 때까지 혼합되었다. 혼합 후에 용해가 완료되지 않았을 경우, 혼합물이 저온 설정에서 가열될 것이다. pH가 측정되고 약 7.30 ± 0.05로 조정되었다. 배지 용액이 WFI로써 약 360 mL가 되었다. 종균 배양 병이 증기 및 기체 이동을 허용하도록 적절히 통기되었다. 10% 글루코오스 용액(w/v)이 100 mL 메스실린더로 약 30 mL의 WFI를 측정하여 제조되고 사전-조립된 글루코오스 첨가 병에 넣어졌고, 상기 병을 교반기에 넣었고 교반기가 가동되었다. 약 4.00 g의 글루코오스가 글루코오스 첨가 병에 첨가되고, 용해될 때까지 혼합되었고 (용해를 위하여 필요할 경우 저온 열이 이용되었음), WFI를 충분한 양(qs, quantity sufficient)으로 첨가하여 글루코오스 용액이 40 mL가 되었다. 이후 글루코오스 첨가 병이 통기 캡으로 느슨하게 닫혔다. 글루코오스 및 종균 배양 병이 모두 멸균을 위하여 123 ℃에서 30 분 동안 오토클레이빙된다. 멸균 후, 두 물품이 오토클레이브로부터 수거되고 생물-안전 캐비닛에서 냉각되도록 방치되었다. 무균 냉각 후, 10%의 글루코오스 용액이 효모 추출물 및 대두 펩톤 II 용액을 수용하는 종균 배양 병에 옮겨지고 혼합되어, 이에 의하여 완료된 종균 배양 병을 제공했다.

[0168] 이 완료된 종균 배양 병은 사전-세정된 MACS(여기에 준비된 혐기성 지시약이 넣어짐ㄴ)에 넣어졌다. 완료된 종균 배양 병의 캡이 느슨해졌다. 이후 완료된 종균 배양 병이 MACS 내의 교반 플레이트에 놓였고 (교반 플레이트는 약 150 rpm으로 가동되었다) 완료된 종균 배양 병 안의 배지가 MACS 내에서 최소 12 시간 동안 약 34.5 ℃ +/- 1 ℃에서 환원되었으며, 그 후 1 mL 배지 블랭크(blank)가 (540 nm에서의 바이오매스 결정을 위한) 광학 밀도 측정을 위하여 채취되었다. 그 후, MACS (혐기성) 안의 완료된 종균 배양 병이 접종되었다. APF WCB 배양 바이알이 냉동 세포 은행으로부터 수득되었고 MACS에 가져와졌다. 바이알은 약 10-15 분 동안 해동되었고, 그 후 약 400 ㎕의 바이알 내용물이 완료된 종균 배양 병 안의 배지에 직접 넣어졌다. 완료된 종균 배양 병 위의 캡이 완전히 느슨해지고, 캡은 병의 위에 놓였으며, 교반 플레이트는 150 rpm으로 설정되었다. MACS에서 최소한 약 11 시간의 인큐베이션 후, 아래 기재되는 바와 같이 발효 생산이 착수되었다.

[0169] 30 L 스테인리스 스틸 발효기와 같은 발효기의 탐침(예를 들어 산화환원 탐침, pH 탐침, 탁도 탐침, 예를 들

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어 Broadley James and Optek에 의하여) 및 시퀀스 구성이 체크되고 보정되었고, 각각의 발효기 포트에 삽입되고 적소에서 죄어졌다. 예를 들어, 발효기는 30 L 부피 발효기 용기, 교반기 구동 시스템, 유틸리티 연결을 위한 파이핑 어셈블리(CIP, 세정 증기, CDA, 질소, 산소, 공정 냉각수, 생물-폐기물, 및 플랜트 증기), 계측 장비 (pH, 온도, 압력, 산화환원, 광학 밀도, 및 질량 유량), 및 네 개의 연동 펌프로 구성된 ABEC 30 L (VT) 발효기 시스템일 수 있다. 바닥 탑재된 교반기 속도가 Allen-Bradley 변환 주파수 드라이브(variable frequency drive, VFD)를 사용하여 제어되었다. 시스템의 반-자동 및 자동 제어가 프로그래밍으로써 Allen-Bradley ControlLogix PLC에 의하여 취급된다. 시스템은 발효 조업 동안 배양 온도, 압력, pH, 및 산화환원의 페루프 PID(비례-적분-미분(proportional-integral-derivative)) 제어를 제공하도록 설계되었다. Allen-Bradley DeviceNet®(오픈 디바이스 레벨 네트워크)가 스키드 상의 디바이스 및 센서와의 소통 및 제어를 위하여 이용된다.

[0170] 멸균 유지, 평형, 런 및 수확 모드에 대하여, 교반, 온도, 압력 및 질소 오버레이가 다음 설정점으로써 조업되었다.

[0171] 멸균 유지 및 평형 모드에 대하여:

표 1

[0172]

제어된 파라미터	설정점 및 범위
교반	100 rpm ± 10
질소 오버레이	12 SLPM ± 2
발효기 압력	5 psig ± 1
발효기 온도	35 ± 1℃
산화환원	-390 내지 - 150 mV

[0173] 런 모드에 대하여:

표 2

[0174]

제어된 파라미터	설정점 및 범위
교반	70 rpm ± 5
질소 오버레이	12 SLPM ± 2
발효기 압력	5 psig ± 1
발효기 온도	35 ± 1℃

[0175] 수확 모드에 대하여:

표 3

[0176]

제어된 파라미터	설정점 및 범위
교반	150 rpm ± 10
질소 오버레이	10 SLPM ± 2
초기 발효기 압력	0 psig
발효기 온도	25 ± 1℃

[0177] 발효 배지를 제조하기 위하여, 필요한 용구는, 표준 저울(standard balance), 카보이(20 L, 예를 들어), 유리 병(5 L), 메스실린더, 교반 막대 및 교반기와 함께, D(+) 글루코오스, Anhydrous(Mallinckrodt Baker, Cat# 7730, 300.0 g), 대두 펩톤 타입 II(SPTII)(Marcor, Cat #1130, 650.0 g), 주사용수(WFI, 13 L) 및 효모 추출물(YE)(BD Cat #212730 , 240.0 g)을 포함한다. 약 10 L의 WFI가 교반 막대와 함께 카보이에 첨가되었다. 카보이가 교반기에 놓였고, 교반기가 작동되었으며, 그 후 약 650.0 g의 대두 펩톤 타입 II가 약 240.00 g의 YE와 함께 첨가되었다. 발효 배지는 WFI를 q.s.로 13 L까지 첨가했고, 카보이가 막아졌다. 이후, WFI가 유리 5 L 병(그 안에 교반 막대를 포함)에 넣어질 경우 약 2 L를 첨가하여 10% 글루코오스 용액(w/v)이 제조되었다. 교반기에 놓이고 막대가 회전하면서, 약 300.00 g의 글루코오스가 병에 첨가되었고, 용해될 때까지 혼합되었

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다. 글루코오스 용액은 WFI를 q.s.로 3 L까지 첨가했고, 병에 넣어지고 막아졌으며, 10% 글루코오스 용액을 제공했다.

[0178] 카보이 안의 발효 배지가 발효기에 첨가되고, 정치증기멸균(steam in place) 전 발효기 부피가 기록되고, 조업의 발효 시퀀스가 진전되었다. SIP(steam in place)(122 ℃, +/- 1 ℃)의 종료 시, SIP 후 발효기 부피가 기록되었다. 튜브 및 인-라인(in-line) 0.2 ㎛ 필터(PALL Corp.)를 가지는 용기 및 연동 펌프를 포함하는 글루코오스 첨가 어셈블리가 발효기에 연결되었고, 라인은 SIP를 거치고 냉각되었다. 첨가 밸브 포트가 개방되고, 약 3 L의 (필터 멸균된) 글루코오스가 첨가되고, 적절한 양의 (필터 멸균된) WFI가 20 L의 총 발효기 부피까지 q.s.로 글루코오스 첨가 병에 첨가되고, 동일한 글루코오스 필터 라인을 통하여 발효기에 펌핑되었다. 첨가 밸브 포트가 폐쇄되었다. 생산 발효 배지는 그 후, 필요한 대로 첨가 라인의 SIP를 이용하여 멸균 1 N 수산화나트륨 또는 1 N 염산으로써 약 pH 7.3 +/- 0.05로 pH 조정되었다. 이후, 멸균 유지를 위한 파라미터가 설정되고 접종 전에 약 12 시간 동안 유지되었다. 배지의 출발 글루코오스 농도가 대사산물 분석기 및 기록된 글루코오스 농도를 사용하여 측정되었다.

[0179] 앞에서 명시된 바와 같이, 종균 배양 인큐베이션의 종료 시 (약 11 ± 1 시간), 1 mL의 샘플이 광학 밀도(OD) 측정을 위해 취해졌다. OD는 적절한 범위 내에서 OD 값이 기록되고 배양이 발효를 위하여 이용되었을 경우 분광광도계를 사용하여 540nm에서 오프라인 측정되었다. 따라서 발효기 탁도 탐침이 영으로 맞춰졌다. 혐기성 챔버로부터의 종균 접종물 병이 발효기에 가져와졌고 및 종균 접종 이송 어셈블리(그 안의 APF 배양 배지를 포함하는 종균 용기, 용기는 PALL Corp. 또는 Millipore로부터 입수 가능한 멸균 Kleenpak™ Connector 어셈블리를 포함하는 배양 접종 이송 라인을 가짐)가 발효기의 밸브를 대체했고, 펌프 1로의 배관이 고정되었다. 발효기 압력은 2 psig로 낮춰졌고, 종균 접종물 병의 전체 부피가 발효기에 펌핑되었다. 접종의 종료 시, 발효기의 온라인 흡광 단위(AU)가 기록되고, 발효기 파라미터가 런 모드로 설정되고 시간이 기록되었다.

[0180] 이후 24 및 48 시간에 샘플이 발효기로부터 취해지면서, 예를 들어, 무균 상태를 유지하면서 발효가 진행되었다 (발효 런은 약 60 시간 내지 약 80 시간, 바람직하게는 약 68 시간 내지 약 76 시간, 가장 바람직하게는 약 72 시간 동안일 수 있다). 발효 동안 취한 최소한 하나의 샘플에 대한 런인 테스트는, 예를 들어 오프-라인 광학 밀도 측정, 글루코오스 측정, ELISA, SDS-PAGE, 웨스턴 블롯을 포함할 수 있지만 이에 제한되지 않는다. 발효의 종료 시 (예를 들어 발효의 종료 시 육즙 부피는 약 18-19 L임), 샘플이 (예를 들어, 오프-라인 광학 밀도 측정, 글루코오스 측정, ELISA, SDS-PAGE, 웨스턴 블롯 및 DNA/RNA 정량화에 의하여) 테스트를 위하여 취해질 수 있다.

[0181] 발효의 종료 시, 온라인 광학 밀도, EFT (경과된 발효 시간), 및 발효 종료 시간 뿐만 아니라 교반 rpm, ℃로 나타낸 온도, 압력 psig 및 질소 오버레이 slpm 그리고 산화환원 mV가 기록되었다. 그 다음, 생산 발효 육즙이 수확을 거쳤다. 즉 생산 발효 육즙이 여과를 통하여 정화되었고, 이에 의하여, 예를 들어, 약 15 L의 여과액이 수집된다. 발효 파라미터는 수확을 위하여 설정되었고, 사전-필터, 심층 필터 및 최소한 하나의 압력 게이지를 포함하는 정화를 위한 필터 어셈블리가 준비되었다 (CUNO, 3M 여과). 사전-필터 및 심층 필터가 약 20 L의 주사용수로써 플러싱되었다. 플러싱 후, 여과 어셈블리가 발효기의 수확/배출 포트에 부착되었다. 발효기 온도가 약 25 ℃로 감소되었고, 그 후 발효 육즙의 정화가 시작되었다 (정화 시작 시간, 초기 온라인 OD, 초기 pH, 초기 온도 및 발효기의 초기 부피를 기록하라). 발효기 내 압력은 약 6 psi의 압력에 도달할 때까지 여과 동안 10 분 마다 약 1 psi(제곱인치당 파운드)의 속도로 증가되었고, 수확 종료까지 압력이 유지되었다. 이 필터는 APF 발효 배지 중의 대략 80%의 RNA/DNA를 제거하고 (나머지는 아래 논의되는 바와 같이 이후의 크로마토그래피 단계 동안 본질적으로 제거되었다), 따라서 발효 육즙으로부터 이러한 성분을 제거하기 위한 종래의 RNase 및/또는 DNase의 의존/사용을 폐지한다. 사전-필터 유입구 압력, 심층 필터 유입구 압력, 발효기 압력, 교반 및 여과액 부피와 같은 공정 파라미터가 수집된 2 L의 여과액마다 모니터링되었고, 이의 종료 시 정화 종료 시간 및 수집된 여과액의 부피가 기록되었다. 수확 단계의 완료 이후, 시스템은 오염제거되고 세정되었다.

[0182] 여과액 카보이가 샘플링을 위하여 BSC에 가져와졌고, 이로부터 약 10 mL 이하(=10 mL)의 여과액이 오프라인 OD 측정 및 다른 분석(예를 들어 ELISA, SDS-PAGE, DNA/RNA 및 웨스턴 블롯)을 위하여 샘플링되었다.

[0183] 이후 여과액은 한외여과/희석을 거쳤다. 접선 흐름 필터 (TFF) 유닛 어셈블리가 조립되었다. TFF 유닛은 분당 약 2 L의 바람직한 속도로 WFI로써 약 90 분 동안 행구어진 다음 TFF 유닛에 이를 통하여 (재순환된) 0.1 N 수산화나트륨이 약 60 분 동안 지나가 위생처리되었고, 그 후 1 L의 10 mM 인산나트륨 완충제, pH 6.5가 이를 통하여 지나갔고, 이어서 WFI로써 약 30 분 동안 행구어졌다. 이후 수확 단계(약 15 L)에서 나온 여과액이 TFF를 통과하여(이는 생물-안전 캐비닛에서 수행된다), 여과액이 약 5 L +/- 0.5 L으로 농축된다 (농축 단계

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는 분당 약 2 L 및 약 5 psig의 막간차압으로 진행한다). 투과액의 샘플이 취해지고 예를 들어 ELISA, dsDNA, SDS-PAGE 및 웨스턴 블롯 테스트를 거칠 수 있다. 약 5 L +/- 0.5 L로 농축되면, 이후 체류액 풀이 약 15 L의 멸균 여과된 10 mM 인산나트륨 완충제, pH 6.5로써 TFF를 통하여 분당 약 2 L의 속도로 약 20 L까지 희석되었다. 이후 샘플이 다시 취해지고 예를 들어 ELISA, DNA/RNA, SDS-PAGE 및 웨스턴 블롯 테스트를 거칠 수 있다. 한외여과/희석 물질(체류액)이 4 ℃에서 보관되었다.

[0184] 사용 이후 모든 시스템은 1N 수산화나트륨 또는 멸균 (증기) 온도를 이용하여 오염제거되고 세정되었다.

[0185] 정제된 보툴리눔 신경독소 A 형 복합체를 수득하도록 실시예 2 공정으로부터 수득된 발효 배지의 정제 중의 예시적인 공정에서 사용하기 위한 아래에 제시된 용액, 완충제 등을 제조하기 위하여, 다음 물질, 기구 및 절차가 사용되었다. 이용된 예시적인 완충제(0.2-마이크론 진공 필터를 통하여 여과되고 이의 전도도가 기록보존을 위하여 mS/cm로 측정됨)10 mM 인산나트륨, pH 6.5; 50 mM 인산나트륨, pH 6.5; 50 mM 아세트산나트륨, pH 4.8; 50 mM 아세트산나트륨, 170 mM 염화나트륨, pH 4.8; 50 mM 아세트산나트륨, 250 mM 염화나트륨, pH 4.8; 50 mM 아세트산나트륨, 1 M 염화나트륨, pH 4.8; 50 mM 아세트산나트륨, pH 4.0 및 10 mM 시트르산염, pH 6.5를 포함한다.

[0186] 다음은 실시예 2 공정으로부터 보툴리눔 신경독소 A 형을 수득하고 정제하기 위한 조업의 예이다. 모든 생성물-접촉 부품은 비-반응성이고 비-흡수성임을 보장하도록 설계되고 구성되었다. 추가적으로, 모든 기구가 단일 사용 일회용 시스템의 이용을 허용하도록 설계되거나, 문서화된 입증 방법에 따라 위생처리, 세정 및 오염제거를 용이하게 하도록 설계되고 구성되었다. 크로마토그래피 컬럼 및 모든 관련 배관을 포함하는 시스템 또는 스키드는 흐름 경로가 단일 사용 일회용으로 설계되는 한, 생성물에 접촉하지 않도록 설계되었다. 크로마토그래피 컴포넌트는 AlphaBio로부터 입수되고 UF/DF 컴포넌트는 Scilog Inc로부터 입수되었다. 사용된 크로마토그래피 셋업은 변환 속도 드라이브(variable speed drive)를 가지는 용액 수송용 연동 펌프, 5 유입구를 가지는 유입구 밸브 매니폴드, 3 자동화 밸브의 배열을 가지는 컬럼 밸브 매니폴드, 3 유출구를 가지는 유출구 밸브 매니폴드, pH, 전도도, 및 UV, UV 흡광도에 기초한 피크 수집을 포함하는 컬럼 유출액 모니터링, 및 정제 조업을 완료하기 위하여 필요한 계측장비 및 제어부를 포함했다. 제어 시스템은 정제 공정을 제거하도록 설계된 소프트웨어 및 하드웨어를 가졌다. 명령 및 데이터가 HMI (인간 기계 인터페이스(Human Machine Interface)) 터미널을 통하여 입력되었다. 작업자는 HMI에서의 명령에 의하여 모든 자동화 공정 기능을 개시하고, 공급 유량, 압력, 전도도, pH, UV 흡광도 및 개별적인 밸브 위치와 같은공정 파라미터를 모니터링하고 조정했다.

[0187] UF/DF 시스템은 재순환 펌프, 정용여과 펌프, 2 저울 및 접선 흐름 필터(TFF) 홀더를 포함했다. 재순환 펌프는 투과액 저장고의 중량에 기초하여 유량을 제어하여 한정된 막간차압을 유지하고 정지하기 위하여 3 개의 일회용 압력 센서 및 (투과액 저장고 아래에 위치된) 저울 중 하나와 접속했다. 정용여과 펌프는 체류액 저장고의 일정한 중량 유지에 기초하여 시작 및 정지하기 위하여 (체류액 저장고 아래에 위치된) 두 번째 저울과 접속했다.

[0188] 수확 단계(동물 단백질이 없는 발효 배지 수확)에서 나온 체류액 물질의 농축 및 희석 후, 물질이 음이온 교환 컬럼에 로딩되었다. 다음은 실시예 2의 2컬럼 공정에서 유용한 음이온 교환 컬럼 패킹 및 테스트를 위하여 이용된 절차이다.

[0189] 사전-패킹된 컬럼이 셋의 모든 크로마토그래피 단계를 위하여 사용되었다. 첫 번째로, 공급 물질(한외여과/희석을 거친 수확된 APF 배지)이 음이온 교환 컬럼(상기한 바와 같이 ABI의 Poros 50HQ)을 통과했다. 최소한 5 컬럼 부피(CV)의 50mM 인산나트륨, pH 6.5가 음이온 교환 컬럼(이 예에서, 포획 컬럼)을 평형시키기 위하여 이용되었다.

[0190] 평형 후, 로딩 단계가 수행되었고, 여기서 공급 물질(예를 들어 약 20 L의 수확 단계 후 수확 발효 육즙)이 예를 들어 약 200 cm/hr의 속도로 음이온 교환 컬럼에 로딩되었다. 0.5 컬럼 부피의 로딩된 물질이 음이온 교환 컬럼을 통과한 후, 독소 복합체가 음이온 교환 컬럼 물질에 결합되는 동안, 흐름 통과(flow through, FT) 풀이 폴리에테르설폰 용기와 같은 저장소에 수집되었다. 이에 세척 단계가 이어졌고, 여기서 최소한 약 15 컬럼 부피의 세척 완충제(예를 들어 pH 6.5의 50 mM 인산나트륨)가 음이온 교환 컬럼을 통과했다. 컬럼 유출구에서 실시간으로 측정된 UV가 약 80 mAU 이하로 감소했을 때 세척 단계가 정지되었다. 세척 완충제 부피 및 흐름 통과/세척 풀 부피가 기록되었고, 1 mL 샘플의 흐름 통과/세척 풀이, 예를 들어 독소 농도, 핵산 함량, 전체 세포 단백질, SDS-PAGE, qPCR, 2D LC 및 ELISA를 위하여 취해지고 테스트되었다.

[0191] 다음 단계는 용리 단계였고, 여기서 용리 완충제(예를 들어 50 mM 아세트산나트륨, pH 4.8)가 음이온 교환 컬

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럼에 펌핑되었다. 컬럼 유출구에서의 UV 눈금이 실시간으로 약 150 mAU 이상까지 증가했을 때, 1 CV의 용리 완충제(50 mM 아세트산나트륨, pH 4.8)로 사전-충전된 용기에서 용리액의 수집이 시작되었다. UV 눈금이 약 200 mAU 이하로 감소되었을 때 용리액 풀의 수집이 정지되었다 (이 시점에서 수집된 부피는 약 1 내지 약 2 CV임). 이후 크로마토그래피 시스템이 1 N 수산화나트륨을 사용하여 오염제거되고 세정되었다.

[0192] 이후 음이온 교환 컬럼에서 나온 용리액 풀이 양이온 교환 컬럼에 첨가하기 위하여 준비되었다. 음이온 교환 용리액 부피, pH, 전도도 및 공급 온도가 기록되었고, 음이온 교환 컬럼에서 나온 용리액 풀이 1 CV의 50 mM 아세트산나트륨, pH 4.8로써 희석되었다.

[0193] 음이온 교환 컬럼의 런-스루(run-through) 이후, 양이온 교환 크로마토그래피 조업이 착수되었다. 양이온 교환 컬럼(예를 들어 Poros® 20HS)이 최소 5 CV의 평형 완충제(50 mM 아세트산나트륨, pH 4.8)로써 평형화되었다. 평형 후, 음이온 교환 컬럼에서 나온 희석된 용리액 풀이 양이온 교환 컬럼에 로딩되고 로딩된 총 부피가 기록되었다. 0.5 컬럼 부피의 로딩된 희석된 용리액 풀이 양이온 교환 컬럼을 통과한 후, 흐름 통과(FT) 풀이 수집되었다. 양이온 교환 컬럼의 제1 세척이 수행되었고 여기서 약 3-5 CV의 50mM 아세트산나트륨, pH 4.8이 양이온 교환 컬럼을 통과했다 (이용된 제1 세척 완충제의 부피가 기록되었다). 제2 세척이 수행되었고, 여기서 약 3 CV의 170 mM 염화나트륨, 50 mM 아세트산나트륨, pH 4.8이 컬럼을 통하여 펌핑되었고, 이 용리액은 "WASH 2 Peak"로 표지된 새로운 용기에 수집되었다. UV 눈금이 50 mAU 이상까지 증가했을 때 수집이 시작되었다. 1 CV가 수집되고 이용된 제2 세척 완충제 부피가 기록되었다.

[0194] 양이온 교환 컬럼으로부터의 벌크 독소 복합체 용리가 양이온 교환 컬럼에 펌핑된 용리 완충제(예를 들어 50 mM 아세트산나트륨 중의 250 mM 염화나트륨, pH 4.8)를 이용하여 수행되었다. 용리의 UV 눈금이 최소한 약 100 mAU에 도달했을 때, 희석 완충제(40 mL의 100 mM 인산칼륨, pH 6.8 및 60 mL의 10 mM 시트르산칼륨, pH 6.5)로 사전-충전된 용기에 용리액 수집이 시작되었다. 양이온 교환 컬럼으로부터의 용리액 수집은 UV 눈금이 약 100 mAU 이하로 감소할 때까지 계속되었다. 희석 후 용리액의 총 부피가 기록되었다. 이후 양이온 교환 크로마토그래피 시스템이 오염제거되고 세정되었다.

[0195] 양이온 교환 컬럼으로부터의 용리 이후, 용리액은 여과를 거쳤다. 서로 적층된 3 개의 100K MWCO 막(Sartorius AG, Goettingen, Germany)을 사용하는 접선 흐름 여과(TFF) 시스템이 이용되었다. 정용여과/평형 및 위생처리 용액 기재가 그러하듯이, 양이온 교환 용리액 풀 초기 부피가 기록되었다. 예를 들어, 정용여과 용액은 10 mM 시트르산칼륨, pH 6.5일 수 있고, 위생처리 용액은 0.1 N 수산화나트륨일 수 있다. 시스템 셋업이 양이온 컬럼(IAPF) 또는 HIC 컬럼(FAPF)에서 나온 보툴리눔 독소 함유 용리액을 수용하는 저장고로부터의 한 튜브를, 접선 흐름 여과막의 유입구로의 한외여과 펌프 헤드를 통하여 연결하여 진행되었다. 접선 흐름 여과막의 투과액 유출구로부터의 제2 튜브는 한외여과 (UF) 투과액 컨테이너에 연결되었다. 체류액 저장고를 향한 접선 흐름 여과막의 체류액 유출구로부터의 튜브가 잠그어졌고, 정용여과 펌프 헤드를 통하고 체류액 저장고를 향한 정용여과(DF) 완충제로부터의 제4 튜브 또한 잠그어졌다. 막이 그러하듯이 시스템의 보관 완충제가 폐기물을 향한 체류액을 포함하는 최소한 약 720 mL의 주사용수(WFI)로써 막을 플러싱하여 플러싱되었고, 그 후 막이 저장고로 재순환되는 체류액을 포함하는 최소한 약 4200 mL의 주사용수로써 추가로 플러싱되었다. 이후, (필요한 경우) 막 위생처리가 폐기물을 향한 체류액을 포함하는 최소한 약 200 mL의 1N 수산화나트륨으로써 막을 플러싱하고, 이어서 저장고로 재순환되는 체류액을 포함하는 최소한 약 200 mL의 1N NaOH로써 막을 플러싱하여 최소 30 분 동안 수행되었다. 이후 체류액 및 투과액 pH가 평형 완충제 pH의 +/- 0.2 유닛 이내 (예를 들어, pH 6.5의 +/- 0.2 유닛 이내)일 때까지 폐기물을 향한 체류액을 포함하는 평형 완충제(pH 6.5의 10 mM 시트르산칼륨)로써 막을 플러싱하여 평형화가 수행되었다.

[0196] 희석 또는 농축(예시적 가공)이 적절했는지를 알아보기 위하여 물질(양이온 교환 컬럼에서 나온 용리액(생성물 풀))의 농도가 결정되었다 (예시적 표적 농도는 약 0.7 mg/mL일 수 있다). 희석이 10 mM 시트르산칼륨, pH 6.5를 이용하여 수행되었다. 표적 부피가 예를 들어 0.7 mg/mL 생성물 농도에 대하여 결정되었다 (표적 부피 = (출발 농도/출발 부피)/0.7 mg/mL).

[0197] 생성물 풀((따라서 가공되거나 가공되지 않은) 양이온 교환 컬럼에서 나온 용리액)이 막에 로딩되고, (폐쇄된 투과액 유출구로써) 시스템(TFF 시스템)의 재순환이 배압(backpressure) 없이 최소한 2 분 동안 가동되었고, 그 후 투과액 밸브가 체류액 배압 밸브를 표적의 약 7 psig 막간차압으로 조정하면서 천천히 개방되었다. 희석을 위하여, 10 mM 시트르산칼륨, pH 6.5가 표적 부피에 첨가되고, 한외여과 없이 정용여과를 착수하고; 농축을 위하여, 한외여과가 시작된다. 정용여과를 위하여: 투과액 폐기물이 새로운 컨테이너에 수집되고 (표적 정용여과 부피는 5X 정용여과 부피이다) 최소한 5 정용여과 부피의 10 mM 시트르산칼륨, pH 6.5로써 정용여과

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되었다. 정용여과 공정 데이터가 최소 10-분 간격으로 수집되었다 (투과액 중량 g/부피 mL, 유입구 압력 (psig), 체류액 압력 (psig), 투과액 압력 (psig) 및 막간차압 (psig)). 재순환/및 헹굼을 위하여: 투과액 유출구 펄터가 폐쇄되어, 시스템이 배압 없이 최소한 2 분 동안 재순환/가동되고, 시스템이 최소한 20 mL의 10 mM 시트르산칼륨, pH 6.5로 헹구어졌다. 생성물 풀은 체류액 및 헹굼액을 포함한다. 샘플이 생성물 풀로부터 취해지고, 예를 들어, 278nm에서의 UV, SDS-Page, LcHPLC. SE-HPLC, qPCR, RP-HPLC, Native-Page, AUC, Limulus amebocyte lysate, 웨스턴 블롯 및 ELISA 테스트를 포함하는 확인 분석을 거칠 수 있다. 사용 후 세정을 위하여, 시스템이 최소한 10 분 동안 재순환된 1N 수산화나트륨으로 플러싱되고, 그 후 시스템이 그 안의 0.1 N 수산화나트륨으로 플러싱되고 보관되었다.

[0198] 이후 벌크 신경독소 보관 및 분할을 위하여 멸균 여과 및 충전이 수행되었다. 농도 조정은 헹굼 후 샘플로써 10 mM 시트르산칼륨, pH 6.5를 사용하여 독소 농도를 약 0.5 mg/mL로 조정하도록 수행되었다. 독소 농도가 약 0.5 mg/mL 미만일 경우, 농도 조정이 필요하지 않다.

[0199] 멸균 피펫을 사용하여, 멸균 mL/1.5mL 샘플 튜브 각각으로의 10mL/0.75mL 분취물이 제조되었다. 생성물 컨테이너는 손으로 부드럽게 교반되고 원하는 양의 용액(벌크 약물 물질, 즉 벌크 보툴리눔 독소 함유)을 각 바이알에 옮겼다. 샘플이 최대 5 일 동안 2 ℃ - 8 ℃ 냉장고에서 보관되거나 0.75 mL의 여과액 생성물 풀이 냉동 바이알로 옮겨졌다. 냉동바이알은 -70 ℃ +/- 5 ℃에서 보관된다.

[0200] 실시예 3

[0201] 조합 방법

[0202] 환자에게 투여하기에 적절한 약제학적 조성물이 실시예 2 공정으로부터 수득된 보툴리눔 신경독소를 한 가지 이상의 부형제와 조합하여 제조될 수 있다. 부형제는 조합 공정 동안 및 추후의 사용 전 보관 기간 동안 보툴리눔 독소를 안정시키는 작용을 할 수 있다. 부형제는 또한 증량제로서 기능을 하거나 및/또는 약제학적 조성물에 얼마간의 장성(tonicity)을 제공하는 기능을 할 수 있다. 조합은 한 가지 이상의 부형제(예컨대 알부민 [예컨대 인간 혈청 알부민 또는 재조합 인간 알부민] 및 염화나트륨)와 혼합하여 실시예 2 공정으로부터 수득된 보툴리눔 신경독소를 수배로 희석하는 것을 필요로 하고, 이에 의하여 독소 조성물을 형성하고, 조성물을 동결건조, 냉동건조 또는 진공건조하여 독소 조성물의 보관 및 출하 안정 형태를 제조한다. 따라서, 약 1.5 내지 1.9 ng의 실시예 2 수득된 보툴리눔 독소 A 형 복합체가 약 0.5 밀리그램의 재조합 인간 알부민 (Delta Biotechnologies) 및 약 0.9 밀리그램의 염화나트륨과 함께, 이들 세 가지 성분을 함께 혼합하고 이어서 진공건조하여 조합된다. 진공건조는 약 20 ℃ 내지 약 25 ℃, 약 80 mm Hg의 압력에서, 약 5 시간 동안 일어날 수 있고, 이때 상기 성분들이 있는 바이알이 진공건조되고 진공하에 밀폐되고 막아져, 이에 의하여 약 100 유닛의 보툴리눔 신경독소 A 형 복합체를 포함하는 바이알을 수득한다. 생성된 고체 (분말화) 진공건조 생성물은 사용 시 일반 (0.9%) 식염수로써 복원되고 경부 근긴장이상증 및 다한증과 같은 다양한 적응증을 가지는 환자 치료에 사용된다. 동결건조, 진공 또는 냉동건조는 보관 및 출하 안정 형태의 복합된 보툴리눔 신경독소를 제조한다.

[0203] 또 다른 예에서, 약 1.5-1.9 ng의 벌크 보툴리눔 독소 A 형이 약 0.5 밀리그램의 인간 혈청 알부민 (Baxter/Immuno, Octapharma, and Pharmacia & Upjohn) 및 약 0.9 밀리그램의 염화나트륨과 함께, 이들 세 가지 성분을 함께 혼합하고 이어서 진공건조하여 조합된다. 예시적인 진공건조가 약 20 ℃ 내지 약 25 ℃, 약 80 ㎛ Hg의 압력에서 약 5 시간 동안 일어날 수 있고, 이때 상기 성분들이 있는 바이알이 진공건조되고 진공하에 밀폐되고 막아져, 이에 의하여 약 100 유닛의 보툴리눔 독소를 포함하는 바이알을 수득한다. 생성된 고체 (분말화) 진공건조된 생성물은 사용 시, 일반 (0.9%) 식염수로써 복원되고 경부 근긴장이상증 및 다한증과 같은 다양한 적응증을 가지는 환자 치료에 사용된다. 추가적으로, 약제학적 보툴리눔 독소 조성물이 예를 들어 인간 혈청 알부민 및/또는 락토오스를 함유할 수 있다. 한 예에서, 예를 들어 약 1.5-1.9 ng의 벌크 보툴리눔 독소 A 형이 약 125 마이크로그램의 인간 혈청 알부민, 및 2.5 밀리그램의 락토오스와 조합되되고 보관 안정성을 위하여 진공 전고, 동결건조 또는 냉동건조될 수 있다. 또 다른 예에서, 본 명세서에 개시된 공정에 의하여 수득된 약 1.5-1.9 ng의 보툴리눔 신경독소가 약 10 mg의 트레할로오스 및 약 0.5 mg의 혈청 알부민 (예컨대 인간 혈청 알부민, 천연 또는 재조합), 그리고 선택적으로 약 1 밀리그램의 메티오닌과 조합되어 약 100 유닛의 보툴리눔 독소 건조 생성물을 제공할 수 있다. 이 조성물은 예를 들어 동결건조되고 사용 전에 약 1 mL의 증류 멸균수 또는 멸균 무보존(unpreserved) 식염수(주사용 0.9% 염화나트륨)로써 복원될 수 있다. 특정 예에서, 약제학적 보툴리눔 독소 조성물은 인간 혈청 알부민 20% 및 수크로오스와 조합된, 본 명세서에 개

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시된 공정에 의하여 수득된 약 1.5-1.9 ng의 보툴리눔 신경독소를 가지는 예시적 제제에서와 같이 수크로오스를 포함할 수 있고, 상기 제제는 또한 동결건조되어 약 100 유닛의 보툴리눔 독소 A 형을 제공하고, 무보존 식염수(예를 들어 약 0.5 mL 내지 약 8.0 mL 부피)로 나중에 복원될 수 있다. 특정 예에서, 200 유닛의 보툴리눔 신경독소가 mL당 약 10 mg의 수크로오스 및 2 mg의 인간 혈청 알부민과 조합될 수 있고, 나중에 사용 전에 생리 식염수로써 복원될 생성된 조성물이 바이알에 넣어지고 냉동건조되었다.

[0204] 추가적으로, 조합이 또한 본 명세서에 개시된 IAPF 공정에 의하여 수득 가능한 보툴리눔 독소 A 형 복합체의 신경독성 성분(즉 복합 단백질이 없는 보툴리눔 독소 A 형 복합체의 약 150 kDa 성분)을 이용할 수 있다. 결합된 비-독성 단백질(예를 들어 HAs, NTNH)로부터 약 150 kDa 신경독성 성분을 정제하는 한 방법에서, A 형 신경독소가 Tse et al. (1982)(Goodnough, M. C., 1994, Thesis, UW, Wis.)의 방법의 변형에 의하여 복합체의 결합된 비-독성 단백질로부터 정제된다. (앞에서 논의한 바와 같이 2-컬럼 음이온-양이온 또는 3-컬럼 음이온-양이온-HIC 단계를 이용하는) 본 발명의 IAPF 공정에 의하여 수득되는 보툴리눔 신경독소 복합체가 DEAE-Sephadex A 50 (Sigma Chemical Co., St. Louis, Mo.), pH 5.5, 컬럼으로부터 회수되고, 39 g의 고체 황산암모늄/100 mL 첨가에 의하여 침전된다. 침전된 독소 복합체가 원심분리에 의하여 수집되고, 25 mM 인산나트륨, pH 7.9에 대하여 투석되고, 동일한 완충제로써 평형화된 DEAE-Sephadex A50 컬럼에 가해진다. 신경독성 성분이 복합체의 비-독성 단백질로부터 분리되고, 선형 0-0.5 M 염화나트륨 구배로 컬럼으로부터 용리된다. 부분적으로 정제된 신경독소 성분은 pH 7.9에서 DEAE-Sephadex A50 컬럼으로부터 회수되고, 25 mM 인산나트륨, pH 7.0에 대하여 투석된다. 투석된 독소는 25 mM 인산나트륨, pH 7.0 중에서 SP-Sephadex C50 (Sigma Chemical Co.)에 가해진다. 오염 물질은 이러한 조건하에 컬럼에 결합하지 않는다. 순수 신경독소(약 150 kDa 성분)가 선형 0-0.25 M 염화나트륨 구배로 용리된다. 약 150-kDa 순수 신경독소는 금속 친화성 크로마토그래피, 겔 여과 또는 다른 단백질 크로마토그래피 방법에 의하여 추가로 정제될 수 있다. 상기와 같이, 이러한 순수 신경독소(보툴리눔 독소 복합체의 약 150 kDa 신경독성 성분)가 앞에서 논의된 다양한 부형제(예를 들어 혈청 알부민, 수크로오스, 락토오스, 염화나트륨, 트레할로오스 등)와 함께 동결건조, 진공 또는 냉동-건조될 수 있다.

[0205] 본 발명의 IAPF 공정에 의하여 수득된 벌크 보툴리눔 신경독소 복합체는, 다양한 방식으로 조합될 수 있다. 보툴리눔 독소의 다양한 제제를 개시하는 대표적인 특허, 예컨대 미국 특허 제6,087,327호 (젤라틴과 함께 제제화된 보툴리눔 독소 A 및 B 형의 조성물을 개시함); 1996년 4월 30일 등록되고 발명의 명칭이 "Pharmaceutical Composition of Botulinum Neurotoxin and Method of Preparation"이며 37 ℃에서 보관 안정성인 알부민 및 트레할로오스 포함 순수 보툴리눔 A 형 제제를 청구하는 미국 특허 제5,512,547호 (Johnson et al); 1998년 5월 26일에 등록되고 25 ℃ 내지 42 ℃에서 보관될 수 있는 티오알킬, 알부민 및 트레할로오스 포함동결건조된 보툴리눔 독소 제제를 청구하는 미국 특허 제5,756,468호 (Johnson et al) ("Pharmaceutical Compositions of Botulinum Toxin or Botulinum Neurotoxin and Method of Preparation"); 1997년 12월 9일에 등록되고 발명의 명칭이 "Pharmaceutical Composition Containing Botulinum B Complex"이며 B 형 복합체 및 단백질 부형제를 포함하는 냉동건조된, 염화나트륨이 없는 보툴리눔 B 형 복합체 제제를 청구하는 미국 특허 제5,696,077호 (Johnson et al); 및 보툴리눔 독소를 안정시키기 위한 재조합 알부민, 콜라겐 또는 녹말과 같은 다양한 부형제의 사용을 개시하는 미국 특허 공개공보 제2003 0118598호 (Hunt) (이러한 공개된 미국 특허 출원 또는 미국 특허 모두가 전체가 참조문헌으로 포함된다), 모두 본 발명의 IAPF 공정에 의하여 제공된 벌크 보툴리눔 신경독소 복합 및 약제학적 조성물 제공에 사용될 수 있는 다양한 유용한 제제/부형제의 예를 제공한다.

[0206] 수득된 보툴리눔 독소 복합체는 pH 7-8 완충제에서 이온 교환 컬럼으로부터 용리되어 보툴리눔 독소 분자로부터 비독소 복합체 단백질을 해리시킬 수 있고, 이에 의하여 (발효된 *클로스트리디움 보툴리눔* 박테리아의 유형에 따라) 대략 150 kDa 분자량, 및 1-2 X 10^8 LD_{50} U/mg 이상의 특이역가를 가지는 보툴리눔 독소 A 형 신경독성 성분; 또는 대략 156 kDa 분자량 및 1-2 X 10^8 LD_{50} U/mg 이상의 특이역가를 가지는 정제된 보툴리눔 독소 B 형, 또는 대략 155 kDa 분자량 및 1-2 X 10^7 LD_{50} U/mg 이상의 특이역가를 가지는 정제된 보툴리눔 독소 F 형을 제공한다.

[0207] 본 발명은 많은 이점을 제공한다. 첫 번째로, 실시예 2의 2 및 3 컬럼 공정은 동물 원천 시약 및 배지(예를 들어 카제인 가수분해물 및 콜롬비아 혈액 한천 플레이트)의 사용을 제거하고, 따라서 프리온과 같은 작용원 또는 다른 감염원에 환자가 노출될 이론적 위험을 현저하게 감소시킨다. 실시예 2의 두 번째로, 2 및 3 컬럼 크로마토그래피 공정 (및 관련 시스템과 장치)은 우수한 배치마다의 일관성에의하여 증명되는 바와 같이 매우

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재현성이다. 이러한 개선은 상용화되어 입수 가능한 화합물 포함 보툴리눔 독소로써 수년에 걸쳐 반복된 치료를 필요로 하는 환자에서 더욱 일관된 임상 프로파일로 전환된다. 본 명세서에 개시된 IAPF 공정(2 및 3 컬럼)의 약물 물질(보툴리눔 신경독소) 분석 연구는 더 낮은 부하의 단백질 및 핵산 불순물을 나타냈다. 이러한 더 낮은 부하의 단백질 불순물은 더 낮은 면역원성 (항체 생산) 위험으로 전환된다. 더욱이, IPAF 공정의 개선된 순도는 생물학적 약물과 통상적으로 관련된 비-특이적 증상(예를 들어, 비인두염, 상기도 증상, 근골격 증상, 두통 등)의 더 낮은 발생으로 전환된다. 더욱이, 이 공정의 개선된 축소 규모는 연구실 및 제조 설비 직원에 대한 BoNT/A 노출 위험을 감소시킨다.

[0208] 본 발명의 대표적인 장점은, 예를 들어 다음을 포함한다:

[0209] 1. 동물 원천(예를 들어 인간 또는 동물)으로부터 유래된 성분 또는 물질이 공정에서 사용되지 않고, DNase 및 RNase, 콜롬비아 혈액 한천 플레이트, 카제인의 사용이 제거되므로 (예를 들어, 정화/수확 단계 및 현대의 크로마토그래피 기법 동안의 하전 여과에 의하여; 제조용 세포 은행으로부터의 세포, 즉 사전에 선택되고 APF 배지에서 증식/유지된 세포로서 배양 배지에 직접 종균하는 것에의하여 대체되고; 배양 병 및 발효 매질이 펩톤원으로서 대두 펩톤 타입 II(SPTII)로 대체됨) 안정성이 개선된다.

[0210] 2. 10 L의 발효 배지당 약 50 mg 내지 약 200 mg의 고품질 보툴리눔 독소 A 형 복합체가 수득될 수 있다.

[0211] 3. 정제된 벌크 독소가 확고성(robust), 일관성(consistent), 규모변화성(scalable), 입증성(validatable), 및 cGMP 순응성(compliant)인 공정으로부터 수득된다. 확고성은 공정 파라미터 중 한 가지 이상의 약 ± 10% 변화 시에도 공정이 재현성임을 의미한다. 입증성은 공정이 재현적으로 일관된 정제된 독소 수율을 제공함을 의미한다. cGMP 순응성은 공정이 FDA 요구의 현행 우수 제조 관리기준(current Good Manufacturing Practices)에 순응하는 제조 공정으로 쉽게 전환될 수 있음을 의미한다.

[0212] 4. 최종 정제된 보툴리눔 독소 복합체의 역가가 Schantz 또는 변형된 Schantz 공정으로부터 수득된 정제된 보툴리눔 독소 복합체의 역가(예를 들어 MLD50 검사에 의하여 결정됨)를 충족시키거나 이를 초과한다.

[0213] 5. 임의의 침전 단계를 벌크 보툴리눔 독소 복합체를 정제하기 위하여 크로마토그래피 단계로 대체하고, 이는 정제 공정의 특이성(specificity)을 개선한다.

[0214] 6. 새로운 개선된 공정은 개선된 취급 및 95% 초과(>95%)의 조업 성공률 달성을 야기하는 규모 축소를 용이하게 한다 (예를 들어, 110 L -120 L의 발효 배지를 이용하는 전형적인 부피로부터 약 10 L 내지 약 50 L로 축소시키고, 심지어 약 2 L 내지 약 30 L의 발효 배지 또는 그 사이의 양으로 축소시킴). 전형적인 현행 벌크 약물 물질 제조 규모는 115 L의 비-APF 발효 배지이고, 본 발명의 한 양태와 같이 20 L의 발효 배지로 축소되었다. 이러한 규모 축소는 BoNT/A 복합체의 합성 및 세포 방출 뿐만 아니라 정제 단계에 걸친 전체 수율을 최적화하여 가능하게 되고, 예를 들어 5X 이상의 발효 부피(예를 들어 115 L)를 필요로 하는 종래 공정에서 수득된 것과 같은 유사한 양의 최종 벌크 보툴리눔 독소(약물 물질)를 야기한다. 이러한 축소된 규모는 발효 작업 부피의 관리를 더 쉽게 만들고, 따라서 조업자가 BoNT/A 복합체에 노출될 잠재적 위험을 최소화하며, 이는 중요한 조업적, 안전성 장점이다.

[0215] 7. BoNT/A 복합체의 잠재적 치사 성질로 인하여, 본 명세서에 개시된 제조 공정 전반에 걸쳐 폐쇄 시스템이 이행되었다. 종래기술 방법과는 달리, 본 발명의 양태에 따라 생성된 약물 물질이 유닛 조업 사이의 이송 동안 환경에 노출되지 않고; 모든 조업이 완전히 포함된다.

[0216] 8. 본 명세서에 개시된 벌크 보툴리눔 독소 제조 공정이 제조 동안 약물 물질의 동일성, 품질, 순도 또는 역가를 희생시키지 않고 모든 단계에서 단순화된다. 비-APF 공정에서 이용된 다수의 단계가 재설계된 IAPF 공정에서 제거되었고, 이에 의하여 생산 시간을 예를 들어, 21 일로부터 6 일 이하로 단축시킨다.

[0217] 9. 냉동 용액으로서 벌크 보툴리눔 독소를 보관하는 조건은 약물 물질 안정성을 크게 개선한다.

[0218] 다양한 간행물, 특허 및/또는 참조문헌이 본 명세서에 언급되었고, 이들의 내용은 전체가 본 명세서에 참조로 포함된다. 본 명세서에 개시된 본 발명의 대안의 요소 또는 구체예의 분류가 제한으로 간주되지 않아야 한다. 각 군 일원이 개별적으로 또는 그룹의 다른 일원 또는 본 명세서에서 발견되는 다른 요소와 임의의 조합으로 지칭되고 청구될 수 있다. 군의 하나 이상의 일원이 편의성 및/또는 특허성의 이유로 군에 포함되거나 군으로부터 제거될 수 있음이 예상된다. 더욱이, 모든 가능한 변형에서 상기 요소의 임의의 조합이, 본 명세서에 달리 지시되지 않고 문맥에 의하여 명백히 부정되지 않으면, 본 발명에 포함된다.

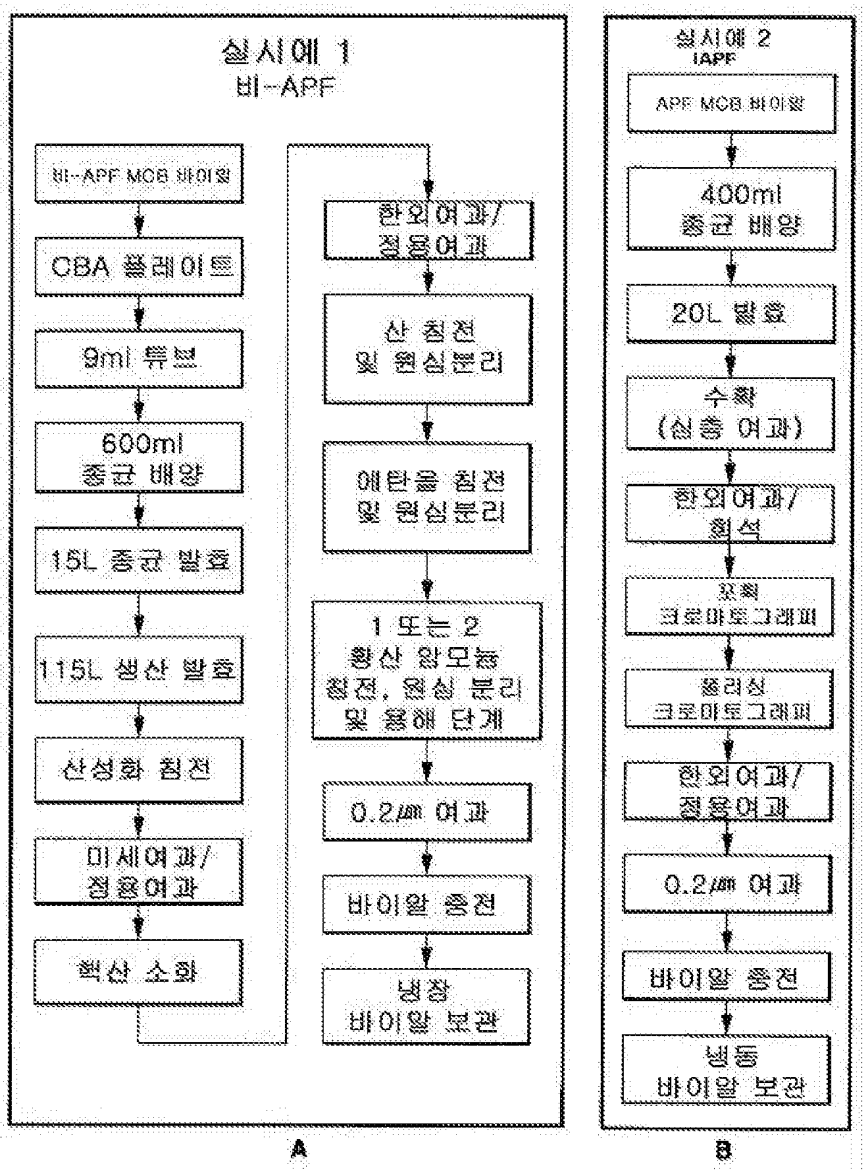
[0219] 비록 본 발명이 특정한 바람직한 방법에 관하여 상세하게 기재되기는 했지만, 본 발명의 범위 내의 다른 구체

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예, 버전, 및 변형이 가능하다. 따라서, 다음 청구항의 사상 및 범위가 앞에서 제시된 구체예의 특정 설명으로 제한되지 않아야 한다.

도면

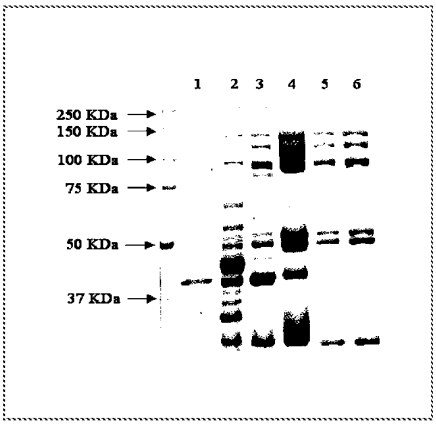
도면1



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Repr. Drawing



Zoom Q

Abstract PURPOSE: A purification method of Clostridium botulinum type A toxin is provided, thereby simply and easily isolating a complex of botulinum type A toxin and a nontoxic unit. CONSTITUTION: A purification method of Clostridium botulinum type A toxin comprises the steps of: (a) adding sulphuric acid into a Clostridium botulinum type A strain cultured medium up to pH 3 to 4 to produce and obtain a precipitate; (b) dissolving the precipitate in a buffer solution and adding ammonium sulfate into the buffer solution to obtain a precipitate; (c) diluting the precipitate in a phosphate buffer solution, treating the diluted solution with RNA hydrolyase and adding ammonium sulfate into the supernatant to obtain a precipitate; (d) dissolving the precipitate in a buffer solution and subjecting the buffer solution to ion exchange chromatography to obtain a fraction having A280/280 value of 0.6 or more; and (e) subjecting the fraction to gel filtration chromatography. copyright KIPO 2003

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(54) 출원명 클로스트리디움 보툴리눔 A형 독소를 정제하는 방법

요약

본 발명은 클로스트리디움 보툴리눔(*Clostridium botulinum*) A형 독소를 정제하는 방법에 관한 것으로, 구체적으로는 (a) 클로스트리디움 보툴리눔(*Clostridium botulinum*) A형 균주의 배양액에 황산을 첨가하여 pH를 3~4가 되도록 하여 침전물을 생성시킨 후, 침전물을 수득하는 단계; (b) 수득된 침전물을 완충용액으로 용해시킨 다음, 용해물을 황산 암모늄을 이용하여 침전시킨 후, 침전물을 수득하는 단계; (c) 상기 침전물을 포스페이트 완충용액으로 현탁한 다음, RNA 가수분해 효소를 처리한 후 불용화된 것을 제거하고 한 후 상등액을 황산암모늄을 이용하여 침전시킨 후 침전물을 수득하는 단계; (d) 수득된 침전물을 완충용액에 용해시킨 다음, 이온교환크로마토그래피를 수행하여 A260/280의 값이 0.6 이상되는 분획만을 수득하는 단계; (e) 수득된 분획에 대하여 겔 투과 크로마토그래피를 수행하는 단계를 포함하여 이루어지는 것을 특징으로 한다.

대표도

도1

색인어

클로스트리디움 보툴리눔, 독소, 이온교환크로마토그래피, 겔 투과 크로마토그래피

명세서

도면의 간단한 설명

도1은 본 발명의 클로스트리디움 보툴리눔 A형 독소의 분리 정제방법의 일 실시예의 각 단계에서 얻어진 시료를 전기영동한 결과를 나타내는 도면이다.

* 도면 기호의 간단한 설명.

1: 최초 배양액의 전기영동 결과.

2: 1단계 실시 후에 얻어진 시료에 대한 전기영동 결과.

3: 2단계 실시 후에 얻어진 시료에 대한 전기영동 결과.

4: 3단계 실시 후에 얻어진 시료에 대한 전기영동 결과.

5: 4단계 실시 후에 얻어진 시료에 대한 전기영동 결과.

6: 5단계 실시 후에 얻어진 시료에 대한 전기영동 결과.

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발명의 상세한 설명

발명의 목적

발명이 속하는 기술 및 그 분야의 종래기술

본 발명의 클로스트리디움 보툴리눔(*Clostridium botulinum*) A형 독소를 정제하는 방법에 관한 것이다.

신경독성을 지닌 독소를 분비하는 클로스트리디움 균주들이 1890년대에서부터 지금까지 발견되었으며, 지난 70년간 이들 균주들이 분비하는 독소에 대한 특성 규명이 연구되어 왔다(Schant, E. J. 등, *Microbiol. Rev.* 56; 80; 1992).

보툴리눔 독소는 그 혈청학적 특징에 따라 A 내지 G형의 7가지 형으로 나뉘어진다. 각 독소는 약 150KDa 정도의 독소 단백질을 가지고 있는데, 자연적으로는 여러 비독성 단백질들과 결합되어 있는 복합체로 이루어져 있다. 중간(Medium) 복합체(300kDa)는 독소 단백질과 비독성-비헤마글루티닌 단백질로 이루어져 있고, 큰(Large; 450kDa) 복합체 및 거대(Large-Large; 900kDa) 복합체는 중간 복합체가 헤마글루티닌과 결합되어 있는 형태를 띠고 있다(Sugiyama, H., *Microbiol. Rev.* , 44, 419; 1980). 이러한 비독성 비헤마글루티닌 단백질들은 장내에서 낮은 pH와 각종 단백질 가수분해 효소로부터 독소를 보호하는 기능을 하는 것으로 알려져 있다(Sugiyama, H., *Microbiol. Rev.* ,44, 419; 1980).

독소는 세포 내에서 분자량이 약 150kDa인 하나의 폴리펩티드로 합성된 후, 세포내 단백질 분해 효소의 작용이나 트립신과 같은 인위적인 효소 처리에 의하여 N말단으로부터 1/3되는 위치에서 절단되어 2개의 단위체인 경쇄(L: light chain)(분자량; 50 kDa)와 중쇄(H: heavy chain)(분자량; 100kDa)로 나뉘어진다. 이렇게 나뉘어진 독소는 단일 폴리펩티드일 때에 비해 그 독성이 크게 증가한다. 두 단위체는 이황화결합에 의해 서로 연결되어 있으며, 각각은 다른 기능을 갖는다. 중쇄는 목표물(target)이 되는 세포의 수용체(receptor)와 결합하고(Park. M.K., 등, *FEMS Microbiol. Lett.* 72, 243; 1990), 낮은 pH(pH4)에서 생체막과 반응하여 채널(channel)을 형성하는 기능을 가지며(Mantecucco, C. 등, *TIBS* 18, 324; 1993), 경쇄는 약리활성를 가지고 있어 세제(detergent)를 사용하여 세포에 투과성을 부여하거나, 전기천공(electroporation) 등으로 세포내 투입되었을 때 신경전달물질의 분비를 방해한다(Poulain, B. 등, *Proc. Natl. Acad. Sci. USA.* 85, 4090 ; 1988).

독소는 신경근육종말(neuromuscular junction)의 콜린성 전시냅스(cholinergic presynapse)에서 아세틸콜린의 엑소시토시스(exocytosis)를 저해하여 전신 무력증을 일으킨다. 극미량의 독소를 처리해도 독성이 나타나는 것으로 보아 이 독소는 어떤 효소 활성을 가질 것으로 생각되어져 왔다(Simpson, L. L. 등, *Ann. Rev. Pharmaeol. Toxicol.* 26, 427; 1986). 최근에 밝혀진 바에 의하면, 독소는 메탈로펩티다제 활성(metallopeptidase activity)을 갖고 있으며 그 기질은 엑소시토시스 장치 복합체(exocytosis machinary complex)를 이루는 단위 단백질들인 시냅토브레빈(Synaptobrevin), 신택신(Syntaxin), 25KDa의 시냅토좀 연계 단백질(Synaptosomal associated protein of 25KDa)(SNAP25) 등이다. 각 형의 독소는 이 세가지 단백질들 중 하나를 기질로 삼고 있는데, B, D, F 및 G형은 시냅토브레빈을, A와 E형은 SNAP25를, C형은 신택신을 특정 부위에서 절단하는 것으로 알려져 있다(Binz, T. 등, *J. Biol. Chem.* 265, 9153; 1994).

발명이 이루고자 하는 기술적 과제

본 발명은 상기와 같은 종래 기술의 문제점을 해결하기 위하여, 간단하고 편리한 방법으로 클로스트리디움 보툴리눔(*Clostridium botulinum*) A형 독소를 정제할 수 있는 방법을 제공하는 것을 그 목적으로 한다.

발명의 구성 및 작용

본 발명의 클로스트리디움 보툴리눔(*Clostridium botulinum*) A형 독소를 정제하는 방법은 다음 단계를 포함하여 이루어지는 것을 특징으로 한다.

(a) 클로스트리디움 보툴리눔(*Clostridium botulinum*) A형 균주의 배양액에 황산을 첨가하여 pH를 3~4가 되도록 하여 침전물을 생성시킨 후, 침전물을 수득하는 단계;

(b) 수득된 침전물을 완충용액으로 용해시킨 다음, 용해물을 황산 암모늄을 이용하여 침전시킨 후, 침전물을 수득하는 단계;

(c) 상기 침전물을 포스페이트 완충용액으로 현탁한 다음, RNA 가수분해 효소를 처리한 후 불용화된 것을 제거하고 한 후 상등액을 황산암모늄을 이용하여 침전시킨 후 침전물을 수득하는 단계;

(d) 수득된 침전물을 완충용액에 용해시킨 다음, 이온교환크로마토그래피를 수행하여 A260/280의 값이 0.6 이상되는 분획만을 수득하는 단계;

(e) 수득된 분획에 대하여 겔 투과 크로마토그래피를 수행하는 단계.

본 발명의 (a) 단계에서, 클로스트리디움 보툴리눔(*Clostridium botulinum*) A형 균주의 배양액에 황산을 가하여 pH를 산성화시킨다. 이때 사용하는 황산은 어떻한 농도의 것이라도 좋으나, 바람직하기로는 3N의 것이 사용될 수 있다. 황산을 pH3~4가 될 때까지 첨가한다. 그 후, 일정한 시간, 바람직하기로는 3~4시간 동안 방치한다. 방치 후 맑은 상층액과 침전물이 있는 하층을 분리하여, 상층을 조심스럽게 제거하고 하층을 원심분리하여 침전물을 수득한다.

본 발명의 (b) 단계에서, 수득된 침전물을 완충용액으로 용해시킨 다음, 용해물을 황산 암모늄을 이용하여 침전시킨 후, 침전물을 수득한다. 상기 완충용액은 0.1M 시트레이트 완충용액(0.1M 시트르산-트리소듐 시트레이트 완충용액, pH5.5)이 바람직하다. 상기 완충용액에 용해된 용액을 원심분리하여 용해액만을 얻는다. 완충용액에의 용해 및 원심분리에 의한 용해액 수득을 과정을 복수회 실시하여도 상관없다. 그 후, 황산 암모늄을 가하여 용해물을 침전시킨다. 이때 사용되는 황산 암모늄의 양은 35.1g/100ml인 것이 바람직하다. 이렇게 황산 암모늄에 의하여 침전된 침전물은 예를 들면, 원심분리를 통하여 수득한다.

본 발명의 (c) 단계에서, 수득된 침전물을 포스페이트 완충용액으로 현탁한 다음, RNA 가수분해 효소를 처리한 후 불용화된 것을

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제거하고 한 후 상등액을 황산암모늄을 이용하여 침전시킨 후 침전물을 수득한다. 상기 포스페이트 완충용액은 예를 들면, 0.005M Na_2HPO_4-NaH_2PO_4, pH 6.0인 완충용액이 사용될 수 있다. 포스페이트 완충용액 현탁액에 RNA 가수분해 효소를 첨가한다. 이때 첨가되는 효소의 양은 예를 들면, 50㎍/ml이 될 수 있다. RNA 가수분해 효소를 첨가한 후, 37℃에서 약 3시간 동안 반응시킨다. 그 후, 원심분리를 이용하여 RNA 가수분해에 의하여 불용화된 물질을 제거한 다음, 상등액을 황산 암모늄을 처리하여 침전시킨다. 황산 암모늄의 사용량은 35.1g/100ml이 바람직하다.

본 발명의 (d) 단계에서, 수득된 침전물을 완충용액에 용해시킨 다음, 이온교환크로마토그래피를 수행한다. 상기 완충용액은 0.05M 포스페이트 완충용액(pH 5.5)가 바람직하다. 완충용액 용해액을 이온교환 크로마토그래피, 예를 들면 DEAE-sephadex A50 칼럼(Pharmacia, 스웨덴)을 이용하여 음이온 교환크로마토그래피를 수행한다. 이때 이온교환 크로마토그래피를 수행하기 전에 완충용액 용해액을 투석막을 통과시키고 원심분리하여 불용성 물질을 미리 제거할 수도 있다. 이온교환 크로마토그래피 결과 나오는 분획 중 A260/A280의 값이 0.6 이상인 분획을 수득한다.

본 발명의 (e) 단계에서, 수득된 분획에 대하여 겔 투과 크로마토그래피를 수행한다. 본 발명에 사용될 수 있는 겔 투과 크로마토그래피용 칼럼으로는 예를 들면, Sephadex G-100 칼럼(Pharmacia, 스웨덴)이 사용될 수 있다. 겔 투과 크로마토그래피를 수행하기 전에 수득된 분획을 황산 암모늄으로 침전시키고, 이를 완충용액에 용해하여 불용성 물질을 제거할 수 있도록 하는 전처리를 할 수 있다. 상기 겔 투과 클로마토그래피 결과 관찰되는 2개의 피크 중 앞의 피크를 수득하여 황산 암모늄(39.1g/100ml)으로 침전시킴으로써 A형 독소와 비독소 단위체만으로 이루어진 복합체를 순수분리할 수 있게 된다.

이하 본 발명을 실시예를 통하여 보다 상세하게 설명한다. 그러나, 이들 실시예는 본 발명을 예시적으로 설명하기 위한 것으로 본 발명의 범위가 이 실시예에 한정되는 것은 아니다.

실시예

1단계: 황산으로 침전물 형성

클로스트리디움 보툴리눔 A형 균주 배양액을 3N 황산용액으로 pH 3.5이 될 때까지 산성화시켰다. 3~4 시간 동안 방치한 다음, 맑은 상층과 탁한 침전물이 있는 하층 중 상층을 조심스럽게 제거하고, 하층을 2,400xg로 4℃에서 20분 동안 원심분리하여 침전물을 수득하였다.

이렇게 얻어진 시료에 대하여 전기영동한 결과는 도1과 같다. 전기영동은 10% 폴리아크릴아미드 겔을 사용하여 수행하였으며, 수행 후 쿠마시블루로 염색하였다.

2단계: 침전물의 용해 및 황산 암모늄을 이용한 침전

0.1M 시트레이트 완충용액(0.1M citric acid-trisodium citrate butter, pH 5.5)으로 침전물을 1시간 가량 용해 및 용출시켰다. 원심분리(9800xg, 20분, 4℃)를 수행하여 용해액을 얻었다. 위의 과정을 한번 더 반복한 후 황산 암모늄(35.1 g/100ml)으로 용출물을 다시 4℃에서 밤새동안 침전시켰다.

이렇게 얻어진 시료에 대하여 전기영동한 결과는 도1과 같다. 전기영동은 10% 폴리아크릴아미드 겔을 사용하여 수행하였으며, 수행 후 쿠마시블루로 염색하였다.

3단계3: RNA 가수분해 효소의 처리

원심분리(12,000xg, 20분, 4℃)하여 얻은 침전물을 포스페이트 완충용액(0.005M Na_2HPO_4-NaH_2PO_4 완충용액, pH 6.0)으로 용해한 뒤, RNA 가수분해 효소(50mg/ml)를 첨가하고 37C에서 3시간 동안 반응시켰다. 다시 이를 23,700g로 20분간 상온에서 원심분리하여 불용화된 물질을 제거하고, 다시 상등액에 황산암모늄(35.1g/100ml)을 처리하여 4℃에서 밤새동안 침전시켰다.

이렇게 얻어진 시료에 대하여 전기영동한 결과는 도1과 같다. 전기영동은 10% 폴리아크릴아미드 겔을 사용하여 수행하였으며, 수행 후 쿠마시블루로 염색하였다.

4단계: 음이온 교환 크로마토그래피

원심분리(17,600xg, 20분, 4℃)하여 얻은 침전물을 0.05M 포스페이트 완충용액(pH 5.5)으로 용출시킨 후, 이를 투석막(Spectrum Co, 미국, MW Cut-off: 12,000)으로 투석시킨 후 다시 원심분리(17,600xg, 15분, 23℃)하여 불용성 물질을 제거한 뒤, 0.05M 시트레이트 완충용액으로 평형화시킨 DEAE-Sephadex A50 칼럼(Pharmacia, 스웨덴)에 분당 0.5ml의 속도로 통과시켰다. 이어서 같은 완충 용액으로 흘려 최초로 나오는 피크(A260/A280의 값이 0.6 이상되는 분획)를 수득하였다.

이렇게 얻어진 분획들에 대하여 전기영동한 결과는 도1과 같다. 전기영동은 10% 폴리아크릴아미드 겔을 사용하여 수행하였으며, 수행 후 쿠마시블루로 염색하였다.

5단계: 겔 투과 크로마토그래피

황산 암모늄(35.1g/100ml)을 처리하고 원심분리(17,600xg, 15분, 4℃)하여 다시 침전시키고, 이를 다시 0.05M 시트레이트 완충용액(pH 5.0)으로 용출시킨 다음, 원심분리(17,600xg, 15분, 23℃)하여 불용성 물질을 제거하였다. 0.05M 시트레이트 완충용액으로 평형화시킨 Sephadex G-100 칼럼(Pharmacia, 스웨덴)에 통과시킨 결과, 2개의 피크(peak)가 관찰되었는데 이 중 앞의 피크를 수득한 다음, 황산 암모늄(39.1g/100 ml)으로 침전시키고 원심분리(17,600xg, 20분, 4℃)하여 보툴리눔 A형 독소와 비독소 단위체만으로 이루어진 복합체를 순수분리할 수 있었다.

이렇게 얻어진 보툴리눔 A형 독소에 대하여 전기영동한 결과는 도1과 같다. 전기영동은 10% 폴리아크릴아미드 겔을 사용하여 수행하였으며, 수행 후 쿠마시블루로 염색하였다.

발명의 효과

본 발명의 방법에 따르면, 간단하고 편리한 방법으로 보툴리눔 A형 독소와 비독소 단위체만으로 이루어진 복합체를 순수분리할 수 있다.

(57) 청구의 범위

청구항 1.

다음 단계를 포함하여 이루어지는 것을 특징으로 하는 클로스트리디움 보툴리눔(*Clostridium botulinum*) A형 독소를 정제하는 방법:

(a) 클로스트리디움 보툴리눔(*Clostridium botulinum*) A형 균주의 배양액에 황산을 첨가하여 pH를 3~4가 되도록 하여 침전물을 생성시킨 후, 침전물을 수득하는 단계;

(b) 수득된 침전물을 완충용액으로 용해시킨 다음, 용해물을 황산 암모늄을 이용하여 침전시킨 후, 침전물을 수득하는 단계;

(c) 상기 침전물을 포스페이트 완충용액으로 현탁한 다음, RNA 가수분해 효소를 처리한 후 불용화된 것을 제거하고 한 후 상등액을 황산암모늄을 이용하여 침전시킨 후 침전물을 수득하는 단계;

(d) 수득된 침전물을 완충용액에 용해시킨 다음, 이온교환크로마토그래피를 수행하여 A260/280의 값이 0.6 이상되는 분획만을 수득하는 단계;

(e) 수득된 분획에 대하여 겔 투과 크로마토그래피를 수행하는 단계.

청구항 2.

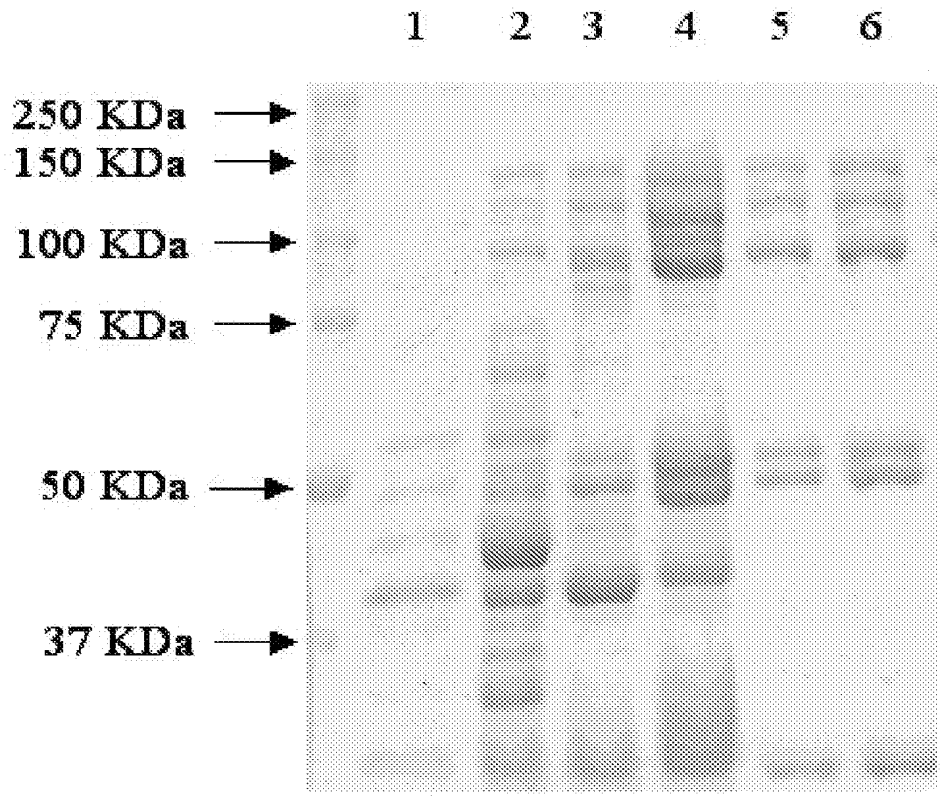
제1항에 있어서, (b) 단계의 상기 완충용액은 0.1M 시트레이트 완충용액(0.1M 시트르산-트리소듐 시트레이트 완충용액, pH5.5)임을 특징으로 하는 클로스트리디움 보툴리눔(*Clostridium botulinum*) A형 독소를 정제하는 방법.

청구항 3.

제1항에 있어서, (b) 단계에서 황산 암모늄은 35.1g/100ml의 양으로 첨가하는 것을 특징으로 하는 클로스트리디움 보툴리눔(*Clostridium botulinum*) A형 독소를 정제하는 방법.

도면

도면 1



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— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

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(54) **Title**: PROCESS AND SYSTEM FOR OBTAINING BOTULINUM NEUROTOXIN

(57) **Abstract**: Rapid, animal protein free, chromatographic processes and systems for obtaining high potency, high yield botulinum neurotoxin for research, therapeutic and cosmetic use.

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PROCESS AND SYSTEM FOR OBTAINING BOTULINUM NEUROTOXIN

by

Jennifer L. Ton, Hemant A. Patel, Ronald C. Bates and Wajdie M. Ahmad

CROSS-REFERENCE

This application claims the benefit of U.S. Patent Application Serial Number 12/502,181 filed on July 13, 2009, the entire disclosure of which is incorporated herein by this specific reference.

BACKGROUND

The present invention relates to systems and processes for obtaining a Clostridial neurotoxin, methods for making a pharmaceutical composition therefrom and to therapeutic and cosmetic uses of the pharmaceutical composition so made. In particular, the present invention relates to a rapid, animal protein free, chromatographic process and system for obtaining a high potency, high purity, and high yield biologically active botulinum neurotoxin.

A pharmaceutical composition suitable for administration to a human or animal for a therapeutic, diagnostic, research or cosmetic purpose comprises an active ingredient and one or more excipients, buffers, carriers, stabilizers, tonicity adjusters, preservatives and/or bulking agents. The active ingredient in a pharmaceutical composition can be a biologic such as a botulinum neurotoxin. Known methods (such as the Schantz method) for obtaining a botulinum neurotoxin useful as the active ingredient in a pharmaceutical composition are multi-week culturing, fermentation and purification processes which use animal-derived proteins, such as meat broth and casein used in culture and fermentation media, and animal derived purification enzymes. Administration to a patient of a pharmaceutical composition made through use of animal derived products can entail risk of administering pathogens or an infectious agent, such as a prion. Additionally, known animal protein free methods for obtaining a botulinum toxin are also time-consuming processes (i.e. take more than a week to complete) with numerous upstream (culturing and fermentation) and downstream (purification) steps, and yet still result in obtaining a botulinum neurotoxin with detectable impurities.

Botulinum toxin

The genus Clostridium has more than one hundred and twenty seven species, grouped by morphology and function. The anaerobic, gram positive bacterium *Clostridium botulinum* produces a potent polypeptide neurotoxin, botulinum toxin (synonymously "toxin"), which causes a neuroparalytic illness in humans and animals known as botulism. Symptoms of botulinum toxin intoxication can progress from difficulty walking, swallowing, and speaking to paralysis of the respiratory muscles and death.

One unit of botulinum toxin is defined as the LD_{50} upon intraperitoneal injection into female Swiss Webster mice weighing about 18-20 grams each. One unit of botulinum toxin is the amount of botulinum toxin that kills 50% of a group of female Swiss Webster mice. Seven generally immunologically distinct botulinum neurotoxins have been characterized, these being respectively botulinum neurotoxin serotypes A, B, C_1, D, E, F and G each of which is distinguished by neutralization with type-specific antibodies. The different serotypes of botulinum toxin vary in the animal species that they affect and in the severity and duration of the paralysis they evoke. The botulinum toxins apparently bind with high affinity to cholinergic motor neurons and translocate into the neuron and block the presynaptic release of acetylcholine.

Botulinum toxins have been used in clinical settings for the treatment of e.g. neuromuscular disorders characterized by hyperactive skeletal muscles. Botulinum toxin type A has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of essential blepharospasm, strabismus and hemifacial spasm in patients over the age of twelve, cervical dystonia, glabellar line (facial) wrinkles and for treating hyperhydrosis. The FDA has also approved a botulinum toxin type B for the treatment of cervical dystonia.

Although all the botulinum toxins serotypes apparently inhibit release of the neurotransmitter acetylcholine at the neuromuscular junction, they do so by affecting different neurosecretory proteins and/or cleaving these proteins at different sites. Botulinum toxin type A is a zinc endopeptidase which can specifically hydrolyze a peptide linkage of the intracellular, vesicle-associated protein (VAMP, also called synaptobrevin) 25 kiloDalton (kDa) synaptosomal associated protein (SNAP-25). Botulinum type E also cleaves SNAP-25 but targets different amino acid sequences within this protein, as compared to botulinum toxin type A. Botulinum toxin types B, D, F and G act on VAMP with each serotype cleaving the protein at a different site.

Finally, botulinum toxin type C_1 has been shown to cleave both syntaxin and SNAP-25. These differences in mechanism of action may affect the relative potency and/or duration of action of the various botulinum toxin serotypes.

The molecular weight of the active botulinum toxin protein molecule (also known as “pure toxin” or as the ”neurotoxic component”) from a botulinum toxin complex, for all seven of the known botulinum toxin serotypes, is about 150 kDa. Interestingly, the botulinum toxins are released by Clostridial bacterium as complexes comprising the 150 kDa neurotoxic component along with one or more associated non-toxin proteins. Thus, the botulinum toxin type A complex can be produced by Clostridial bacterium as 900 kDa, 500 kDa and 300 kDa forms (approximate molecular weights). Botulinum toxin types B and C_1 are apparently produced as only a 500 kDa complex. Botulinum toxin type D is produced as both 300 kDa and 500 kDa complexes. Finally, botulinum toxin types E and F are produced as only approximately 300 kDa complexes. The complexes (i.e. molecular weight greater than about 150 kDa) contain hemagglutinin (HA) proteins and a non-toxin non-hemagglutinin (NTNH) protein. Thus, a botulinum toxin complex can comprise a botulinum toxin molecule (the neurotoxic component) and one or more HA proteins and/or NTNH protein. These two types of non-toxin proteins (which along with the botulinum toxin molecule can comprise the relevant neurotoxin complex) may act to provide stability against denaturation to the botulinum toxin molecule and protection against digestive acids when toxin is ingested. Additionally, it is possible that the larger (greater than about 150 kDa molecular weight) botulinum toxin complexes may result in a slower rate of diffusion of the botulinum toxin away from a site of intramuscular injection of a botulinum toxin complex. The success of botulinum toxin type A to treat a variety of clinical conditions has led to interest in other botulinum toxin serotypes. Thus, at least botulinum toxins types, A, B, E and F have been used clinically in humans. Additionally, a formulation of the neurotoxic component (i.e. without the associated non-toxin proteins) is sold in Europe under the tradename XEOMIN (Merz Pharmaceuticals, Frankfurt, Germany).

The botulinum toxin type A is known to be soluble in dilute aqueous solutions at pH 4-6.8. At pH above about 7 the stabilizing non-toxin proteins dissociate from the neurotoxin, resulting in a gradual loss of toxicity, particularly as the pH and temperature rise (Schantz E.J., et al *Preparation and characterization of botulinum*

toxin type A for human treatment (in particular pages 44-45), being chapter 3 of Jankovic, J., et al, Therapy with Botulinum Toxin, Marcel Dekker, Inc, 1994).

As with enzymes generally, the biological activities of the botulinum toxins (which are intracellular peptidases) are dependant, at least in part, upon their three dimensional conformation. Dilution of the toxin from milligram quantities to a solution containing nanograms per milliliter presents significant difficulties, such as, for example, tendency for toxin to adhere to surfaces and thus reduce the amount of available toxin. Since the toxin may be used months or years after the toxin containing pharmaceutical composition is formulated, the toxin is stabilized with a stabilizing agent such as albumin, sucrose, trehalose and/or gelatin.

A commercially available botulinum toxin containing pharmaceutical composition is sold under the trademark BOTOX® (botulinum toxin type A purified neurotoxin complex) available commercially from Allergan, Inc., of Irvine, California. Each 100 unit vial of BOTOX® consists of about 5 ng of purified botulinum toxin type A complex, 0.5 mg human serum albumin, and 0.9 mg sodium chloride, vacuum-dried form and intended for reconstitution with sterile normal saline without a preservative (0.9% sodium chloride injection). Other commercially available, botulinum toxin-containing pharmaceutical compositions include Dysport® (*Clostridium botulinum* type A toxin hemagglutinin complex with human serum albumin and lactose in the botulinum toxin pharmaceutical composition), available from Ipsen Limited, Berkshire, U.K. as a powder to be reconstituted with 0.9% sodium chloride before use), and MyoBloc™ (an injectable solution comprising botulinum toxin type B, human serum albumin, sodium succinate, and sodium chloride at about pH 5.6, available from Solstice Neurosciences of San Diego, California. The neurotoxic component (the 150 kDa toxin molecule) and botulinum toxin complexes (300 kDa to 900 kDa) can be obtained from, for example, List Biological Laboratories, Inc., Campbell, California; the Centre for Applied Microbiology and Research, Porton Down, U.K.; Wako (Osaka, Japan), as well as from Sigma Chemicals of St Louis, Missouri.

Animal protein free and/or chromatographic methods for obtaining a botulinum neurotoxin are disclosed in U.S. patents 7,445,914; 7,452,697; 7,354,740; 7,160,699; 7,148,041, and; 7,189,541. Also of interest are U.S. patent applications 11/609,449 entitled “Media for Clostridium Bacterium”, filed December 12, 2006; 12/098,896 entitled “Animal Product Free Media and Processes for Obtaining a Botulinum Toxin”,

filed April 7, 2008; 11/932,689 entitled “Chromatographic Method and System for Purifying a Botulinum Toxin”, filed October 31, 2007; 11/932,789 entitled “Chromatographic Method and System for Purifying a Botulinum Toxin” filed October 31, 2007, and;12/234,537, entitled “Animal Product Free Media And Processes For Obtaining A Botulinum Toxin”, filed September 19, 2008.

Botulinum toxin for use in a pharmaceutical composition can be obtained by anaerobic fermentation of *Clostridium botulinum* using the well known Schantz process (see e.g. Schantz E.J., et al., *Properties and use of botulinum toxin and other microbial neurotoxins in medicine,* Microbiol Rev 1992 Mar;56(1):80-99; Schantz E.J., et al., *Preparation and characterization of botulinum toxin type A for human treatment*, chapter 3 in Jankovic J, ed. Neurological Disease and Therapy. Therapy with botulinum toxin (1994), New York, Marcel Dekker;1994, pages 41-49, and; Schantz E.J., et al., *Use of crystalline type A botulinum toxin in medical research,* in: Lewis GE Jr, ed. Biomedical Aspects of Botulism (1981) New York, Academic Press, pages 143-50). The Schantz process for obtaining a botulinum toxin makes use of animal products for example as reagents and as part of the culture and fermentation media.

A number of steps are required to make a Clostridial toxin pharmaceutical composition suitable for administration to a human or animal for a therapeutic, diagnostic, research or cosmetic purpose. These steps can include obtaining a purified Clostridial toxin and then compounding the purified Clostridial toxin. A first step can be to plate and grow colonies of Clostridial bacteria, typically on blood agar plates, in an environment conducive to anaerobic bacterial growth, such as in a warm anaerobic atmosphere. This step allows Clostridial colonies with desirable morphology and other characteristics to be obtained. In a second step selected Clostridial colonies can be fermented in a first suitable medium and if additionally desired, into a second fermentation medium. After a certain period of fermentation, the Clostridial bacteria typically lyse and release Clostridial toxin into the medium. Thirdly, the medium can be purified so as to obtain a bulk toxin. Typically medium purification to obtain bulk toxin is carried out using, among other reagents, animal-derived enzymes, such as DNase and RNase, which are used to degrade and facilitate removal of nucleic acids. The resulting bulk toxin can be a highly purified toxin with a particular specific activity. After stabilization in a suitable buffer, the bulk

toxin can be compounded with one or more excipients to make a Clostridial toxin pharmaceutical composition suitable for administration to a human. The Clostridial toxin pharmaceutical composition can comprise a Clostridial toxin as an active pharmaceutical ingredient (API). The pharmaceutical composition can also include one or more excipients, buffers, carriers, stabilizers, preservatives and/or bulking agents.

The Clostridium toxin fermentation step can result in a fermentation medium solution that contains whole Clostridium bacteria, lysed bacteria, culture medium nutrients and fermentation by-products. Filtration of this culture solution so as to remove gross elements, such as whole and lysed bacteria, provides a harvest/clarified medium. The clarified medium comprises a Clostridial toxin and various impurities and is processed to obtain a concentrated Clostridial toxin, which is called bulk toxin.

Fermentation and purification processes for obtaining a bulk Clostridial toxin using one or more animal derived products (such as the milk digest casein, DNase and RNase) are known. An example of such a known non-animal product free ("NAPF") process for obtaining a botulinum toxin complex is the Schantz process and modifications thereto. The Schantz process (from initial plating, cell culture through to fermentation and toxin purification) makes use of a number of products derived from animal sources such as, for example, animal derived Bacto Cooked Meat medium in the culture vial, Columbia Blood Agar plates for colony growth and selection, and casein in the fermentation media. Additionally, the Schantz bulk toxin purification process makes use of DNase and RNase from bovine sources to hydrolyze nucleic acids present in the toxin containing fermentation medium. Concerns have been expressed regarding a potential for a viral and transmissible spongiform encephalopathy (TSE), such as a bovine spongiform encephalopathy (BSE), contamination when animal products are used in a process for obtaining an API and/or in a process for making (compounding) a pharmaceutical composition using such an API.

A fermentation process for obtaining a tetanus toxoid that uses reduced amounts of animal-derived products (referred to as animal product free or "APF" fermentation processes; APF encompasses animal protein free) is known, see e.g. U.S. patent 6,558,926. An APF fermentation process for obtaining a Clostridial toxin,

has the potential advantage of reducing the (the already very low) possibility of contamination of the ensuing bulk toxin with viruses, prions or other undesirable elements which can then accompany the active pharmaceutical ingredient, Clostridial toxin, as it is compounded into a pharmaceutical composition for administration to humans.

Chromatography, such as column chromatography for example, can be used to separate a particular protein (such as a botulinum neurotoxin) from a mixture of proteins, nucleic acids, cell debris, etc. in a process known as fractionation or purification. The protein mixture typically passes through a glass or plastic column containing, for example, a solid, often porous media (often referred to as beads or resin). Different proteins and other compounds pass through the matrix at different rates based on their specific chemical characteristics and the way in which these characteristics cause them to interact with the particular chromatographic media utilized.

The choice of media determines the type of chemical characteristic by which the fractionation of the proteins is based. There are four basic types of column chromatography; ion-exchange, gel filtration, affinity and hydrophobic interaction. Ion-exchange chromatography accomplishes fractionation based on surface electrostatic charge using a column packed with small beads carrying either a positive or a negative charge. In gel filtration chromatography, proteins are fractionated based on their size. In affinity chromatography, proteins are separated based on their ability to bind to specific chemical groups (ligand) attached to beads in the column matrix. Ligands can be biologically specific for a target protein. Hydrophobic interaction chromatography accomplishes fractionation based on surface hydrophobicity.

Column chromatography to purify (fractionate) a Clostridial toxin is well known. See for example the following publications:

1. Ozutsumi K., et al, *Rapid, simplified method for production and purification of tetanus toxin*, App & Environ Micro, Apr. 1985, p 939-943, vol 49, no. 4.(1985) discloses use of high pressure liquid chromatography (HPLC) gel filtration to purify tetanus toxin.

2. Schmidt J.J., et al., *Purification of type E botulinum neurotoxin by high-performance ion exchange chromatography*, Anal Biochem 1986 Jul;156(1):213-219

discloses use of size exclusion chromatography or ion exchange chromatograph to purify botulinum toxin type E. Also disclosed is use of protamine sulfate instead of ribonuclease (RNase).

3. Simpson L.L., et al., *Isolation and characterization of the botulinum neurotoxins Simpson LL; Schmidt JJ; Middlebrook JL,* In: Harsman S, ed. Methods in Enzymology. Vol. 165, Microbial Toxins: Tools in Enzymology San Diego, CA: Academic Press;vol 165:pages 76-85 (1988) discloses purification of botulinum neurotoxins using gravity flow chromatography, HPLC, capture steps using an affinity resin, size exclusion chromatography, and ion (anion and cation) exchange chromatography, including use of two different ion exchange columns. Various Sephadex, Sephacel, Trisacryl, S and Q columns are disclosed.

4. Zhou L., et al., *Expression and purification of the light chain of botulinum neurotoxin A: A single mutation abolishes its cleavage of SNAP-25 and neurotoxicity after reconstitution with the heavy chain,* Biochemistry 1995;34(46):15175-81 (1995) discloses use of an amylose affinity column to purify botulinum neurotoxin light chain fusion proteins.

5. Kannan K., et al., *Methods development for the biochemical assessment of Neurobloc (botulinum toxin type B),* Mov Disord 2000;15(Suppl 2):20 (2000) discloses use of size exclusion chromatography to assay a botulinum toxin type B.

6. Wang Y-c, *The preparation and quality of botulinum toxin type A for injection (BTXA) and its clinical use,* Dermatol Las Faci Cosm Surg 2002;58 (2002) discloses ion exchange chromatography to purify a botulinum toxin type A. This reference discloses a combination of precipitation and chromatography techniques.

7. Johnson S.K., et al., *Scale-up of the fermentation and purification of the recombination heavy chain fragment C of botulinum neurotoxin serotype F, expressed in Pichia pastoris* , Protein Expr and Purif 2003;32:1-9 (2003) discloses use of ion exchange and hydrophobic interaction columns to purify a recombinant heavy chain fragment of a botulinum toxin type F.

8. Published U.S. patent application 2003 0008367 A1 (Oguma) discloses use of ion exchange and lactose columns to purify a botulinum toxin.

The purification methods summarized above relate to small-scale purification of the neurotoxic component of a botulinum toxin complex (i.e. the approximately 150

kDa neurotoxic molecule), or a specific component of the neurotoxic component, as opposed to purification of the entire 900 kDa botulinum toxin complex.

Furthermore, existing processes, including commercial scale processes, for obtaining a botulinum toxin suitable for compounding into a botulinum toxin pharmaceutical composition typically include a series of precipitation steps to separate the toxin complex from impurities that accompany the botulinum toxin from the fermentation process. Notably, precipitation techniques are widely used in the biopharmaceutical industry to purification a botulinum toxin. For example, cold alcohol fractionation (Cohn's method) or precipitation is used to remove plasma proteins. Unfortunately, previous precipitation techniques for purifying a botulinum toxin have the drawbacks of low resolution, low productivity, difficulty of operation, difficulty to control and/or validate and/or difficulty to scale-up or scale-down. Previously published U.S. Patent Application Serial No. 11/452,570, published October 12, 2006, discloses steps such as centrifugation, acid precipitation, ethanol precipitation, acidification steps, and ammonium sulfate precipitation utilized in various animal-protein free and NAPF processes (for a detailed discussion, see U.S. Published Patent App. No. 2006/0228780, herein incorporated by reference in its entirety). Some distinctions between a non-animal protein free process and an animal protein free processes for obtaining a botulinum neurotoxin are shown therein.

What are needed therefore are rapid, relatively smaller scale yet high yield systems and processes for obtaining high purity, highly potent botulinum neurotoxin, which can be used for research purposes and/or to make a pharmaceutical composition.

SUMMARY

The present invention meets this need and provides high purity, highly potent botulinum neurotoxins obtainable by rapid, smaller scaled, commercially useful, high yield, animal protein free, chromatographic systems and processes. The resultant botulinum neurotoxin is useful for making a pharmaceutical composition. The Clostridial toxin obtained by the practice of our invention is preferably a botulinum neurotoxin and most preferably a botulinum neurotoxin type A complex of about 900 kDa or the 150 kDa neurotoxic component therefrom. Our invention does not require NAPF reagents, such as DNase and RNase.

Definitions

The following words and terms used herein have the following definitions.

“About” means that the item, parameter or term so qualified encompasses a range of plus or minus ten percent above and below the value of the stated item, parameter or term.

"Administration,” or "to administer" means the step of giving (i.e. administering) a pharmaceutical composition or active ingredient to a subject. The pharmaceutical compositions disclosed herein are "locally administered" by e.g. intramuscular (i.m.), intradermal, subcutaneous administration, intrathecal administration, intracranial, intraperitoneal (i.p.) administration, topical (transdermal) and implantation (i.e. of a slow-release device such as polymeric implant or miniosmotic pump) routes of administration.

"Animal product free" or "substantially animal product free" encompasses, respectively, “animal protein free” or "substantially animal protein free" and means the absence or substantial absence of blood derived, blood pooled and other animal derived products or compounds. “Animal” means a mammal (such as a human), bird, reptile, fish, insect, spider or other animal species. “Animal” excludes microorganisms, such as bacteria. Thus, an APF medium or process or a substantially APF medium or process within the scope of the present invention can include a botulinum toxin or a *Clostridial botulinum* bacterium. For example, an APF process or a substantially APF process means a process which is either substantially free or essentially free or entirely free of animal-derived proteins, such as immunoglobulins, meat digest, meat by products and milk or dairy products or digests.

"Botulinum toxin" or "botulinum neurotoxin: means a neurotoxin produced by *Clostridium botulinum*, as well as modified, recombinant, hybrid and chimeric botulinum toxins. A recombinant botulinum toxin can have the light chain and/or the heavy chain thereof made recombinantly by a non-Clostridial species. “Botulinum toxin,” as used herein, encompasses the botulinum toxin serotypes A, B, C, D, E, F and G. "Botulinum toxin,” as used herein, also encompasses both a botulinum toxin complex (i.e. the 300, 600 and 900 kDa complexes) as well as pure botulinum toxin (i.e. the about 150 kDa neurotoxic molecule), all of which are useful in the practice of the present invention. "Purified botulinum toxin" means a pure botulinum toxin or a

botulinum toxin complex that is isolated, or substantially isolated, from other proteins and impurities which can accompany the botulinum toxin as it is obtained from a culture or fermentation process. Thus, a purified botulinum toxin can have at least 90%, preferably more than 95%, and most preferably more than 99% of the non-botulinum toxin proteins and impurities removed. The botulinum C_2 and C_3 cytotoxins, not being neurotoxins, are excluded from the scope of the present invention.

"Clostridial neurotoxin" means a neurotoxin produced from, or native to, a Clostridial bacterium, such as C*lostridium botulinum, Clostridium butyricum or Clostridium beratti*, as well as a Clostridial neurotoxin made recombinantly by a non-Clostridial species.

"Entirely free" ("consisting of" terminology) means that within the detection range of the instrument or process being used, the substance cannot be detected or its presence cannot be confirmed.

"Essentially free" (or "consisting essentially of"') means that only trace amounts of the substance can be detected.

"Modified botulinum toxin" means a botulinum toxin that has had at least one of its amino acids deleted, modified, or replaced, as compared to a native botulinum toxin. Additionally, the modified botulinum toxin can be a recombinantly produced neurotoxin, or a derivative or fragment of a recombinantly made neurotoxin. A modified botulinum toxin retains at least one biological activity of the native botulinum toxin, such as, the ability to bind to a botulinum toxin receptor, or the ability to inhibit neurotransmitter release from a neuron. One example of a modified botulinum toxin is a botulinum toxin that has a light chain from one botulinum toxin serotype (such as serotype A), and a heavy chain from a different botulinum toxin serotype (such as serotype B). Another example of a modified botulinum toxin is a botulinum toxin coupled to a neurotransmitter, such as substance P.

"Pharmaceutical composition" means a formulation in which an active ingredient can be a botulinum toxin. The word "formulation" means that there is at least one additional ingredient (such as, for example and not limited to, an albumin [such as a human serum albumin or a recombinant human albumin] and/or sodium chloride) in the pharmaceutical composition in addition to a botulinum neurotoxin active ingredient. A pharmaceutical composition is therefore a formulation which is

suitable for diagnostic, therapeutic or cosmetic administration (e.g. by intramuscular or subcutaneous injection or by insertion of a depot or implant) to a subject, such as a human patient. The pharmaceutical composition can be: in a lyophilized or vacuum dried condition, a solution formed after reconstitution of the lyophilized or vacuum dried pharmaceutical composition with saline or water, for example, or; as a solution that does not require reconstitution. The active ingredient can be one of the botulinum toxin serotypes A, B, C_1, D, E, F or G or a botulinum toxin, all of which can be made natively by Clostridial bacteria. As stated, a pharmaceutical composition can be liquid or solid, for example vacuum-dried. Exemplary methods for formulating a botulinum toxin active ingredient pharmaceutical composition are disclosed in published U.S. patent publication 20030118598, filed November 5, 2002, herein incorporated by reference in its entirety.

"Substantially free" means present at a level of less than one percent by weight of a culture medium, fermentation medium, pharmaceutical composition or other material in which the weight percent of a substance (such as an animal product, animal protein or animal derived product or protein) is assessed.

"Therapeutic formulation" means a formulation can be used to treat and thereby alleviate a disorder or a disease and/or symptom associated thereof, such as a disorder or a disease characterized by hyperactivity (e.g. spasticity) of a peripheral muscle or gland, (e.g. sweat gland).

"Therapeutically effective amount" means the level, amount or concentration of an agent (e.g. such as a botulinum toxin or pharmaceutical composition comprising botulinum toxin) needed to treat a disease, disorder or condition without causing significant negative or adverse side effects.

"Treat", "treating", or "treatment" means an alleviation or a reduction (which includes some reduction, a significant reduction a near total reduction, and a total reduction), resolution or prevention (temporarily or permanently) of an disease, disorder or condition, such as a buttock deformity, so as to achieve a desired therapeutic or cosmetic result, such as by healing of injured or damaged tissue, or by altering, changing, enhancing, improving, ameliorating and/or beautifying an existing or perceived disease, disorder or condition. A treatment effect, such as an alleviating effect from administration of a botulinum neurotoxin may not appear clinically for between 1 to 7 days after administration of the botulinum neurotoxin to a patient for

example and can have a duration of effect of from about 1 month to about 1 year or any range of time therebetween, for example, depending upon the condition and particular case being treated.

Percentages are based on weight per volume unless otherwise noted.

APF means animal product/protein free

CV means column volume

DF means diafiltration

ELISA means enzyme-linked immunosorbent assay.

IAPF, as in "IAPF system" or "IAPF process", means "improved animal protein free" system or process. An IAPF system or process includes the use of either two chromatography media or three chromatography media to purify a botulinum toxin or neurotoxin component, as specifically detailed herein. Chromatography media includes chromatography resins, as known in the art. Batches of botulinum neurotoxin obtained by use of two chromatography media are herein designated as IAPF.

FAPF, as in "FAPF system" or "FAPF process", means "further improved animal protein free" system or process. Accordingly, FAPF is an IAPF process, and a FAPF system or process means that three chromatography media are used to purify a botulinum toxin or neurotoxin component. Batches of botulinum neurotoxin obtained by use of three chromatography media are herein designated as FAPF.

NAPF means non-animal protein free

SDS-PAGE means sodium dodecylsulfate polyacrylamide gel electrophoresis

SEC-HPLC means size exclusion high performance liquid chromatography

UF means ultrafiltration

In one embodiment of the invention, a substantially APF chromatographic process for obtaining a biologically active botulinum neurotoxin is provided, the process comprising the following steps of (a) providing a substantially APF fermentation medium; (b) fermenting *Clostridium botulinum* bacteria in the fermentation medium, and; (c) recovering the biologically active botulinum neurotoxin from the fermentation medium by contacting the fermentation medium with an anion exchange chromatography media followed by contacting an eluent from the anion exchange chromatography medium with a cation exchange chromatography media, to thereby obtain the biologically active botulinum neurotoxin from the substantially

APF chromatographic process. In particular embodiments, the process can provide a botulinum neurotoxin that comprises less than one part per million (ppm) residual nucleic acid which is one nanogram or less of residual nucleic acid for each milligram of the botulinum neurotoxin obtained. In still another aspect, the process is carried out in one week or less.

In one example, media having a ratio of 3:1:1 means a botulinum toxin culture/fermentation medium containing 3% HySoy, 1% HyYeast, and 1% glucose. HySoy (Quest product no. 5X59022) is a source of peptides made by enzymatic hydrolysis of soy. HyYeast (HyYest, Quest product no. 5Z10102 or 5Z10313 is a baker's yeast extract. In another example, media having a ratio of 5:1:1 means a botulinum toxin culture/fermentation medium containing 5% HySoy, 1% HyYeast, and 1% glucose.

Another embodiment provides a substantially APF chromatographic process for obtaining a biologically active botulinum neurotoxin type A complex, the process comprising the following sequential steps of culturing *Clostridium botulinum* bacteria in a substantially APF culture medium; fermenting *Clostridium botulinum* bacteria from the culture medium in about 2 L to about 75 L of a substantially APF fermentation medium, more preferably in about 2 L to about 50 L of a substantially APF fermentation medium, even more preferably in about 2 L to about 30 L of a substantially APF fermentation medium (particular embodiments have at least one of the culture medium and the fermentation medium including a vegetable protein and/or a vegetable protein derivative, for example a hydrolyzed vegetable protein), harvesting the fermentation medium by removing cellular debris present in the fermentation medium using filtration or centrifugation; concentrating the harvested fermentation medium by filtration, such as by ultrafiltration (UF) for example; diluting the concentrated fermentation medium by adding a buffer. Following dilution with the buffer, a first contacting step is undertaken in which the diluted harvested fermentation medium is contacted with an anion exchange media so that the biologically active botulinum neurotoxin becomes captured by the anion exchange media; followed by elution of the captured botulinum neurotoxin from the anion exchange media to thereby obtain a first eluent containing the botulinum toxin; performing a second contacting step in which the first eluent is contacted with a cation exchange media to remove impurities from the first eluent, to thereby obtain a

second eluent containing the botulinum toxin; followed by processing the second eluent by diafiltration (DF); and filtering the processed second eluent, thereby obtaining biologically active botulinum neurotoxin type A complex using a substantially APF chromatographic process. The botulinum neurotoxin type A complex obtained can have a potency of about 2.0 x 10^7 units/mg to about 6.0 x 10^7 units/mg of botulinum neurotoxin type A complex. In particular examples botulinum neurotoxin type A complex having a potency of between about 2.4 x 10^7 units/mg to about 5.9 x 10^7 units/mg, for example, can be obtained.

In a particular embodiment, the process utilizes fermentation medium comprising no more than about 5% w/v of a vegetable-derived protein product, no more than about 2% w/v of a yeast extract and no more than about 2% w/v glucose, and wherein the pH level of the fermentation medium is from about 6.5 to about pH 8.0, more preferably from about pH 6.8 to about pH 7.6, at the commencement of the fermenting step. In a particular embodiment, the culturing step is carried out until the optical density of the culture medium at about 540 nanometers (nm) is between about 0.8 absorbance units (AU) and about 4.5 AU. The culturing step is preferably initiated by introducing a *Clostridium botulinum* APF working cell bank content to the culture medium, where the working cell bank content comprises at least about 1 x 10^4 colony-forming units, preferably from about 1 x 10^4 to about 5 x 10^7 colony-forming units of *Clostridium botulinum* per milliliter of the working cell bank, and where the *Clostridium botulinum* bacterium in the working cell bank have a substantially uniform morphology. In still yet another embodiment, the fermenting step is carried out for about 60 to 80 hours and until an optical density of the fermentation medium at about 890 nm decreases to between about 0.05 AU to about 0.7 AU. In one aspect, the botulinum neurotoxin obtained by a substantially APF chromatographic process comprises less than 1 ppm of residual nucleic acid and the process is carried out in one week or less.

In yet another embodiment, an APF process utilizing chromatography for obtaining a biologically active botulinum neurotoxin is provided, comprising the sequential steps of: (a) adding *Clostridium botulinum* bacteria from an APF working cell bank to an APF culture medium; (b) culturing the *Clostridium botulinum* bacteria in the culture medium; (c) fermenting the *Clostridium botulinum* bacteria from step (b) in an APF fermentation medium until *Clostridium botulinum* cell lysis occurs; (d)

harvesting the fermentation culture to provide a harvested fermentation medium; (e) subjecting the harvested fermentation medium to concentration by filtration; (f) diluting the filtered fermentation medium by addition of a buffer to obtain a diluted fermentation medium; (g) a first contacting step in which the diluted fermentation medium is contacted with a capture chromatography media, wherein the capture chromatography media is an anion exchange media; (h) a second contacting step wherein an eluent from the first contacting step is contacted with a polishing chromatography media, wherein the polishing chromatography media is a cation exchange media, and (i) filtering eluent from the second contacting step, thereby obtaining biologically active botulinum neurotoxin by the improved APF process, wherein the botulinum neurotoxin obtained comprises 1 ppm of residual nucleic acid or less than 1 ppm of residual nucleic acid and the process is carried out in one week or less.

In one aspect, a substantially animal product free (APF) chromatographic system for obtaining a biologically active botulinum neurotoxin is provided, comprising a substantially APF fermentation medium, *Clostridium botulinum* bacteria for fermenting in the fermentation medium, an anion exchange chromatography medium for recovering biologically active botulinum neurotoxin from the fermentation medium, and a cation exchange chromatography medium for recovering further biologically active botulinum neurotoxin from an eluent from the anion exchange chromatography medium, thereby obtaining biologically active botulinum neurotoxin from a substantially APF chromatography process. In particular configurations, the system can further comprise a first apparatus for anaerobically culturing the *Clostridium botulinum* bacteria in a substantially APF culture medium, and can further be comprised of a second apparatus for anaerobically fermenting the *Clostridium botulinum* bacteria in the substantially APF fermentation medium, wherein the *Clostridium botulinum* bacteria are obtained from the first apparatus. For clarification, the system can include a harvesting apparatus for removing cellular debris from the fermentation medium obtained from the second apparatus, to thereby provide a harvested fermentation medium. The harvested fermentation medium can be passed through a concentration and diluting apparatus to concentrate then subsequently dilute the harvested fermentation medium. In a particular example, the system can also include hydrophobic interaction medium for recovering further purified

biologically active botulinum neurotoxin from an eluent from the cation exchange chromatography medium. Additionally, a filtration apparatus for reducing bioburden in the obtained biologically active botulinum neurotoxin can also make up the system, for reducing the bioburden of the biologically active botulinum neurotoxin obtained by utilizing either two or three chromatography medium. In a specific example, an anaerobic chamber having an integrated high efficiency particulate air filter within its workspace, for culturing *Clostridium botulinum* bacteria in the substantially APF culture medium, can be utilized. Exemplary systems can provide botulinum neurotoxin having a potency of at least about 2.0×10^7 units/mg of botulinum neurotoxin and the botulinum neurotoxin obtained comprises one ng or less than one ng of residual nucleic acid for each mg of the botulinum neurotoxin obtained. In particular embodiments, the substantially APF fermentation medium is provided in an amount of from about 2 L to about 75 L; and from about 200 mL to about 1 L of substantially APF culture medium is utilized.

In another aspect of our invention, a substantially APF system using chromatography for obtaining a biologically active botulinum neurotoxin is provided, the system comprising a first apparatus for culturing *Clostridium botulinum* bacteria, the first apparatus capable of containing a substantially APF culture medium; a second apparatus for fermenting *Clostridium botulinum* bacteria which have been cultured in the first apparatus, the second apparatus capable of containing a substantially APF fermentation medium; a third apparatus for harvesting the fermentation medium; a fourth apparatus for concentrating the harvested fermentation medium and diluting the filtered fermentation medium; a fifth apparatus for carrying out a first purification of the botulinum neurotoxin from the harvested medium, wherein the fifth apparatus comprises an anion exchange chromatography media, thereby obtaining a first purified botulinum neurotoxin; and a sixth apparatus for carrying out a second purification of the botulinum neurotoxin wherein the sixth apparatus comprises a cation exchange chromatography media, to thereby obtain a second purified botulinum neurotoxin, wherein the botulinum neurotoxin obtained has a potency of at least about 2.0×10^7 units/mg of botulinum neurotoxin to about 5.9×10^7 units/mg of botulinum neurotoxin, the botulinum neurotoxin obtained comprises one ng or less than one ng of residual nucleic acid for each mg of the botulinum neurotoxin obtained and the process is carried out in one week or less. In particular

embodiments, the botulinum neurotoxin obtained can have a potency of at least 4.4 x 10^7 units/mg of botulinum neurotoxin. In a particular embodiment of the system, the system can further comprise a seventh apparatus for carrying out a further purification of the botulinum neurotoxin obtained from the sixth apparatus, wherein the seventh apparatus comprises a hydrophobic interaction media, thereby obtaining a third purified botulinum neurotoxin. In an additional embodiment, the system can further comprise an eighth apparatus comprising a membrane for filtering eluent from the seventh apparatus.

Another aspect of our invention includes a substantially APF chromatographic system for obtaining a biologically active botulinum neurotoxin comprising a first apparatus for anaerobic culturing *Clostridium botulinum* bacteria, the first apparatus capable of containing from about 200 mL to about 1 L of a substantially APF culture medium; a second apparatus comprising an anaerobic chamber having an integrated high efficiency particulate air filter within the chamber capable of containing the first apparatus; a third apparatus for anaerobic fermentation of *Clostridium botulinum* bacteria which has been cultured in the first apparatus, the third apparatus capable of containing from about 2 L to about 75 L of a substantially APF fermentation medium, preferably from about 2 L to about 30 L of a substantially APF fermentation medium and including at least one disposable probe selected from the group consisting of a reduction-oxidation probe, a pH probe and a turbidity probe; a fourth apparatus for harvesting the fermentation medium; a fifth apparatus for concentrating the harvested fermentation medium and diluting the filtered fermentation medium; a sixth apparatus for carrying out a first purification of botulinum neurotoxin obtained from the harvested fermentation medium, the sixth apparatus comprising an anion exchange chromatography media, thereby obtaining a first purified botulinum neurotoxin; a seventh apparatus for carrying out a second purification of the botulinum neurotoxin the seventh apparatus comprising a cation exchange chromatography media, thereby obtaining a second purified botulinum neurotoxin; an eighth apparatus for carrying out a third purification of the second purified botulinum neurotoxin, the eighth apparatus comprising hydrophobic interaction media to thereby obtain a third purified botulinum neurotoxin; and a ninth apparatus for filtering the third purified botulinum neurotoxin, the ninth apparatus comprising a filtration membrane, wherein the botulinum neurotoxin obtained has a potency of about 2.4 x 10^7 units/mg of botulinum

neurotoxin to about 5.9 x 10^7 units/mg of botulinum neurotoxin, the botulinum neurotoxin obtained comprises one ng or less than one ng of residual nucleic acid for each mg of the botulinum neurotoxin obtained and the process is carried out in one week or less. In accordance with these processes, a biologically active botulinum neurotoxin is thereby obtained, and in particular examples, the botulinum neurotoxin obtained has a potency of at least about 4.4 x 10^7 units/mg of botulinum neurotoxin.

In accordance with processes and systems herein disclosed, biologically active botulinum neurotoxin is thereby obtained. In particular embodiments, the biologically active botulinum neurotoxin obtained by the process and systems herein disclosed has a molecular weight of about 900 kDa.

Our invention further includes a method for making a substantially APF pharmaceutical composition in which the active ingredient is a biologically active botulinum neurotoxin, the method comprising the steps of: (a) obtaining a biologically active botulinum neurotoxin by: (i) providing a fermentation medium which is substantially free of an animal product; (ii) fermenting *Clostridium botulinum* bacteria in the fermentation medium, and; (iii) recovering the biologically active botulinum neurotoxin from the fermentation medium, using an anion exchange chromatography media followed by use of a cation exchange chromatography media, wherein the botulinum neurotoxin recovered has a potency of at least about 2.0 x 10^7 units/mg of botulinum neurotoxin, preferably about 2.4 x 10^7 units/mg of botulinum neurotoxin to about 5.9 x 10^7 units/mg of botulinum neurotoxin, in some embodiments at least about 4.4 x 10^7 units/mg of botulinum neurotoxin, the botulinum neurotoxin comprises one ng or less than one ng of residual nucleic acid for each mg of the botulinum neurotoxin, and steps (i) to (iii) are completed in one week or less, and; (b) compounding the botulinum neurotoxin with at least one suitable excipient, thereby making a substantially APF pharmaceutical composition. In a particular embodiment, the compounding step comprises the step of drying the botulinum neurotoxin by a process selected from the group of processes consisting of freeze drying, lyophilization and vacuum drying and wherein the suitable excipient is selected from the group consisting of albumin, human serum albumin, recombinant human serum albumin, gelatin, sucrose, trehalose, hydroxyethyl starch, collagen, lactose, sucrose sodium chloride, polysaccharide, caprylate, polyvinylpyrrolidone and sodium. Accordingly, one aspect our invention also provides substantially APF pharmaceutical

compositions made by compounding the biologically active botulinum neurotoxin obtained by the processes and systems herein disclosed.

Additionally, our invention also includes a method for treating a condition in a patient, the method comprising the step of administering to the patient a therapeutically effective amount of a pharmaceutical composition made by methods for making a substantially APF pharmaceutical composition in which the active ingredient is a biologically active botulinum neurotoxin obtained by the APF processes (i.e. IAPF and FAPF processes) herein disclosed. Examples of conditions to be treated are selected from the group consisting of a headache, a migraine headache, tension headache, a sinus headache, a cervicogenic headache, a sweating disorder, axillary hyperhidrosis, palmar hyperhidrosis, plantar hyperhidrosis, Frey's syndrome, a hyperkinetic skin line, a facial wrinkle, glabellar lines, crow's feet, marionette lines, a nasolabial fold, a skin disorder, achalasia, strabismus, chronic anal fissure, blepharospasm, musculoskeletal pain, fibromyalgia, pancreatitis, tachycardia, prostatic enlargement, prostatitis, urinary retention, urinary incontinence, overactive bladder, hemifacial spasm, tremors, myoclonus, gastrointestinal disorders, diabetes, sialorrhea, detrusor-sphincter dyssynergia, post stroke spasticity, wound healing, juvenile cerebral palsy, smooth muscle spasm, restenosis, a focal dystonia, epilepsy, cervical dystonia, thyroid disorder, hypercalcemia, an obsessive compulsive disorder, arthritic pain, Raynaud's syndrome, striae distensae, peritoneal adhesion, vasospasms, rhinorrhea, muscle contracture, an injured muscle, laryngeal dystonia, writer's cramp and carpel tunnel syndrome, for example.

In one embodiment, a method for treating a condition in a patient, the method comprising the step of locally administering to the patient an effective amount of a substantially APF pharmaceutical composition made by a method including the steps of: (a) obtaining a biologically active botulinum neurotoxin by (i) providing a fermentation medium which is substantially free of an animal product; (ii) fermenting *Clostridium botulinum* bacteria in the fermentation medium, and; (iii) recovering the biologically active botulinum neurotoxin from the fermentation medium, using an anion exchange chromatography media followed by use of a cation exchange chromatography media, wherein the botulinum neurotoxin recovered has a potency of at least about 2.0×10^7 units/mg of botulinum neurotoxin, the botulinum neurotoxin

comprises one ng or less than one ng of residual nucleic acid for each mg of the botulinum neurotoxin, and steps (i) to (iii) are completed in one week or less, and; (b) compounding the botulinum neurotoxin with at least one suitable excipient, thereby making a substantially APF pharmaceutical composition, whereby local administration of the substantially APF pharmaceutical composition treats the condition.

Local administration of therapeutically effective amounts of a pharmaceutical compositions, comprising a biologically active botulinum neurotoxin provided by the IAPF process/systems/method herein disclosed, can be repeated at intervals of from about 2 months to about 6 months or at intervals of about 2 months to about 3 months, for example. Exemplary useful dosages locally administered to the patient of a therapeutically effective amount of a substantially APF pharmaceutical composition made in accordance with the present disclosure, can have botulinum neurotoxin unit amounts of between about 0.01 unit and about 10,000 units. In particular instances, the botulinum neurotoxin is administered in an amount of between about 0.01 unit and about 3000 units. In particular examples, the biologically active botulinum neurotoxin that is the active pharmaceutical ingredient in the pharmaceutical composition is botulinum neurotoxin type A or type B, for example.

Our invention includes a substantially APF process, utilizing chromatography, for obtaining a biologically active botulinum neurotoxin. The process can comprise the sequential steps of providing a substantially APF fermentation medium, followed by fermenting *Clostridium botulinum* bacteria in the fermentation medium and recovering the biologically active botulinum neurotoxin from the fermentation medium using an anion exchange chromatography media followed by use of a cation exchange chromatography media to thereby obtain the biologically active botulinum neurotoxin from the substantially APF chromatographic process. The recovering step can also include the use of a hydrophobic interaction media after the use of cation exchange chromatography media. The biologically active botulinum neurotoxin obtained can be a botulinum neurotoxin complex or a botulinum toxin neurotoxic component isolated therefrom with a molecular weight of about 150 kDa free of the complexing proteins of a botulinum toxin complex. The APF processes (utilizing 2-columns (IAPF), e.g. anion followed by cation chromatography; or 3-columns (FAPF), e.g. anion followed by cation followed by hydrophobic interaction chromatography)

can be used to obtain a biologically active botulinum neurotoxin such as botulinum neurotoxins type A, B, C_1, D, E, F and G. The botulinum neurotoxin obtained is preferably a botulinum neurotoxin type A complex.

In one aspect of our invention, the amount of fermentation medium used can comprise from about 2 L to about 75 L of substantially APF fermentation medium, preferably from about 2 L to about 30 L of substantially APF fermentation medium. As an example, from about 100 mg to about 5 grams, preferably from about 100 mg to about 3 grams, more preferably from about 100 mg to about 1 gram of the biologically active botulinum neurotoxin is obtained from the process. As an example, from about 20 mg to about 100 mg or from about 20 mg to about 80 mg of the biologically active neurotoxin may be obtained per liter of the fermentation medium used. Fermentation medium can comprise vegetable derived protein product, yeast extract and glucose, for example. As an example, the fermentation medium comprises about 5% w/v or less of a vegetable derived protein product. In yet another example, the fermentation medium comprises about 2% w/v or less of a yeast extract. In a further embodiment, the fermentation medium comprises about 2% w/v or less of glucose. In a particular example, fermentation medium comprises about 5% w/v or less of a vegetable-derived protein product, about 2% w/v or less of a yeast extract and about 2% w/v or less of glucose, the vegetable-derived protein product, yeast extract and glucose being in any ratio in accordance with the recited w/v percentage amounts. In some embodiments, the fermenting step proceeds for between about 60 hours to about 80 hours.

In one embodiment, a substantially APF process utilizing chromatography for obtaining a biologically active botulinum neurotoxin, the process comprising the following sequential steps, is provided where culturing *Clostridium botulinum* bacteria in a substantially APF culture medium, then fermenting *Clostridium botulinum* bacteria from the culture medium in about 2 L to about 75 L of a substantially APF fermentation medium, more preferably in about 2 L to about 30 L of a substantially APF fermentation medium, where at least one of the substantially APF culture medium and substantially APF fermentation medium include a vegetable protein, followed by harvesting the fermentation medium by removing cellular debris present in the fermentation medium and concentrating the harvested fermentation medium by filtration, and diluting the concentrated fermentation medium by adding a buffer.

Once buffered, a first contacting step is executed, in which the diluted harvested fermentation medium is contacted with an anion exchange media so that the biologically active botulinum neurotoxin is associated with the anion exchange media, then eluting the captured botulinum neurotoxin from the anion exchange media proceeds to thereby obtain a first eluent, followed by a second contacting step in which the first eluent is contacted with a cation exchange media to remove impurities from the first eluent, thereby obtaining a second eluent; which is then processed, such as by UF and/or DF; and then filtering the processed second eluent, thereby obtaining biologically active botulinum neurotoxin using a substantially APF process that utilizes chromatography.

As an example, the time for completion of the process, from culturing the bacteria to obtaining the biologically active botulinum neurotoxin can be from between about 50 hours to about 150 hours, more preferably about 80 hours to about 120 hours, for example. In particular embodiments, the culture medium comprises no more than about 4% w/v of a vegetable-derived protein product, in another, the culture medium comprises no more than about 2% w/v of a yeast extract and yet in still another, the culture medium comprises no more than about 2% w/v glucose. The culture medium can comprise the vegetable-derived protein product, yeast extract and glucose in any ratio in accordance with the recited w/v percentage amounts. In a specific example, the pH level of the culture medium can be from about pH 6.5 to about pH 8.0, preferably about pH 6.8 to about pH 7.6, more preferably 7.3 at the commencement of the culturing step. The culturing step can be carried out for between about 8 hours and about 14 hours, about 10 hours to 12 hours, preferably about 11 hours, at a temperature of from about 33 °C to about 37 °C, preferably at about 34.5 °C, in an anaerobic chamber. In a particular example, the anaerobic chamber can contain an integral high efficiency particular filter within its workspace, where culturing is conducted. The fermenting step can be carried out for between about 60 hours and about 80 hours, preferably about 72 hours at a temperature of from about 33 °C to about 37 °C, preferably at 35 °C. In accordance with one aspect of our invention, the harvesting step can remove at least about 80% of RNA and DNA contained in the fermentation medium and the anion exchange media can remove all measurable remaining DNA and RNA (below limit of detection) in the harvested fermentation medium. In another aspect, the harvesting step can be carried out for

between about 1 hour and about 3 hours, preferably about 2.5 hours. In particular examples, the harvesting step can be carried out until 75% of the original fermentation medium volume has been collected. In one aspect of an embodiment, the concentrating step can be carried out for between about 30 minutes and about 2 hours, preferably about 0.75 hour. In another aspect, the diluting step dilutes the harvested fermentation medium back up to the initial weight of the fermentation medium at the commencement of the harvesting step. A first contacting step can be carried out for between about 4 hours and about 5 hours, for example. In one example, the first eluate from the anion exchange resin is collected at spectrophotometer readings of from about 150 mAU or greater, until spectrophotometer readings at 280 nm decrease from peak apex back to about 150 mAU. The second contacting step can be carried out for between 1 hour and about 3 hours, preferably for about 2 hours. This second eluate can be collected from the cation exchange resin at spectrophotometer readings from about 100 mAU or greater, until spectrophotometer readings decrease from peak apex to about 100 mAU, for example. A step of processing this second eluent by concentration and diafiltration can be carried out for between about 1 hour and about 2 hours, preferably for about 1.5 hours. In a particular embodiment, the filtering step includes bioburden reduction by passing the second eluent through a bioburden reduction filter. The bioburden reduction filter can have a pore size of from about 0.1 μm to about 0.3 μm, preferably 0.2 μm. In particular embodiments, the process can further comprise a third contacting step after the second contacting step, by contacting the second eluent to a hydrophobic interaction media to further remove impurities from the second eluent and to thereby obtain a third eluent. This third contacting step can be carried out for between about 1 hour and 3 hours, preferably for about 2 hours. The third eluate can be collected from the hydrophobic interaction media at spectrophotometer readings from about 50 mAU or greater, until spectrophotometer readings decrease from the peak apex back to about 50 mAU, for example. Where there is a third contacting step, the step of processing by concentration and diafiltration is applied to the third eluent and is carried out for between about 2 hours and about 4 hours. Bioburden reduction by passing the eluent that is concentrated and diafiltered (either from a 2 or 3-column process utilized) through a bioburden reduction filter can accordingly be performed. In particular embodiments, the process

further comprises a step of freezing the biologically active botulinum neurotoxin obtained.

In particular embodiments, the substantially APF culture medium comprises a volume of between about 100 mL and about 500 mL. Particular culturing steps are initiated by introducing between about 100 µL and about 500 µL of a *Clostridium botulinum*-containing APF working cell bank media to the substantially APF culture medium. The culturing step can then take place in an anaerobic chamber for at least about 8 hours, preferably about 11 hours, at a temperature of about 34.5 °C ± 1 °C, for example. In one example, the working cell bank media can have a viable cell count assay of at least about 1 x 10^4 colony forming units/mL of working cell bank media, for example about 1 x 10^5 to about 5 x 10^7 colony forming units/mL of working cell bank media, and the *Clostridium botulinum* bacterium in the working cell bank can have been selected to have a substantially uniform morphology.

In one embodiment, the working cell bank media includes about 20% by volume glycerol, such as sterile glycerol, for example. The working cell bank media can be made by (a) growing *Clostridium botulinum* bacterium in an APF medium containing about 2% w/v soy peptone, about 1% w/v yeast extract, and about 1% w/v glucose in an anaerobic chamber, at a temperature of about 34.5 °C ± 1 °C until an optical density of an aliquot of the medium measured at a wavelength of about 540 nm is about 2.5 ± 1.0 AU, and; adding glycerol to obtain a concentration of glycerol in the medium of about 20%, thereby obtaining a working cell bank. A storage form of the working cell bank can be prepared by freezing the working cell bank at about below -135 °C, for example. The storage form of the working cell bank, for use in an exemplary process in accordance with the present disclosure, can be thawed at ambient temperature and used to initiate the culturing step.

The culturing step can be carried out for between about 8 hours and about 14 hours, preferably about 11 hours at a temperature of from about 33 °C to about 37 °C, preferably at about 34.5 °C, in an anaerobic chamber, such as, for example an anaerobic chamber/cabinet having an integrated high efficiency particulate air (HEPA) filter, preferably within its workspace. The fermenting step can be carried out for between about 20 hours and about 80 hours, preferably from about 60 hours to about 80 hours, more preferably for about 72 hours at a temperature of from about 33 °C to about 37 °C, preferably at 35 °C. The process can further comprise, for

example and before the culturing step, a step of allowing for oxidative reduction of the substantially APF culture medium by exposing the medium to the atmosphere of an anaerobic chamber. The process can also include before the fermenting step, a step of allowing for oxidative reduction of the substantially APF fermentation medium by also exposing the fermentation medium to the atmosphere of an anaerobic chamber. As one example, the step of allowing for oxidative reduction of the substantially APF culture medium can be carried out for between about 10 hours and about 14 hours in the anaerobic chamber. Similarly, the step of allowing for oxidative reduction of the substantially APF fermentation medium in the fermentor can be carried out for between about 10 hours and about 14 hours before the beginning of the fermenting step.

In one embodiment, an APF process, including chromatography, for obtaining a biologically active botulinum neurotoxin is disclosed, comprising the following sequential steps of adding *Clostridium botulinum* bacteria from an APF working cell bank to an APF culture medium; culturing the *Clostridium botulinum* bacteria in the culture medium; fermenting *Clostridium botulinum* bacteria from the culturing step in an APF fermentation medium until *Clostridium botulinum* cell lysis occurs; harvesting the APF fermentation culture to provide a harvested fermentation medium; subjecting the harvested fermentation medium to concentration by filtration; diluting the filtered fermentation medium by addition of a buffer to obtain a diluted fermentation medium; a first contacting step in which the diluted fermentation medium is contacted with a capture chromatography media, wherein the capture chromatography media is an anion exchange media; a second contacting step wherein an eluent from the first contacting step is contacted with a polishing chromatography media, wherein the polishing chromatography media is a cation exchange media, and filtering the eluent from the second contacting step, thereby obtaining biologically active botulinum neurotoxin by the improved APF process. In particular embodiments, the process can further comprise the step of conducting a third contacting step, after the second contacting step and before the filtering step, by contacting eluent from the second contacting step with a hydrophobic interaction media. The *Clostridium botulinum* lysis phase can occur between about 35 hours and about 70 hours after commencement of the fermenting step, for example. The fermentation medium can have a volume of between about 2 L and about 75 L, between about 2 L and about 30 L, or between

about 2 L and 20 L of fermentation medium, for example. The whole of this process can be carried out for between about 50 hours to about 150 hours, more preferably from about 80 hours to about 120 hours. The biologically active botulinum neurotoxin thus obtained by this process can have a potency of about 2.4 x 10^7 to about 5.9 x 10^7 units/mg of biologically active botulinum neurotoxin, for example.

In accordance with one aspect, at the end of fermentation, from about 40 mg to about 85 mg of botulinum neurotoxin per liter of fermentation medium can be obtained. Subsequent to various stages of processing (filtration/chromatography/filtration runs), from about 30 mg to about 60 mg of botulinum neurotoxin per liter of fermentation medium; from about 5 mg to about 25 mg of botulinum neurotoxin per liter of fermentation medium; from about 6 mg to about 20 mg of botulinum neurotoxin per liter of fermentation medium can be obtained.

As one embodiment, the pH of the fermentation medium can be adjusted to be between about pH 6.0 and about pH 8, preferably between about pH 6.8 and about pH 7.6 at commencement of the fermenting step, more preferably about pH 7.3. As another example, substantially APF chromatographic process for obtaining a biologically active botulinum neurotoxin is also provided, the process comprising the steps of obtaining a substantially APF fermentation medium containing a botulinum neurotoxin; contacting the medium with an anion exchange chromatography resin to provide a purified eluent containing a botulinum neurotoxin; contacting the eluent with an cation exchange chromatography resin to thereby obtain a further purified eluent, and filtering the further purified eluent to thereby obtain a biologically active botulinum neurotoxin purified from a substantially APF chromatographic process. In particular configurations, an anion chromatography column can be utilized which contains from about 600 mL to about 800 mL of anion exchange chromatography resin. The anion chromatography column can have a diameter of about 8 cm to about 10 cm and an anion exchange chromatography resin bed height in the column of from about 9 cm to about 16 cm, for example. A flow rate of fermentation medium through the anion exchange chromatography resin can be from about 140 cm/hour to about 250 cm/hour, or from about 150 cm/hour to about 160 cm/hour, for example. In another aspect, from about 150 mL to about 300 mL of cation exchange chromatography resin in a chromatography column can be utilized in the process, where the cation

chromatography column has a diameter of about 5 cm to about 8 cm and a cation exchange chromatography resin bed height of from about 5 cm to about 11 cm, for example. The process can include at least one of a diafiltration step and/or a bioburden reduction step. The bioburden reduction step can utilize a capsule filter. The diafiltration of purified eluent is preferably performed before a bioburden reduction step. In one example, the step of diafiltering the further purified eluent is either preceded or followed by adjusting the concentration of the diafiltered further purified eluent, and passing the concentration-adjusted diafiltered further-purified eluent through a bioburden reduction filter. The process can provide a botulinum neurotoxin obtained having potency, as determined by a mouse LD50 bioassay, of from at least about 2.0 x10^7 units/mg of botulinum toxin, such as about 2.4 x 10^7 to about 6.0 x 10^7 units/mg of botulinum neurotoxin. Exemplary recovery at the end of the process of from about 4 mg to about 25 mg of botulinum toxin can be recovered per liter of fermentation media, for example.

In another embodiment, an essentially APF process for purifying a biologically active botulinum neurotoxin can comprise the steps of obtaining from about 2 L to about 30 L an APF fermentation medium that includes a botulinum neurotoxin; harvesting the APF fermentation medium step to provide a harvested APF fermentation medium; performing anion exchange chromatography upon the harvested APF fermentation medium to thereby provide a first eluent; contacting the eluent from the anion exchange chromatography with cation exchange chromatography media to perform cation exchange chromatography to thereby provide a second eluent; and filtering the second eluent from the cation exchange chromatography media, thereby obtaining a purified botulinum neurotoxin, wherein the purified botulinum neurotoxin obtained has a potency of from about 2.4 x 10^7 to about 5.9 x 10^7 units/mg of biologically active botulinum neurotoxin and can be obtained in a quantity of between about 4 mg to about 25 mg per liter of APF fermentation medium used.

Our invention also comprises a compounding method for making a substantially APF pharmaceutical composition in which the active ingredient is a biologically active botulinum neurotoxin, comprising the steps of obtaining a biologically active botulinum neurotoxin by (i) providing a fermentation medium which is substantially free of animal products; (ii) fermenting *Clostridium botulinum* bacteria

in the fermentation medium, and (iii) recovering the biologically active botulinum neurotoxin from the fermentation medium, using an anion exchange chromatography media followed by use of a cation exchange chromatography media; and then compounding the botulinum neurotoxin with at least one suitable excipient to thereby making a substantially APF pharmaceutical composition. In one example, the method includes the step of drying the compounded botulinum neurotoxin and at least one suitable excipient to obtain a stable form for shipment or storage, by freeze drying or lyophilization or vacuum drying, in which the active ingredient is the biologically active botulinum neurotoxin, where the fermentation medium comprises a protein product obtained from a vegetable. The vegetable from which the protein product can obtained can be a soy, corn or malt, debittered seed of *Lupinus campestris*, or hydrolyzed products therefrom. The botulinum neurotoxin obtained can have a potency between about 2.0 x 10^7 units/mg of botulinum neurotoxin to about 6.0 x 10^7 units/mg of botulinum neurotoxin. The botulinum neurotoxin is selected from the group consisting of botulinum neurotoxins types A, B, C_1, D, E, F and G, preferably botulinum neurotoxin type A. In particular instances the botulinum neurotoxin is obtained as a botulinum toxin neurotoxic component with a molecular weight of about 150 kDa free of the complexing proteins of a botulinum toxin complex. In particular embodiments, the suitable excipient is selected from the group consisting of albumin, human serum albumin, recombinant human serum albumin, gelatin, sucrose, trehalose, hydroxyethyl starch, collagen, lactose, sucrose, amino acid, sodium chloride, potassium chloride, polysaccharide, caprylate, polyvinylpyrrolidone and potassium citrate. Obtaining the biologically active botulinum neurotoxin can further comprise the step of using a hydrophobic interaction media following use of the cation exchange media. In particular examples, vacuum drying takes place at a temperature of about 20 °C to about 25 °C. In some embodiments, the vacuum drying takes place at a pressure of about 70 mmHg to about 90 mmHg, for example. The time for vacuum drying can be from about 4 hours to about 5 hours, for example.

Particular aspects of the present disclosure are directed to providing a pharmaceutical composition, which can, for example, comprise a biologically active botulinum neurotoxin complex and an excipient is selected from the group consisting of albumin, human serum albumin, recombinant human serum albumin, gelatin,

sucrose, trehalose, hydroxyethyl starch, collagen, lactose and sucrose, where the pharmaceutical composition is essentially free of nucleic acid.

In particular examples, a pharmaceutical composition is provided that comprises a biologically active botulinum neurotoxin wherein the botulinum neurotoxin obtained has a potency between about 2.0x 10^7 to about 6.0 x 10^7 units/mg of biologically active botulinum neurotoxin and at least one excipient, where the composition comprises less than about 12 ppm of nucleic acid, preferably less than 1 ppm of nucleic acid per mg of botulinum neurotoxin complex.

In a particular embodiment, a substantially APF chromatographic process for obtaining a biologically active botulinum neurotoxin comprises the following sequential steps of: culturing *Clostridium botulinum* bacteria in a substantially APF culture medium for between about 10 hours and about 12 hours, or until a biomass measurement of culture medium has an optical density, at a wavelength of about 540 nanometers (nm), of between about 0.8 AU and about 4.5 AU; fermenting *Clostridium botulinum* bacteria from the culture medium in a substantially APF fermentation medium for between about 65 hours to about 75 hours or until a biomass measurement is taken at the end of fermentation by measuring the optical density of the fermentation medium using a online biomass probe at a wavelength of about 890 nm is between about 0.05 AU and about 0.7 AU; harvesting the fermentation medium for about 2.5 hours, whereby cellular debris in the fermentation medium is removed and the weight of fermentation medium is reduced to about three quarters of its starting weight at the beginning of the harvesting step; concentrating the harvested fermentation medium by tangential flow filtration to about one quarter of its starting volume at the beginning of the harvesting step; diluting the concentrated fermentation medium by adding a buffer, wherein the concentrating and diluting steps take place for between about 0.5 hour to about 2 hours, whereby during concentration the fermentation medium is reduced to about one quarter of its starting weight at the beginning of the harvesting step, and is then diluted, by the addition of the buffer, back up to its original starting weight at the beginning of the harvesting step; contacting the diluted fermentation medium with a capture chromatography media, to capture the biologically active botulinum neurotoxin, for a time period of about 4 hours to about 5 hours; contacting eluent from the capture chromatography media with a first polishing chromatography media to conduct a first polishing run to remove

impurities therefrom for a time period of about 1.5 hours to about 2.5 hours; conducting a second polishing run by passing eluent from the polishing chromatography media through a hydrophobic interaction media for a time period of about 1.5 hours to about 2.5 hours; processing eluent from the hydrophobic interaction media by diafiltration, for a time period of about 1 hour to about 4 hours; and filtering the processed eluent through a bioburden reduction filter, for about 0.5 hour, thereby obtaining biologically active botulinum neurotoxin.

In another aspect, a substantially APF chromatographic system for obtaining a biologically active botulinum neurotoxin is disclosed, the system comprising: a first apparatus for culturing *Clostridium botulinum* bacteria, the first apparatus capable of containing a substantially APF culture medium; a second apparatus for fermenting *Clostridium botulinum* bacteria which have been cultured in the first apparatus, the second apparatus capable of containing a substantially APF fermentation medium; a third apparatus for harvesting the fermentation medium; a fourth apparatus for carrying out concentrating and diluting the harvested medium from the third apparatus; the fourth apparatus comprising tangential flow filtration (TFF); a fifth apparatus for carrying out a first purification of the botulinum neurotoxin from the concentrated and diluted medium, the fifth apparatus comprising an anion exchange chromatography media, thereby obtaining a first purified botulinum neurotoxin; and a sixth apparatus for carrying out a second purification of the first purified botulinum neurotoxin, the sixth apparatus comprising a cation exchange chromatography media, and thereby obtaining a second purified botulinum neurotoxin.

In a particular embodiment, the system can further comprise a seventh apparatus for carrying out a further purification, by purifying the second purified botulinum neurotoxin obtained from the sixth apparatus, wherein the seventh apparatus comprises a hydrophobic interaction media, thereby obtaining a third purified botulinum neurotoxin. The system can also further be comprised of an eighth apparatus having a filtration membrane for filtering eluent from the sixth or seventh apparatus.

In still yet another embodiment, a chromatography column with a diameter of between about 8 cm and about 15 cm contains the anion exchange chromatography media and the anion exchange chromatography media can have a bed height in the column of between about 8 cm and about 15 cm, for example. In still another

example, the system's fourth apparatus comprises a chromatography column that is operated at a flow rate of between about 125 cm/hour and about 200 cm/hour, and the column can have a column volume between about 500 mL and about 1 L. In one aspect, the fifth apparatus can have a column volume of from about 50 mL and about 500 mL, and a bed height of from about 8 cm and about 15 cm, for example. In some examples, the fifth apparatus' chromatography column has a column diameter from about 2 cm and about 10 cm, for example. The fifth apparatus' chromatography column can have an exemplary flow rate of between about 100 cm and about 200 cm/hour. The seventh apparatus of the system can comprise a filtration membrane.

In another embodiment, the system can further comprise a ninth apparatus, the ninth apparatus comprising an anaerobic chamber for providing an anaerobic atmosphere where the first apparatus for culturing *Clostridium botulinum* bacteria is contained therein. This ninth apparatus preferably includes an integrated high efficiency particulate air (HEPA) filter located within its chamber/workstation. The second apparatus of the system (for fermentation) can include at least one probe for detecting oxidation-reduction potential or pH or optical density. In a particular example, an at least one disposable probe is selected from the group consisting of a reduction-oxidation probe, a pH probe and a turbidity probe. A eighth apparatus of the system can comprise a tangential flow filtration apparatus for concentration and buffer exchange. In a further embodiment, the system can comprise an tenth apparatus that includes a bioburden reduction apparatus for reducing bioburden. In one example, the bioburden reduction apparatus comprises a filter having a pore size of about between about 0.1 μm and 0.3 μm, preferably 0.2 μm. The system can also include a eleventh apparatus for use after obtaining the second purified botulinum neurotoxin, for storing the purified botulinum neurotoxin. In one example, this storage apparatus provides a storage temperature between about -25 °C to about -80 °C

In another aspect, a biologically active botulinum toxin is provided by an APF process having the following steps of providing a substantially APF fermentation medium; fermenting a *Clostridium botulinum* bacteria in the fermentation medium; recovering the biologically active botulinum neurotoxin from the fermentation medium using an anion exchange chromatography media followed by use of a cation exchange chromatography media, where the biologically active botulinum toxin obtained has a potency between about 2.0×10^7 units/mg of botulinum neurotoxin to

about 6.0 x 10^7 units/mg of botulinum neurotoxin. In one embodiment, the process further comprises the step of further purifying the botulinum neurotoxin by using a hydrophobic interaction media following use of the cation exchange media.

In accordance with another aspect, a method for treating a condition in a patient is provided, utilizing a pharmaceutical composition comprising the botulinum neurotoxin obtained in accordance with the methods herein disclosed. A condition can include a disease, ailment, sickness, or cosmetic deformity or appearance. In one example, the method of treating a condition in a patient comprises the step of administering to the patient a therapeutically effective amount of a pharmaceutical composition comprising a botulinum neurotoxin and at least one suitable excipient, where the botulinum toxin has a potency of about 1 unit ≥ about .02 picograms to thereby treat the condition of the patient.

In a particular example, the botulinum neurotoxin for treating these conditions can be obtained by a process of culturing *Clostridium botulinum* bacteria in a substantially APF culture medium; obtaining a substantially APF fermentation medium containing the botulinum neurotoxin; contacting the medium with an anion exchange chromatography media to provide a purified eluent containing the botulinum neurotoxin; contacting the eluent with an cation exchange chromatography media to thereby obtain a further purified eluent, and filtering the further purified eluent to thereby obtain the biologically active botulinum neurotoxin purified from a substantially APF chromatographic process.

In one embodiment a substantially APF chromatographic system for obtaining a biologically active botulinum neurotoxin is included, the system comprising a first apparatus for anaerobic culturing *Clostridium botulinum* bacteria, the first apparatus capable of containing from about 200 mL to about 1 L of a substantially APF culture medium; a second apparatus for anaerobic fermentation of *Clostridium botulinum* bacteria which has been cultured in the first apparatus, the second apparatus capable of containing from about 5 L to about 75 L, or from about 2 L to about 75 L, or from about 2 L to about 30 L of a substantially APF fermentation medium and including at least one disposable probe selected from the group consisting of a reduction-oxidation probe, a pH probe and a turbidity probe; a ninth apparatus for providing an anaerobic atmosphere and capable of containing the first apparatus, the ninth apparatus comprising an anaerobic chamber having an integrated high

efficiency particulate air filter within the chamber, wherein said chamber can contain the first apparatus for anaerobic culturing *Clostridium botulinum* bacteria; a third apparatus for harvesting the fermentation medium; a fourth apparatus for carrying out concentration and dilution of the harvested medium, a fifth apparatus for carrying out a first purification of botulinum neurotoxin obtained from the fourth apparatus, the fifth apparatus comprising an anion exchange chromatography media, thereby obtaining a first purified botulinum neurotoxin; a sixth apparatus for carrying out a second purification of the first purified botulinum neurotoxin, the sixth apparatus comprising a cation exchange chromatography media, thereby obtaining a second purified botulinum neurotoxin; a seventh apparatus carrying out a third purification of the second purified botulinum neurotoxin, the seventh apparatus comprising hydrophobic interaction media, thereby obtaining a third purified botulinum neurotoxin; and an eighth apparatus for concentration and buffer exchange of the third purified botulinum neurotoxin, the eighth apparatus comprising a TFF membrane.

In particular examples, the fermentation medium comprises no more than about 5% w/v of a vegetable-derived protein product, no more than about 2% w/v of a yeast extract and no more than about 2% w/v glucose, and where the pH level of the fermentation medium is from about pH 6.8 to about 7.6, preferably about pH 7.3 at the start of an about 72 hour fermenting step, for example. In one embodiment, the method can further comprise the step of contacting the further purified eluent with a hydrophobic interaction media to obtain an even further purified eluent containing the botulinum neurotoxin. In a particular example, the method of treating the conditions can be by using a botulinum neurotoxin that is obtained as a botulinum toxin neurotoxic component with a molecular weight of about 150 kDa free of the complexing proteins of a botulinum toxin complex. Exemplary administration steps can be selected from the group of administration routes consisting of intramuscular, intradermal, subcutaneous, intraglandular, intrathecal, rectal, oral and transdermal administration, and the botulinum neurotoxin is selected from the group consisting of botulinum toxin type A, B, C_1, D, E, F or G. Preferably, the botulinum neurotoxin is botulinum neurotoxin type A.

In some examples, the system can facilitate a process whereby a biologically active botulinum neurotoxin complex can be obtained for use as part of pharmaceutical composition that comprises less than about 12 ng of nucleic acid per

mg of botulinum neurotoxin complex, preferably below 1 ng of nucleic acid per mg of botulinum neurotoxin complex, more preferably having no measurable a nucleic acid (e.g. below a limit of detection).

DRAWING

Figure 1A is a flow chart showing major steps in the Example 1 NAPF process. Figure 1B is a flow chart showing major steps in the Example 2 IAPF process, wherein the capture and polishing chromatography steps can utilize either a 2-columns (anion exchange followed by cation exchange) or 3-columns (FAPF) (anion exchange followed by cation exchange followed by a hydrophobic interaction column).

DESCRIPTION

Our invention is based on the discovery that a high potency, high purity biologically active Clostridial neurotoxin, such as a botulinum neurotoxin, can be obtained by use of a simple, fast and economical APF chromatographic system and process. Significantly, use of our system and process can result in a purified botulinum neurotoxin comprising 1 ng (or less than 1 ng) of nucleic acid (RNA and DNA) impurities per 1 mg of the purified botulinum neurotoxin obtained, even though no animal derived enzymes, such as RNase and DNase, are used to purify the fermented botulinum neurotoxin. For example, use of our system and process can result in a purified botulinum neurotoxin comprising less than about 0.6 ng of nucleic acid (RNA and DNA) impurities per milligram of purified botulinum neurotoxin, obtained. The botulinum neurotoxin obtained can be a botulinum toxin type A complex, such as a 300 kDa, 500 kDa or 900 kDa (approximate molecular weights) complex or mixtures thereof. The botulinum neurotoxin obtained can also be a botulinum toxin type neurotoxic component (i.e. without the complex proteins) with a molecular weight of about 150 kDa. The botulinum neurotoxin can be any one of the serotypes A, B, C, D, E, F or G or mixtures thereof. Additionally, the improved systems and processes can be practiced in conjunction with a recombinant, hybrid, chimeric or modified botulinum toxin (light chain, heavy chain, or both chains together).

An important aspect of our invention is use of an anion exchange (capture) media chromatography followed by use of cation exchange (polishing) media chromatography to purify botulinum neurotoxin from an APF fermentation medium in which *Clostridium botulinum* bacterium have been fermented. We found that use of anion exchange followed by use of cation exchange chromatography media provides an effective and rapid method for obtaining high purity, high yield botulinum neurotoxin. Previously, it had been thought that use of anion exchange chromatography has a detrimental effect on gel banding patterns of botulinum neurotoxin, thereby discouraging use of anion exchange chromatography for botulinum neurotoxin purification. See e.g. U.S. patent 7,452,697 at column 55, lines 53-57.

Another important aspect of our invention is that it results in high purity botulinum neurotoxin (i.e. $\leq$ 1 ng nucleic acid/ mg botulinum neurotoxin obtained), as set forth above. A further important aspect of our invention is that whereas the known Schantz process requires several weeks (i.e. typically about 18 to about 22 days) to culture, ferment and purify the botulinum neurotoxin, a system and process within the scope of our invention permits all culturing, fermentation and purification steps to be completed in one week or less. In a preferred embodiment of our invention all culturing, fermentation and purification steps can be completed in six days or less. In a more preferred embodiment of our invention all culturing, fermentation and purification steps can be completed in about four days or less (e.g. within about 80 to about 144 hours or within a time/range therebetween). We invented this rapid, more embodiment of our invention by developing an eight or nine step process (and the system for accomplishing the process) and by finding that each of the eight or nine steps in a particular embodiment can be completed within the time periods set forth below:

about 8 hours to about 14 hours for culturing;
about 60 hours to about 80 hours for fermenting;
about 2.5 hours for harvesting;
about 2 hours to about 4 hours for concentrating and diluting;
about 4 hours to about 6 hours for anion exchange chromatography (this includes time for eluting captured botulinum toxin)
about 2 hours for cation exchange chromatography;

about 2 hours for an optional third chromatography step (i.e. hydrophobic interaction chromatography;
about 2 hours to about 4 hours for concentration and diafiltration, and;
about 1/2 hour for further filtration. Thus, the total time required to complete our 8 or 9 step rapid, more preferred embodiment of our invention is from about 75 hours to about 150 hours.

Our invention is more efficient and time saving. In one aspect, our new process utilizes pre-selected and verified cell lines, and thus does away with the prior art Shantz process steps of plating and growing cells, selecting and harvesting colonies, and step-up cell-line expansion of the harvested colonies (prior to cell culturing and fermentation steps) that were needed to culture and then inoculate fermentation medium. In one aspect, our invention begins straight away with culturing pre-selected cells for inoculation of an APF culture medium, thus saving time and process steps.

Through experimentation we developed two chromatography column ("IAPF") and three column ("FAPF" / “FIAPF") chromatography systems and processes for purifying the botulinum neurotoxin present in the fermentation medium, the fermentation medium resulting from an APF fermentation of *Clostridium botulinum* bacterium. Significantly, while an APF fermentation process can reduce or eliminate animal derived products (such as casein and meat broth) as nutrients from the media used to culture and ferment Clostridial bacteria, known APF fermentation processes are typically followed by one or more purification steps which make use of animal derived products, such as the enzymes DNase and RNase. Our systems and processes for purifying the botulinum neurotoxin present in an APF fermentation medium do not use animal derived enzymes.

Our invention can encompass loading a harvested fermentation medium (e.g. clarified by filtration) onto an anion exchange column such as a POROS® 50HQ anion exchange chromatography resin from Applied Biosystems. In one aspect, a strong anion exchange media can be used, having a base matrix of polystyrene/divinylbenzene and particle diameter of about 50 µm and dynamic capacity (BSA mg/ml) of about 60–70. The anion exchange column captures the Clostridial neurotoxin (such as a botulinum toxin complex) and reduces impurity levels. It was found that an anion exchange column provided an efficient capture of a

botulinum toxin complex from harvested fermentation medium with retention of the biological activity of the botulinum toxin complex, while also separating many impurities present with the botulinum toxin in the fermentation medium. A suitable buffer is used to elute the captured (bound) Clostridial neurotoxin from the anion exchange column.

In a two-column embodiment of our invention, eluent (containing the botulinum neurotoxin) from the anion exchange column is loaded onto a cation exchange column to further purify the botulinum neurotoxin from impurities. The cation exchange column can be a POROS® 20HS cation exchange resin from Applied Biosystems. In one aspect, a strong cation exchange media can be used, having a base matrix of polystyrene/divinylbenzene and particle diameter of about 20 µm and dynamic binding capacity (lysosyme mg/ml) of about >75. In a three-column embodiment (FAPF) of our invention, eluent from the cation exchange column is loaded onto a hydrophobic interaction column such as Phenyl Sepharose HP resin from GE Healthcare to further purify the botulinum neurotoxin. In one aspect, a matrix of highly cross-linked agarose beads with a particle size of about 34 µm, which have been derivitized with phenyl groups and have a dynamic binding capacity (chymotrypsinogen mg/ml) of about 45, may be used.

After either the two column or three column process, eluent from the last used column can be further processed to obtain highly purified bulk botulinum toxin complex. Post-chromatography processing steps can include concentration and buffer exchange by ultrafiltration and diafiltration, sterile filtration and preparation of a solution of purified botulinum toxin complex instead of a suspension (prior art), preferably in potassium citrate, and in one example, at a concentration of 10mM potassium citrate at a pH of about 6.5.

In certain preferred embodiments, the media for the growth (anaerobic culturing and anaerobic fermentation) of *Clostridium botulinum* and production of botulinum toxin can comprise soy based products to replace animal derived products so that media used are substantially or entirely free of animal-derived products. The culture step increases the quantity of microorganism for subsequent fermentation. Culturing permits dormant, previously frozen bacteria to rejuvenate into actively growing cultures. Additionally, the volume and quantity of viable microorganisms used to inoculate the fermentation medium can be controlled more accurately from an

actively growing culture than it can be from a stored, non-propagating *Clostridium botulinum* cell bank. Thus, a sample of a working cell bank in APF media is thawed and placed in the selected APF culture medium. Upon obtaining a suitable level of bacterial growth the culture medium is used to inoculate the fermentation medium. As one example, from about 1% to about 5%, or an amount therebetween, of the culture medium having *Clostridium botulinum* from the growth phase is used to inoculate the fermentation medium. Fermentation is carried out to produce the maximum amount of microbial cells in a large-scale anaerobic environment (Ljungdahl et al., *Manual of industrial microbiology and biotechnology* (1986), edited by Demain et al, American Society for Microbiology, Washington, D.C. page. 84). Alternately, growth of *Clostridium botulinum* in the fermentation medium can proceed by adding the sample of the working cell bank directly to the fermentation medium.

In the prior art, growth of *Clostridium botulinum* in the culture medium typically proceeds in two stages, a first stage of cell plating, cell colony growth, selection and growth, followed by a second stage of inoculation of culture medium (typically a two stage step-up culture) and inoculation of fermentation medium and botulinum toxin production. Preferably, growth in the culture media in any stage does not result in cell lysis before inoculation of fermentation media with the final growth in culture medium. Thus, prior to our invention it took about four days to culture *Clostridium botulinum* bacteria before the fermentation step was begun. In accordance with our invention we are able complete all culturing in only 8 to 14 hours because there is no need for the previously utilized steps of plating cells, subsequent waiting time for colony growth on blood agar plates, selection of colonies from the plates for growth in small volumes of culture (e.g. 8-9 mL) that then provide an inoculum for the culturing medium. In accordance with one aspect of our invention, pre-selected cells are directly utilized to inoculate the culture medium that is then utilized to inoculate the full-scale fermentation medium from which botulinum toxin is eventually purified, thus eliminating the plating, colony formation, selection and step up steps previously utilized to grow cells that would inoculate a culture medium which is then itself utilized to inoculate fermentation medium.

Animal-based (non-APF or “NAPF”) culture media generally include brain heart infusion media (BHI), bacto-peptone, NaCl, and glucose. Culture media within the scope of our invention are APF culture media. For example, a soy-based product can

be used instead of BHI and bacto-peptone in the culture and fermentation media. Preferably, the soy-based product is soluble in water and comprises hydrolyzed soy, although *Clostridium botulinum* can grow in media containing insoluble soy. Any source of soy-based products may be used in accordance with the present invention. Preferably, the soy is hydrolyzed soy and the hydrolyzation has been carried out using non-animal enzymes. Sources of hydrolyzed or soluble soy include Hy-Soy (Quest International), Soy peptone (Gibco) Bac-soytone (Difco), AMISOY (Quest), NZ soy (Quest), NZ soy BL4, NZ soy BL7, SE50M (DMV International Nutritionals), and SE50MK (DMV).

EXAMPLES

The following examples set forth particular embodiments of our invention and are not intended to limit the scope of our invention. Unless otherwise set forth in the examples "toxin" or "botulinum toxin" means a botulinum toxin type A complex with a molecular weight of about 900 kDa. Systems and method disclosed herein for purifying a botulinum toxin type A complex with a molecular weight of about 900 kDa, have ready applicability to the purification of about 150 kDa, about 300 kDa, about 500 kDa as well as other molecular weight toxins, complexes, botulinum toxin serotypes and botulinum toxin neurotoxic component.

Example 1
Non-APF (Schantz) Process for Obtaining a Botulinum Toxin

This example sets forth the prior art Schantz process for obtaining botulinum neurotoxin. The process is a non-APF process using animal derived media and reagents (i.e. beef blood agar plates for culturing, casein in the fermentation medium and use of RNase and DNase enzymes for botulinum neurotoxin purification). Figure 1A is a flow chart showing the major steps of the Schantz process. The Schantz process has about 16 to 20 major steps, for production scale work uses a 115 L fermentor and takes about 3 weeks to complete. The Schantz process is commenced by thawing a non-APF *Clostridium botulinum* master cell bank (MCB) vial to room temperature followed by four cultivation steps. First to select colonies with a suitable morphology, aliquots from the thawed MCB vial were streaked on pre-reduced Columbia blood agar (CBA) plates and anaerobically incubated for 30-48 hours at

34 °C ± 1 °C. Second, selected colonies were inoculated into 9 mL test tubes containing a casein growth medium for 6-12 hours at 34 °C. The contents of the 9 mL tube with the most rapid growth and highest density (growth selection step) were then further cultivated through two step-up anaerobic incubations (the third and fourth cultivation steps), being a 12-30 hour incubation at 34 °C in a 600 mL to 1 L seed cultivation bottle, followed by a cultivation in a 15 L to 25 L seed fermentor containing a casein growth medium for 6-16 hours at 35 °C. These two step-up cultivations were carried out in a nutritive media containing 2% casein hydrolysate (a casein [milk protein] digest), 1% yeast extract and 1% glucose (dextrose) in water at pH 7.3.

The step-up cultivations were followed by a further incubation for 60-96 hours at 35 °C in a commercial scale (i.e. 115 L) production fermentor in a casein containing medium under a controlled anaerobic atmosphere. Growth of the bacterium is usually complete after 24 to 36 hours, and during the fermentation step carried out for about 65 to about 72 hours where most of the cells undergo lysis and release botulinum neurotoxin. It is believed that toxin is liberated by cell lysis and activated by proteases present in the media. A filtrate of the culture medium can be prepared using a single layer depth filter to remove gross impurities (i.e. whole and ruptured cells) thereby obtaining a clear solution referred to as a clarified culture. Collection of botulinum neurotoxin from clarified culture was accomplished by lowering the pH of the clarified culture to pH 3.5 with 3M sulfuric acid to precipitate the raw toxin at 20 °C (acidification precipitation). The raw botulinum neurotoxin was then concentrated (to achieve a volume reduction) by ultramicrofiltration (microfiltration) (referred to as MF or UF) followed by diafiltration (DF). A 0.1 μm filter was used for the microfiltration step.

The harvested crude or raw toxin was then transferred to a digestion vessel and stabilized by addition of the protease inhibitor benzamidine hydrochloride. DNase and RNase were added to digest (hydrolyze) nucleic acids. Hydrolyzed nucleic acids and low molecular weight impurities were then removed by further UF and DF steps. The toxin was then extracted with pH 6.0 phosphate buffer and cell debris removed by clarification. Next three sequential precipitation steps (cold ethanol, hydrochloric acid and ammonia sulfate precipitations) were carried out. The purified botulinum neurotoxin complex (bulk toxin) was stored as a suspension in a sodium phosphate/ammonium sulfate buffer at 2 °C to 8 °C.

Completion of this Example 1 Schantz (non-APF) process, including the harvesting and purification steps, takes about two to three weeks. The resulting bulk botulinum neurotoxin was a high quality suspension of 900 kDa botulinum toxin type A complex made from the Hall A strain of *Clostridium botulinum* with a specific potency of $\geq 2 \times 10^7$ U/mg, an A_{260}/A_{278} of less than 0.6 and a distinct pattern of banding on gel electrophoresis, and suitable for use for the compounding of a botulinum toxin pharmaceutical composition.

Botulinum neurotoxin can also be obtained from an APF, non-chromatographic process, as set forth in Example 7 of U.S. patent 7,452,697, the complete APF, non-chromatographic process (from beginning of culturing to end of all purification and processing steps) taking about two to three weeks to complete. Alternately, botulinum neurotoxin can also be obtained from an APF, chromatographic process, as set forth in Example 16 of U.S. patent 7,452,697, the APF, chromatographic process (from beginning of culturing to end of all purification and processing steps) taking a week or longer to complete.

Example 2
APF, Two and Three Column Chromatographic Systems and Processes for Obtaining a Botulinum Neurotoxin

We developed rapid APF, anion-cation chromatographic based systems and processes for obtaining high yield, high purity botulinum neurotoxin. The process of this Example 2 had only 8-10 major steps, for production purposes (that is to obtain gram quantities of the final botulinum neurotoxin) used a 20 L fermentation vessel and takes only 4-7 days, preferably about 4 to about 6 days, to complete all step of the process from initiation of culturing to completion of final purification and toxin storage. Apparatus utilized in the systems herein disclosed are discussed below. Both a two chromatographic media process and a three chromatographic media process were developed and are set forth herein. The two media process used anion exchange chromatography followed by cation exchange chromatography. The three media process used anion exchange chromatography followed by cation exchange chromatography followed by hydrophobic interaction chromatography (HIC). The HIC removed further impurities such as a 49 kDa impurity (which turns out to be a host cell glucose phosphate isomerase, as discussed below).

Preparation of Working Cell Bank

We developed a new *Clostridium botulinum* cell bank (for use to initiate the culturing step) without use of Columbia blood agar plates, and which removed the need for colony selection prior to cultivation and also eliminated the need to carry out the Shantz process step up tube cultivation and multiple seed (cultivation) steps.

For this purpose, a previously established Schantz master cell bank (MCB) was used to create an APF research cell bank (RCB) from which a new APF master cell bank (MCB) and a subsequent working cell bank (WCB) were generated. A research cell bank (RCB) was made from a colony from the Schantz (NAPF) MCB. To remove the animal-derived protein from the MCB vial, the cells were washed twice in APF medium containing 2% w/v SPTII (Soy Peptone type II), 1% w/v yeast extract, and 1% w/v glucose. The cells were plated on APF medium under strict anaerobic conditions using a Modular Atmosphere Controlled System (MACS) anaerobic chamber. An isolated colony was further expanded and stored in APF medium containing about 20% glycerol below -135 °C.

The APF-MCB was made under GMP conditions by expanding the RCB into oxygen-free APF medium (200 mL, reduced for a minimum of 12 hours in an anaerobic chamber) and cultured in a MACS anaerobic chamber at 34.5 °C ±1 °C (stirred at 60 rpm) until the OD_{540} of the culture reached 2.5 ± 1.0 AU. Sterile glycerol was added to the resultant culture to a final concentration of about 20% after which the mixture was transferred into cryovials at 1 mL/vial (APF-MCB vials). The vials were flash frozen in liquid nitrogen, and then stored below -135 °C. An APF-WCB was made under GMP conditions by expanding as above. The resultant APF cell banks were characterized for identity, purity, viability and genetic stability.

Upstream Steps (Culturing and Fermentation)

Our Example 2 process had two general stages; an upstream stage and a downstream stage. The upstream stage includes expansion of a starting cell line (growth and reproduction of *Clostridium botulinum* bacteria in a substantially APF culture medium), fermentation, harvest (removal of cellular debris) to provide a clarified, harvested culture that is then concentrated and diluted. Thus, in this example the nine steps of our two column process are culturing, fermentation,

harvest filtration, concentration, capture (anion) chromatography, polishing (cation) chromatography, buffer exchange, bioburden reduction and vial fill.

The upstream stage included use of a culture medium in a 1 L bottle containing 400 mL of reduced (in an anaerobic chamber) seed APF culture medium (2% w/v SPTII, 1% w/v yeast extract, (adjusted to pH 7.3 with 1 N sodium hydroxide and/or 1 N hydrochloric acid prior to autoclaving)) 1% w/v sterile glucose added post autoclaving of culture media). The culture (seed) medium was inoculated with 400 µL of a thawed *Clostridium botulinum* WCB. Incubation/culturing occurred at 34.5 °C ± 1.0 °C with 150 rpm agitation in an anaerobic chamber.

When the optical density of the culture medium at 540 nm was 1.8 ± 1.0 AU, the entire contents of the 1 L bottle (approximately 400 mL) were transferred to a 20 L production fermentor containing APF fermentation medium adjusted with 1 N sodium hydroxide and/or 1 N hydrochloric acid post-steam sterilization to pH 7.3, fermentation medium composed of 3.25% w/v SPTII, 1.2% w/v yeast extract, 1.5% w/v sterile glucose (added post sterilization; sterilization, e.g. at about 122 °C for 0.5 hour). The temperature and agitation were controlled at 35 °C ± 1 °C and 70 rpm, respectively. Nitrogen overlay was set at 12 slpm and headspace pressure set at 5 psig to maintain an anaerobic environment for cell growth. Fermentation pH and cell density were monitored by pH and online turbidity probes, respectively. The three phases for the production fermentation include exponential growth, stationary, and autolysis phases. Cellular autolysis, which releases active BoNT/A complex into the culture medium, was observed to occur consistently between 35 hours and the end of fermentation. At the end of fermentation, the culture was cooled to 25 °C for harvest.

Once the fermentation medium was cooled to 25 °C, the cell debris was separated from the botulinum neurotoxin type A complex containing lysate by depth filtration, first through a 5 – 0.9 µm nominal retention rating gradient pre-filter to remove cell debris, and then through a positively charged 0.8 – 0.2 µm nominal retention rating gradient to remove DNA (removal of up to about 80%). Both filters were rinsed together with 20 L of water for injection (WFI) before use. A minimum of 15 L of the filtrate was required for further processing, and any excess material was decontaminated after in-process sampling is complete. The filtrate was stored at 4 °C if not immediately processed by ultrafiltration.

Within a biosafety cabinet (BSC) the filtrate from the harvest step was concentrated from 15 L to 5 ± 0.5 L using a hollow fiber, tangential flow filtration (TFF) membrane from GE Healthcare. The ultrafiltered material was then diluted with 10 mM sodium phosphate pH 6.5 buffer to a final volume of 20 L. This material was purified by use of either 2 column (anion then cation) or three chromatography columns (anion, cation, and then hydrophobic interaction). The diluted, ultrafiltered harvest material was stored at 4 °C if not immediately processed by purification.

In the Schantz process the culture step is ended and the fermentation step begun based on time and visual observation of culture growth. In contrast, in our Example 2 processes determination of when to end the culturing step is based on analysis of culture fluid optical density, which ensures that the culture is in the logarithmic growth phase at the time of commencement of the fermentation step, and permits reduction of duration of the culturing step to about 8 hours to about 14 hours. Our OD parameter terminated culture step maximized the health of the cultured cells and encouraged robust and abundant botulinum toxin resulting from the fermentation step. The average optical density (at 540 nm) of the culture medium at conclusion of culturing was 1.8 AU. The average duration of the fermentation step 72 hours and the average final turbidity (A_{890}) of the fermentation medium at conclusion of the fermentation step was 0.15 AU. The average amount of botulinum toxin type A complex present (as determined by ELISA) in the 20 L fermentation medium (whole broth) at the end of the fermentation step for was about 64 μg botulinum toxin type A complex/mL fermentation medium.

The harvest step used depth filtration to remove cell debris and nucleic acids, followed by ultrafiltration and dilution to prepare the fermentation medium for the next step in the process. This harvesting/cell debris clearing is fundamentally different from the Schantz harvest process, which uses precipitation by acidification followed by microfiltration and diafiltration to concentrate and exchange buffers in preparation for further processing.

Downstream Steps (Purification)

Downstream steps included capture of the botulinum neurotoxin on an anion exchange column, elution from the column and further separation from impurities by polishing on a cation exchange column, and preferably (in the three column process), passage of eluent containing desired botulinum neurotoxin through a third column,

preferably a hydrophobic interaction column (e.g. chromatography), followed by concentration and buffer exchange using tangential flow filtration (TFF), and bioburden reduction (e.g. by further filtration using a 0.2 µm filter) to a final botulinum neurotoxin type A complex optimized for cold storage, preferably freezing, and eventual compounding into a botulinum neurotoxin type A complex pharmaceutical composition. The sequence of the chromatography and filtration stages was intended to remove product and process-related impurities, to remove potential adventitious agents and to control the botulinum neurotoxin type A complex concentration and buffer matrix of the final botulinum neurotoxin type A in order to provide a more stable drug substance.

A more detailed embodiment of the three column downstream process carried out is as follows. Clarified (diluted) ultrafiltered material (20 L, as disclosed above) was passed through a POROS® 50HQ anion exchange chromatography resin, the captured botulinum neurotoxin was eluted from the anion exchange column and then run through a POROS® 20HS cation exchange chromatography resin, the eluent from which was run through a Phenyl Sepharose HP chromatography resin. Eluent from the HIC column was subjected to 100 kDa tangential flow filtration, followed by 0.2 µm filtration. The resulting botulinum neurotoxin type A complex was frozen for storage.

In this Example, we used in the first chromatography step of the downstream process a POROS® 50HQ anion exchange chromatography resin packed into a column with an inner diameter of about 8 cm and a column height of about 15 cm. The entire POROS® 50HQ column operation was completed at ambient temperature, and the flow was in the downward direction. The botulinum neurotoxin type A complex was eluted from the anion column using a pH step change where the more negatively charged components such as nucleic acids (e.g. DNAs and RNAs) and other host cell proteins remained bound to the anion exchange column.

Particulars of the anion exchange step were: use of the POROS® 50HQ column using 0.1 N sodium hydroxide for a minimum contact time of 30 minutes (at least about 3 column volumes, at 230 cm/hour). The column was then equilibrated with a 50 mM sodium phosphate, pH 6.5 buffer (at least 5 column volumes). Next the clarified ultrafiltered and diluted material (i.e. processed lysate APF fermentation material) was loaded at 230 cm/hour onto the POROS® 50HQ anion exchange

column, followed by washing with at least about 20 column volumes of 50 mM sodium phosphate, pH 6.5 at 230 cm/hour until absorbance at 280 nm of column effluent decreases to 0.10 AU, followed by eluting with 50 mM sodium acetate, pH 4.8 at 230 cm/hour. The product pool was collected, when the absorbance at 280 nm (A_{280}) increases to at least about 0.15 AU and through the peak maximum to equal or less than about 0.2 AU on the trailing edge, into a vessel containing 1 column volume of 50 mM sodium acetate, pH 4.8. This elution pool was stored at about 2 °C to about 8 °C for up to 48 hours.

The second chromatography step in the downstream process of this Example 2 used a POROS® 20HS cation exchange chromatography resin packed into a column with an inner diameter of 8 cm and a column height of 5 cm. The entire POROS® 20HS column operation was completed at ambient temperature, and the flow was in the downward direction. The botulinum neurotoxin type A complex associates with the POROS® 20HS column resin. The botulinum neurotoxin type A complex was then eluted from the column using a salt step change. The product-related impurities were eluted with the wash buffer and decontamination solution.

Particulars of the cation exchange step were: use of the POROS® 20HS column using 0.1 N sodium hydroxide solution for a minimum contact time of 30 minutes (at least about 3 column volumes, at 230 cm/hour). The column was then equilibrated with a 50 mM sodium acetate, pH 4.8 buffer (at least about 5 column volumes). Next the POROS® 50HQ product pool (collected as described above, fresh or from refrigeration) was loaded onto the POROS® 20HS column. The column was then washed with a 50 mM sodium acetate, pH 4.8 buffer (at least about 3 column volumes) and then washed again with a 50 mM sodium acetate, 150 mM sodium chloride, pH 4.8 buffer. The botulinum neurotoxin type A complex was eluted from the POROS® 20HS column with a 50 mM sodium acetate, 250 mM sodium chloride, pH 4.8 buffer at 200 mL/min, the eluate was diverted into a bioprocess collection bag (containing 1 column volume of 50 mM $NaH_3C_2O_2$, pH 4.8) when the A_{280} increases to about $\geq$ 0.1 AU through peak maximum until the A_{280} of the trailing edge of the elution peak decreases to a trailing edge value of $\leq$ 0.1 AU. The POROS® 20HS product pool was stored in the collection bag at ambient temperature for up to about 6 hours.

In the three-column chromatography media process of this Example 2, eluent from the second (cation exchange) column was passed through a HIC column. The HIC column used was a Phenyl Sepharose HP hydrophobic interaction chromatography resin packed into a column with an inner diameter of about 8 cm and a column height of about 5 cm. The entire Phenyl Sepharose HP column operation was completed at ambient temperature, and the flow was in the downward direction. The botulinum neurotoxin type A complex was eluted from the column using a decreasing salt step change. The impurities were eluted during the load and with the wash buffer and decontamination solution.

Particulars of the hydrophobic interaction chromatography step were: a Phenyl Sepharose HP column was initially sanitized with a 0.1 N sodium hydroxide solution for a minimum contact time of 30 minutes (with at least about 3 column volumes of a 0.1 N sodium hydroxide solution at 200 cm/hour). The column was then equilibrated with at least about 5 column volumes of 50 mM sodium acetate, 0.4 M ammonium sulfate, pH 4.8 buffer. Next the POROS® 20HS (cation exchange column) product pool (from above) was combined 1:1 with a 50 mM sodium acetate, 0.8 M ammonium sulfate, pH 4.8 buffer and loaded onto the Phenyl Sepharose HP column. The column was first washed with at least about 3 column volumes of a 50 mM sodium acetate, 0.4 M ammonium sulfate, pH 4.8 buffer, and then washed with a 50 mM sodium phosphate, 0.4 M ammonium sulfate, pH 6.5 buffer. Botulinum neurotoxin type A complex was eluted from the column with a 10 mM sodium phosphate, 0.14 M ammonium sulfate, pH 6.5 buffer. The eluate was diverted into a bioprocess collection bag when the A_{280} increased to $\geq$ 0.05 AU. The eluate was collected until the A_{280} of the trailing edge of the elution peak decreased to a value of $\leq$ 0.05 AU. The Phenyl Sepharose HP product pool was stored in the collection bag at ambient temperature for up to 6 hours.

A tangential flow filtration system was used to concentrate and diafilter the Phenyl Sepharose HP chromatography step product pool into the drug substance formulation buffer. Pall® Filtron Minimate cassettes with a 100 kDa molecular weight cut off membrane were used for the concentration and diafiltration steps. The formulated material was then passed through a Pall Mini Kleenpak ® 0.2 µm filter to reduce the potential bioburden. As stated previously, the UF/DF step concentrated the Phenyl Sepharose HP product pool (eluent of the HIC column) to a BoNT/A

complex concentration of 0.7 g/L and diafilters the concentrated material with a 10 mM potassium citrate, pH 6.5 buffer.

Particulars of the ultrafiltration/diafiltration process used were as follows. The UF/DF unit and Pall 100 kDa polyether sulfone membrane was initially flushed with a minimum of 5 L of water for injection (WFI) to remove the packing solution and sanitized with a minimum of 200 mL of a 1 N sodium hydroxide solution under recirculation conditions for a minimum of 10 minutes, preferably at least 30 minutes, to sanitize the UF/DF unit. Next the membrane and UF/DF system were equilibrated with sufficient volumes of the 10 mM potassium citrate, pH 6.5 formulation buffer until permeate and retentate pH was pH 6.5. After that the Phenyl Sepharose HP product pool was loaded onto the Minimate® tangential flow filtration cassette and the HIC eluate concentrated to 0.7 g/L. Following the concentration step, the retentate pool was diafiltered against a minimum of 5 diafiltration volumes of the drug substance formulation buffer (10 mM potassium citrate, pH 6.5) at a transmembrane pressure of 7.5 psig (pounds per square inch gauge). The permeate outlet was then closed and the UF/DF system run for at least 2 minutes and the system rinsed with 50 mL of 10 mM potassium citrate, pH 6.5 formulation buffer. After the rinse, the concentration of BoNT/A complex in the retentate pool was determined by measuring the offline A_{278} and based on the A_{278} reading, the concentration of the retentate pool was adjusted to 0.5 g/L with 10 mM potassium citrate, pH 6.5 buffer. The concentration-adjusted retentate pool was then filtered through a Pall Mini Kleenpak 0.2 µm filter to reduce potential bioburden. The filtered concentration-adjusted retentate pool was stored in a collection bag at 2 °C – 8 °C for up to 2 days.

The final purified botulinum neurotoxin type A complex obtained was filled into 1 mL Nunc® cryovials at 700 µL per vial and stored frozen. The filling operation was carried out in a class 100 biosafety cabinet at ambient temperature.

The downstream process (including use of 2 or 3 chromatography columns) was completed in only 1 to 3 days and the botulinum neurotoxin type A complex obtained was stored frozen in a potassium citrate, pH 6.5 buffer at a concentration of 0.5 g/L as a solution. In comparison, the prior art Schantz downstream (toxin purification) process uses multiple filtration, precipitation, extraction and centrifugation steps to purify the botulinum neurotoxin type A complex and requires 1-2 weeks to complete just the downstream steps, and the resultant drug substance (recovered

botulinum neurotoxin) is stored refrigerated as an ammonium sulfate suspension at a concentration of approximately 2.7 g/L. The use of chromatography instead of precipitation and the reduced processing time resulted in a significantly improved, consistent downstream process, as herein disclosed.

In accordance with one aspect, concentrations of vegetable-based products, such as soy-based products, can be Soy Peptone Type II Hy-Soy® or SE50MK(a Kosher soy peptone) in culture and fermentation media. Hy-Soy® in the seed culture medium can range between 10-200 g/L. Preferably, the concentration of Hy-Soy® in the seed medium ranges between 15-150 g/L. Most preferably, the concentration of Hy-Soy® in the seed medium is approximately between about 20-30 g/L or an amount therebetween. The concentration of glucose in seed medium can range between 0.1 g/L and 20 g/L. Preferably, the concentration of glucose ranges between 0.5-15 g/L. Most preferably, the concentration of glucose in the culture medium is approximately 10 g/L. Yeast extract amounts can be from about 5-20 g/L, more preferably from about 10-15 g/L or an amount therebetween. For example, the pH of the culture medium prior to growth of *Clostridium botulinum* can be approximately pH 7.0-7.5, or therebetween, preferably pH 7.3.

As an example, Hy-Soy® amounts in the production fermentation medium can range between 10-200 g/L. Preferably, the concentration of Hy-Soy® in the fermentation medium ranges between 15-150 g/L. Most preferably, the concentration of Hy-Soy® in the fermentation medium is approximately between about 20-40 g/L or an amount therebetween. The concentration of glucose in fermentation medium can range between 0.1 g/L and 20 g/L. Preferably, the concentration of glucose ranges between 0.5-15 g/L or an amount therebetween. Not necessarily, but as above, the glucose can be sterilized by autoclaving together with the other components of the fermentation medium. The pH level of the fermentation medium prior to growth can be pH 7.0-7.8, preferably about 7.0-7.5 or therebetween, more preferably pH 7.3.

As shown by the right hand side of FIG. 1, the two column APF process used in this Example 2 for obtaining a biologically active botulinum neurotoxin complex comprised the following steps: (a) culturing bacteria, such as *Clostridium botulinum* bacteria from an APF WCB vial, in a seed/culturing bottle, (b) then fermenting *Clostridium botulinum* bacteria in a fermentor (toxin production fermentor) having APF fermentation medium to expand the cell line, proceeding with fermentation and

botulinum toxin production until a desired cell lysis phase is reached. Next, (c) harvesting (e.g. clarifying by filtration,) the APF fermentation medium to obtain a harvested fermentation medium, (d) proceeding with concentration and dilution resulting in a diluted harvested fermentation medium that is (e) passed through a capture column to remove impurities, (f) contacting eluent from the capture column with a polishing column to further remove impurities, and optionally a second polishing column (g) concentration and buffer exchange of the polishing column eluent, (h) followed by bioburden reduction filtration and the (i) filling of vials.

In one example, the fermentation volume is 20 L, the total process time for all steps was only 4 to 6 days, and high botulinum neurotoxin yield was obtained.

The following provides more details of a particular embodiment within the scope of our invention. The fermentation step was carried out in APF medium using a 30 L stainless steel fermentor.

In this example below, a much-reduced volume of fermentation medium was used while still providing a high yield of high potency botulinum neurotoxin type A complex. By using the following protocol, only 20 L or less, for example, of APF fermentation medium was required, instead of the typically larger, previous volumes (e.g. 115 L) of fermentation medium required for producing commercially useful amounts for obtaining a botulinum neurotoxin.

The MACS anaerobic workstation (Don Whitley) with airlock provided an oxygen-deficient environment in which to manipulate anaerobic organisms. Access to and egress from the chamber was via a porthole system, comprised of inner and outer doors. The unit was temperature controlled to maintain a user setting within the chamber. A humidistat-controlled condensing plate ensured the effective removal of excess moisture in the chamber. The chamber was illuminated for operator use and alarm for: low gas pressure, continuous gas flow, and loss of power conditions. The chamber was equipped with a HEPA filter to reduce viable and non viable particulate levels in the anaerobic chamber. Anaerobic conditions were maintained utilizing the “Anotox” and Palladium Deoxo “D” Catalyst atmospheric scrubbing system. Condensate water from the condensing plate was collected and piped to an external reservoir where it is removed.

As disclosed above, an APF process was used for preparation of an APF WCB, having cell bank vials stored below -135 °C. An APF WCB cell bank vial was

thawed at room temperature for about 15 min before culture medium inoculation, followed by a single cultivation step as disclosed above to establish a "seed" culture. This was carried out in a modular atmospheric controlled system utilizing aseptic techniques throughout, to minimize bioburden. The modular atmospheric controlled system was cleaned before undertaking inoculation of the completed seed culture vial with APF WCB vial contents. Culture medium was prepared using 1 N hydrochloric acid and 1 N sodium hydroxide (for pH adjustment), D(+) Glucose, Anhydrous (Mallinckrodt Baker, Cat# 7730, 4.00 g), Soy Peptone Type II (SPTII) (Marcor, Cat #1130, 8.00 g), Water for Injection (WFI) 400.0 mL and Yeast Extract (YE) (BD Cat #212730 , 4.00 g). The soy peptone Type II and yeast extract solution was made by measuring 300 mL of WFI with a 500 mL graduated cylinder and poured into a seed culture bottle. The seed culture bottle was placed onto a stirrer and the stirrer activated. 8.00 g of SPTII and 4.00 g of yeast extract was added to the seed culture bottle and mixed until dissolved. If dissolution was not complete after mixing, the mixture would be heated on low setting. The pH was measured and adjusted to about 7.30 ± 0.05. The medium solution was brought up to about 360 mL with WFI. The seed culture bottle was adequately vented to allow steam and gas transfer. A 10% Glucose solution (w/v) was prepared by measuring about 30 mL of WFI with a 100 mL graduated cylinder and placed into the pre-assembled glucose addition bottle, which was placed onto a stirrer and the stirrer activated. About 4.00 g of glucose was added to the glucose addition bottle and mixed until dissolved (low heat was used if necessary to a dissolution) and qs (quantity sufficient) glucose solution to 40 mL with WFI. The glucose addition was then capped loosely with vent cap. Both the glucose and seed culture bottles are autoclaved at 123 °C for 30 minutes for sterilization. After sterilization, both items were removed from the autoclave and left to cool in a bio-safety cabinet. After cooling aseptically, 10% of the glucose solution was transferred into the seed culture bottle containing the yeast extract and soy peptone II solution and mixed, thereby providing a completed seed culture bottle.

This completed seed culture bottle was placed into the pre-cleaned MACS (wherein a prepared anaerobic indicator was placed). The cap of the completed seed culture bottle was loosened. The completed seed culture bottle was then placed on a stir plate within the MACS (stir plate activated to about 150 rpm) and the medium in the completed seed culture bottle was reduced for a minimum of 12 hours at about

34.5 °C +/- 1 °C within the MACS, after which a 1 mL medium blank was sampled for optical density measurement (for biomass determination at 540 nm). Afterwards, the completed seed culture bottle, in the MACS (anaerobic) was inoculated. An APF WCB culture vial was obtained from the frozen cell bank and brought into the MACS. The vial was thawed for about 10-15 minutes, after which about 400 µL of the vial contents were placed directly into the medium in the completed seed culture bottle. The cap on the completed seed culture bottle was loosened completely and the cap was rested on top of the bottle and the stir pace was set to 150 rpm. After at least about 11 hours of incubation in the MACS, fermentation production was undertaken, as described below.

Probes (e.g. redox probe, pH probe, turbidity probe, e.g. by Broadley James and Optek) and sequence configuration of the fermentor, such as a 30 L stainless steel fermentor, were checked and calibrated, and inserted into their respective fermentor ports and tightened in place. For example, a fermentor can be a ABEC 30 L (VT) Fermentor System consisting of a 30 L volume fermentor vessel, an agitator drive system, piping assembly for utility connections (CIP, clean steam, CDA, Nitrogen, Oxygen, Process Chilled Water, bio-waste, and plant steam), instrumentation (pH, temperature, pressure, ReDox, optical density, and mass flow), and four peristaltic pumps. The bottom mounted agitator speed was controlled using an Allen-Bradley variable frequency drive (VFD). Semi-automatic and automatic control of the system is handled by an Allen-Bradley ControlLogix PLC with programming. The system was designed to provide closed-loop PID (proportional-integral-derivative) control of culture temperature, pressure, pH, and redox during fermentation operations. An Allen-Bradley DeviceNet® (an open device level network) is utilized for control and communication with devices and sensors on the skid.

For sterile hold, equilibrium, run and harvest modes, agitation, temperature, pressure and Nitrogen overlay are operated with the following set points.

For sterile hold and equilibrium mode:

Controlled Parameter	Set Points and Range
Agitation	100 rpm ± 10
Nitrogen Overlay	12 SLPM ± 2

Fermentor Pressure	5 psig ± 1
Fermentor Temperature	35 ± 1°C
Redox	-390 to - 150 mV

For RUN mode:

Controlled Parameter	Set Points and Range
Agitation	70 rpm ± 5
Nitrogen Overlay	12 SLPM ± 2
Fermentor Pressure	5 psig ± 1
Fermentor Temperature	35 ± 1°C

For Harvest mode:

Controlled Parameter	Set Points and Range
Agitation	150 rpm ± 10
Nitrogen Overlay	10 SLPM ± 2
Initial Fermentor Pressure	0 psig
Fermentor Temperature	25 ± 1°C

To prepare fermentation medium, material needed include D(+) Glucose, Anhydrous (Mallinckrodt Baker, Cat# 7730, 300.0 g), Soy Peptone Type II (SPTII) (Marcor, Cat #1130, 650.0 g), Water for Injection (WFI, 13 L) and Yeast Extract (YE) (BD Cat #212730 , 240.0 g), along with standard balances, a carboy (20 L, for example), glass bottle (5 L), graduated cylinders, stir bars and stirrers. About 10 L of WFI were added into the carboy along with a stir bar. The carboy was placed onto a stirrer and the stirrer was activated, after which about 650.0 g of soy peptone type II was added, along with about 240.00 g of YE. The fermentation medium was q.s. (quantity sufficient) to 13 L with WFI, and the carboy was capped. A 10% glucose solution (w/v) was then prepared by adding about 2 L if WFI into a glass 5 L bottle (with stir bar therein). Placed onto a stirrer and with the bar spinning, about 300.00 g of glucose was added into the bottle, and mixed until dissolved. The glucose solution was q.s. to 3 L with WFI and the bottled capped, thus providing a 10% glucose solution.

The fermentation medium in the carboy was added to the fermentor and pre-steam in place fermentor volume recorded and the fermentation sequence of operation was advanced. At the end of the SIP (steam in place)(122 °C, +/- 1 °C),

the post-SIP fermentor volume was noted. A glucose addition assembly, comprising a vessel having tube therefrom with and in-line 0.2 µm filter (PALL Corp.) and peristaltic pump, was connected to the fermentor and the line was subjected to SIP and allowed to cool. An addition valve port was opened and about 3 L of glucose (filter sterilized) was added, and the appropriate amount of WFI (filter sterilized) to q.s. the total fermentor volume to 20 L was added to the glucose addition bottle and pumped into the fermentor through the same glucose filter line. The addition valve port was closed. The production fermentation medium had its pH adjusted thereafter, to about pH 7.3 +/- 0.05, with sterile 1 N sodium hydroxide or 1 N hydrochloric acid, utilizing SIP of addition lines, as required. Afterwards, parameters for sterile hold were set and held for about 12 hours before inoculation. The medium's starting glucose concentration was measured using a metabolite analyzer and glucose concentration recorded.

As stated above, at the end of seed culture incubation (about 11 ± 1 hours), 1 mL of sample was taken for optical density (OD) measurement. OD was measured offline at 540nm using a spectrophotometer and if within the appropriate range the OD value was recorded and culture was used for fermentation. The fermentor turbidity probe was accordingly zeroed. The seed inoculum bottle, from the anaerobic chamber, was brought over to the fermentor and a seed inoculum transfer assembly (a seed vessel with APF culture medium therein, the vessel having a culture inoculum transfer line with a sterile Kleenpak™ Connector assembly available from PALL Corp. or Millipore replaced the a valve of the fermentor, and tubing to Pump 1 was fixed. The fermentor pressure was lowered to 2 psig and entire volume of the seed inoculum bottle was pumped into the fermentor. At the end of inoculation, the online Absorbance Units (AU) from the fermentor was recorded, fermentor parameters were set to RUN mode and time was recorded.

Fermentation then proceeded (fermentation runs can be from about 60 hours to about 80 hours, preferably from about 68 hours to about 76 hours, most preferably for about 72 hours) while samples were taken from the fermentor, at 24 and 48 hours, for example, while maintaining aseptic conditions. Tests that were run on at least one sample taken during fermentation can include, but are not limited to, off-line optical density measurements, glucose measurements, ELISA, SDS-PAGE, Western blot, for example. At the end of the fermentation (end of fermentation broth volume is from

about 18-19 L, for example), a sample may be taken (for testing by, for example, off-line optical density measurements, glucose measurements, ELISA, SDS-PAGE, western blot and DNA/RNA quantification.

At the end of the fermentation, online optical density, EFT (elapsed fermentation time), and fermentation end time was recorded, as well as agitation rpm, temperature in °C, pressure psig and Nitrogen overlay slpm and redox mV. Next, the production fermentation broth was subjected to harvesting, i.e. the production fermentation broth is clarified through filtration whereby, for example, about 15 L of filtrate is collected. The fermentation parameters were set for HARVEST and the filter assembly for clarification was prepared (CUNO, 3M filtration) which includes a pre-filter, depth filter and at least one pressure gauge. The pre-filter and depth filter were flushed with about 20 L of water for injection. After flushing, the filtration assembly was attached to the harvest/drain port of the fermentor. The fermentor temperature was decreased to about 25 °C, after which clarification of the fermentation broth begins (record clarification start time, initial online OD, initial pH, initial temperature and initial volume of fermentor). The pressure in the fermentor was increased at a rate of about 1 psi (pound per square inch) about every 10 minutes during filtration, until a pressure of about 6 psi was reached, at which the pressure was held until the end of harvesting. This filter removes approximately 80% of the RNA/DNA in the APF fermentation medium (the remainder essentially removed during later chromatography steps, as discussed below), thus doing away with prior reliance/use of RNase and/or DNase to remove such components from the fermentation broth. Process parameters, such as pre-filter inlet pressure, depth filter inlet pressure, fermentor pressure, agitation and filtrate volume were monitored at every 2 L of filtrate collected, at the end of which the clarification end time and volume of filtrate collected was recorded. Following completion of harvest step, the systems were decontaminated and cleaned.

The filtrate carboy was brought into the BSC for sampling, from which about ≤10 mL of filtrate was sampled for offline OD measurements and other analysis (e.g. ELISA, SDS-PAGE, DNA/RNA and western blot).

The filtrate was then subjected to ultrafiltration/dilution. A tangential flow filter (TFF) unit assembly was assembled. The TFF unit was rinsed for about 90 minutes with WFI at a preferred rate of about 2 L per minute and then the TFF unit was

sanitized by running 0.1 N sodium hydroxide (re-circulated) therethrough for about 60 minutes, after which 1 L of 10 mM sodium phosphate buffer, pH 6.5 was run therethrough, followed by a rinse with WFI for about 30 minutes. The filtrate from the harvest step (about 15 L) was then passed through the TFF (this is carried out in a bio-safety cabinet), concentrating the filtrate down to about 5 L +/- 0.5 L (the concentration step proceeds at about 2 L per minute and at a trans-membrane pressure of about 5 psig). A sample of the permeate can be taken and subjected to ELISA, dsDNA, SDS-PAGE and western blot tests, for example. Once concentrated to about 5 L +/- 0.5 L, the retentate pool was then diluted up to about 20 L with about 15 L of sterile filtered 10 mM sodium phosphate buffer, pH 6.5, through the TFF, at about a rate of 2 L per minute. A sample can be then again be taken and subjected to ELISA, DNA/RNA, SDS-PAGE and western blot tests, for example. The ultrafiltration/dilution material (retentate) was stored at 4 °C.

Following use all systems were decontaminated using either 1N sodium hydroxide or sterilization (steam) temperatures and cleaned.

The following materials, equipment and procedures were used to make the solutions, buffers, etc, set forth below for use in an exemplary process, that is in the purification of the fermentation medium obtained from the Example 2 processes so as to obtain a purified botulinum neurotoxin type A complex. Exemplary buffers utilized (filtered through a 0.2-micron vacuum filter and their conductivity measured in mS/cm, for recordkeeping) include:10 mM sodium phosphate, pH 6.5; 50 mM sodium phosphate, pH 6.5; 50 mM sodium acetate, pH 4.8; 50 mM sodium acetate, 170 mM sodium chloride, pH 4.8; 50 mM sodium acetate, 250 mM sodium chloride, pH 4.8; 50 mM sodium acetate, 1 M sodium chloride, pH 4.8; 50 mM sodium acetate, pH 4.0 and 10 mM citrate, pH 6.5.

The following is an example of operations for purification and obtaining botulinum neurotoxin type A from the Example 2 processes. All product-contact parts were designed and constructed to ensure that they are non-reactive and non-absorptive. Additionally, all equipment was designed to allow the utilization of single use disposable systems or was designed and constructed to facilitate sanitization, cleaning and decontamination as per documented, validated methods. The systems or skids were designed to be non-product contacting while the flow paths are designed to be single use disposable, including the chromatography columns and the

all associated tubing. Chromatography components were obtained from AlphaBio and UF/DF components were obtained from Scilog Inc. The chromatography set ups used included a peristaltic pump for solution delivery with variable speed drive, inlet valve manifold with 5 inlets, a column valve manifold with an array of 3 automated valves, outlet valve manifold with 3 outlets, column effluent monitoring, including pH, conductivity, and UV, peak collection based on UV absorbance, and instrumentation and controls required to complete the purification operations. The control system had both the software and hardware designed to control the purification process. Commands and data were entered via a HMI (Human Machine Interface) terminal. The operator initiated all automated process functions by commands at the HMI and monitored and adjusted process parameters such as feed flow rates, pressure, conductivity, pH, UV absorbance and individual valve positions.

The UF/DF system included of a recirculation pump, diafiltration pump, 2 balances and a tangential flow filter (TFF) holder. The recirculation pump interfaced with 3 disposable pressure sensors and one of the balances (located under the permeate reservoir) to control the flow rate to maintain a defined transmembrane pressure and stop, based on the weight of the permeate reservoir. The diafiltration pump interfaced with the second balance (located under the retentate reservoir) to start and stop, based on maintaining a constant weight of the retentate reservoir.

After concentration and dilution of retentate material from the harvesting step (harvesting the animal protein free fermentation medium), the material was loaded onto an anion exchange column. The following is the procedure used for packing and testing the anion exchange column useful in the Example 2 two column process.

Pre-packed columns were used for all three chromatographic steps. First, feed material (harvested APF media that had been subjected to ultrafiltration/dilution) was passed through the anion exchange column (Poros 50HQ, from ABI as described above). At least 5 column volumes (CVs) of 50mM sodium phosphate, pH 6.5, were utilized to equilibrate the anion exchange column (in this example, a capture column).

After equilibration, the loading step was performed, where feed material (post harvesting step harvested fermentation broth, of about 20 L, for example)) was loaded onto the anion exchange column at a rate of about 200 cm/hr for example. After 0.5 column volume of loaded material had passed through the anion exchange

column, the flow through (FT) pool was collected into a receptacle such as a polyethersulfone vessel, while toxin complex is bound to the anion exchange column material. This was followed by a wash step, where at least about 15 column volumes of the wash buffer (e.g. 50 mM sodium phosphate at a pH of 6.5) was passed through the anion exchange column. The wash step was stopped when the UV, measured at the column outlet, in real time, decreased to less than or equal to about 80 mAU. The wash buffer volume and the flow through/wash pool volume were recorded, and a 1 mL sample of the flow through/wash pool is taken and tested, for example, for toxin concentration, nucleic acid content, whole cell proteins, SDS-PAGE, qPCR, 2D LC and ELISA.

The next step was the elution step, where elution buffer (e.g. 50 mM sodium acetate, pH 4.8) was pumped onto the anion exchange column. When the UV reading at the column outlet, in real-time, increased to about 150 mAU or more, collection of eluate in a container pre-filled with 1 CV of elution buffer (50 mM sodium acetate, pH 4.8) was begun. Collection of eluate pool was stopped when the UV reading decreases to less than or equal to about 200 mAU (volume collected at this point is between about 1 to about 2 CVs). The chromatography system was then decontaminated and cleaned using 1 N sodium hydroxide.

The eluate pool from the anion exchange column was then prepared for addition onto the cation exchange column. The anion exchange eluate volume, pH, conductivity and feed temperature were recoded and the eluate pool from the anion exchange column was diluted with 1 CV of 50 mM sodium acetate, pH 4.8.

Following the run-through of the anion exchange column, cation exchange chromatography operation was undertaken. The cation exchange column (e.g. Poros® 20HS) was equilibrated with a minimum of 5 CVs of equilibration buffer (50 mM sodium acetate, pH 4.8). After equilibration, the diluted eluate pool from the anion exchange column was loaded onto the cation exchange column and the total volume loaded was recorded. After 0.5 column volume of loaded diluted eluate pool had passed through the cation exchange column, the flow through (FT) pool was collected. A first wash of the cation exchange column was conducted where about 3-5 CVs of 50mM sodium acetate, pH 4.8, was passed through the cation exchange column (volume of first wash buffer utilized was recorded). A second wash was performed, where about 3 CVs of 170 mM sodium chloride, 50 mM sodium acetate,

pH 4.8, was pumped through the column, this eluate being collected in a new container labeled “WASH 2 Peak”. Collection was begun when the UV readings increase to greater than or equal to 50 mAU. 1 CV was collected and the second wash buffer volume utilized was recorded.

Elution of bulk toxin complex from the cation exchange column was carried out utilizing elution buffer (e.g. 250 mM sodium choride in 50 mM sodium acetate, pH 4.8) which was pumped onto the cation exchange column. When the UV reading of the elution reached at least about 100 mAU, eluate collection begun into containers pre-filled with dilution buffers (40 mL of 100 mM potassium phosphate, pH 6.8 and 60 mL of 10 mM potassium citrate, pH 6.5). Collection of eluate from the cation exchange column continued until UV readings decreased to about 100 mAU or less. The total volume of elute, after dilution, was recorded. The cation exchange chromatography system was then decontaminated and cleaned.

Following elution from the cation exchange column, the eluate was subjected to filtration. A tangential flow filtration (TFF) system was utilized, using three 100K MWCO membranes (Sartorius AG, Goettingen, Germany) stacked one atop the other. The cation exchange eluate pool initial volume was noted, as are the diafiltration/equilibration and sanitation solution descriptions. For example, the diafiltration solution can be 10 mM potassium citrate, pH 6.5 and the sanitation solution can be 0.1 N sodium hydroxide. System set up proceeded with connection of one tube from the reservoir containing either eluate from the cation column (IAPF) or HIC column (FAPF) , the eluate containing botulinum toxin, through the ultrafiltration pump head into the inlet of the tangential flow filtration membrane. A second tube from the permeate outlet of the tangential flow filtration membrane was connected to the ultrafiltration (UF) permeate container. A tube from the retentate outlet of the tangential flow filtration membrane to the retentate reservoir was secured, and a fourth tube from the diafiltration (DF) buffer through the diafiltration pump head and into the retentate reservoir was also secured. The storage buffer of the system was flushed, as is the membrane, by flushing the membrane with at least about 720 mL of water for injection (WFI) with the retentate directed to waste, after which the membrane was further flushed with at least about 4200 mL of water for injection with the retentate recirculating to the reservoir. After this, membrane sanitation (if necessary) was carried out by flushing the membrane with at least about

200 mL of 1N sodium hydroxide with the retentate directed to waste, followed by a flushing of the membrane with at least about 200 mL of 1N NaOH with the retentate recirculating to the reservoir for a minimum of 30 minutes. Equilibration was then performed, by flushing the membrane with equilibration buffer (10 mM potassium citrate at a pH of 6.5), with retentate directed to waste until the retentate and permeate pH was within +/- 0.2 units of the pH of the equilibration buffer (for example, within +/- 0.2 units of pH 6.5).

The concentration of the material (eluate (product pool) from the cation exchange column) was determined, to see if dilution or concentration (exemplary processing) was appropriate (an example target concentration can be about 0.7 mg/mL). Dilution was accomplished utilizing 10 mM potassium citrate, pH 6.5. A target volume was determined, for example for a 0.7 mg/mL product concentration (target vol= (starting concentration/starting vol)/0.7 mg/mL).

The product pool (eluate (accordingly processed or not) from cation exchange column) was loaded onto the membrane and recirculation (with permeate outlet closed) of the system (TFF system) was run for at least 2 minutes with no backpressure, after which the permeate valve was slowly opened while adjusting the retentate back pressure valve to a target of about 7 psig transmembrane pressure. For dilution, 10 mM potassium citrate, pH 6.5 is added to target volume, and moved onto diafiltration without ultrafiltration; for concentration, ultrafiltration is begun. For diafiltration: permeate waste was collected in a new container (target diafiltration volume is 5X diafiltration volume) and diafiltered with at least 5 diafiltration volumes of 10 mM potassium citrate, pH 6.5. Diafiltration process data was collected at a minimum of 10-minute intervals (permeate weight g/vol mL, inlet pressure (psig), retentate pressure (psig), permeate pressure (psig) and transmembrane pressure (psig)). For recirculation/and rinse: with the permeate outlet filter closed, the system was recirculated/run for at least 2 minutes with no backpressure and the system was rinsed with at least 20 mL of 10 mM potassium citrate, pH 6.5. The product pool includes the retentate and the rinse. A sample can be taken from the product pool and subjected to verification analysis including, for example, UV at 278nm, SDS-Page, LcHPLC. SE-HPLC, qPCR, RP-HPLC, Native-Page, AUC, Limulus amebocyte lysate, Western Blot and ELISA tests. For post-use cleaning, the system was flushed

with 1N sodium hydroxide, recirculated for at least 10 minutes, after which the system was flushed and stored with 0.1 N sodium hydroxide therein.

Sterile filtration and filling was then conducted for storing and dividing the bulk neurotoxin. Concentration adjustment was performed to adjust toxin concentration, using 10 mM potassium citrate, pH 6.5, to about 0.5 mg/mL with the post rinse sample. If toxin concentration was less than about 0.5 mg/mL, then no concentration adjustment is needed.

Using a sterile pipette, 10mL/0.75mL aliquots into each of sterile15 mL/1.5mL sample tubes were made. The product container was gently stirred by hand and transfer the required amount of solution (containing bulk drug substance, i.e. bulk botulinum toxin) into each vial. The samples were stored a maximum of 5 days at 2 °C – 8 °C refrigerator or 0.75 mL of the filtrate product pool was transferred to cryovials. The cryovials are stored at -70 °C +/- 5 °C.

Example 3

Compounding Method

A pharmaceutical composition suitable for administration to a patient can be made by compounding a botulinum neurotoxin obtained from an Example 2 process with one or more excipients. An excipient can act to stabilize the botulinum toxin during the compounding process and during a subsequent period of storage before use. An excipient can also function as a bulking agent and/or to provide a certain tonicity to the pharmaceutical composition. Compounding requires a many fold dilution of the botulinum neurotoxin obtained from an Example 2 process, mixing with one or more excipients (such as albumin [such as a human serum albumin or a recombinant human albumin] and sodium chloride) to thereby form a toxin composition, and preparation of a storage and shipment stable form of the toxin composition, as by lyophilizing, freeze drying or vacuum drying the composition. Thus, about 1.5 to 1.9 ng of the Example 2 obtained botulinum toxin type A complex is compounded with about 0.5 milligrams of recombinant human albumin (Delta Biotechnologies) and about 0.9 milligram of sodium chloride by mixing these three ingredients together followed by vacuum drying. Vacuum drying can take place from about 20 °C to about 25 °C, at a pressure of about 80 mm Hg, for about 5 hours, at which time vials in which these components are vacuum dried are sealed under vacuum and capped, thereby obtaining a vial with about 100 units of botulinum

neurotoxin type A complex. The resulting solid (powdered) vacuum dried product is, upon use, reconstituted with normal (0.9%) saline and used to treat patients with various indications, such as cervical dystonia and hyperhidrosis. Lyophilizing, vacuum or freeze drying prepares a storage and shipment stable form of the compounded botulinum neurotoxin.

In another example, from about 1.5-1.9 ng of the bulk botulinum toxin type A is compounded with about 0.5 milligrams of human serum albumin (Baxter/Immuno, Octapharma, and Pharmacia & Upjohn) and about 0.9 milligram of sodium chloride by mixing these three ingredients together followed by vacuum drying. Exemplary vacuum drying can take place from about 20 °C to about 25 °C, at a pressure of about 80 μm Hg, for about 5 hours, at which time the vials in which these components are vacuum dried are sealed under vacuum and capped, thereby obtaining a vial with about 100 units of botulinum toxin. The resulting solid (powdered) vacuum dried product is, upon use, reconstituted with normal (0.9%) saline and used to treat patients with various indications, such as cervical dystonia and hyperhydrosis. Additionally, a pharmaceutical botulinum toxin composition can contain human serum albumin and/or lactose for example. In one example, about 1.5-1.9 ng of the bulk botulinum toxin type A can be compounded with about 125 micrograms of human serum albumin, and 2.5 milligrams of lactose and vacuum dried, lyophilized or freeze dried for storage stability, for example. In still another example, about 1.5-1.9 ng of botulinum neurotoxin obtained by the processes disclosed herein can be combined with about 10 mg of trehalose and about 0.5 mg of serum albumin (such as human serum albumin, native or recombinant), and optionally, about 1 milligram of methionine to provide about 100 units of botulinum toxin dried product. This composition can be lyophilized and be reconstituted later with, before use, about 1 mL of distilled sterile water or sterile unpreserved saline (0.9% sodium chloride for injection), for example. In particular examples, pharmaceutical botulinum toxin compositions can include sucrose, such as in an exemplary formulation having about 1.5-1.9 ng of botulinum neurotoxin obtained by the processes disclosed herein combined with human serum albumin 20% and sucrose, which can also be lyophilized to provide about 100 units of botulinum toxin type A, and later reconstituted with unpreserved saline (in a volume of about 0.5 mL to about 8.0 mL for example). In a particular example, 200 units of botulinum

neurotoxin can be combined with about 10 mg of sucrose and 2 mg of human serum albumin per mL, and the resultant composition placed into vials and freeze-dried, to be later reconstituted before use with physiological saline.

Additionally, compounding can also utilize the neurotoxic component (i.e. the about 150 kDa component of the botulinum toxin type A complex, free of complexing proteins) of the botulinum toxin type A complex obtainable by the IAPF processes herein disclosed. In one method of purifying the about 150 kDa neurotoxic component from the associated non-toxic proteins (e.g. HAs, NTNH), type A neurotoxin is purified from the associated non-toxic proteins of the complex by a modification of the method of Tse et al. (1982) (Goodnough, M. C., 1994, Thesis, UW, Wis.). Botulinum neurotoxin complex obtained by our IAPF process (which utilizes either the 2-column anion-cation or 3-column anion-cation-HIC steps, as discussed above) is recovered from an DEAE-Sephadex A 50 (Sigma Chemical Co., St. Louis, Mo.), pH 5.5, column and is precipitated by addition of 39 g of solid ammonium sulfate/100 mL. The precipitated toxin complex is collected by centrifugation, dialyzed against 25 mM sodium phosphate, pH 7.9, and applied to a DEAE-Sephadex A50 column equilibrated with the same buffer. The neurotoxic component is separated from the non-toxic proteins of the complex and eluted from the column with a linear 0-0.5 M sodium chloride gradient. Partially purified neurotoxin component is recovered from the DEAE-Sephadex A50 column at pH 7.9 and dialyzed against 25 mM sodium phosphate, pH 7.0. The dialyzed toxin is applied to SP-Sephadex C50 (Sigma Chemical Co.) in 25 mM sodium phosphate, pH 7.0. Contaminating material does not bind to the column under these conditions. The pure neurotoxin (the about 150 kDa component) is eluted with a linear 0-0.25 M sodium chloride gradient. The about 150-kDa pure neurotoxin can be further purified by metal affinity chromatography, gel filtration or other methods of protein chromatography. As above, this pure neurotoxin (the about 150 kDa neurotoxic component of a botulinum toxin complex) can be lyophilized, vacuum or freeze-dried with the various excipients (e.g. serum albumin, sucrose, lactose, sodium chloride, trehalose, etc.) discussed above.

The bulk botulinum neurotoxin complex obtained by our IAPF process, can be compounded in numerous ways. Exemplary patents that disclose various formulations of botulinum toxins, such as U.S. Pat. No. 6,087,327 (discloses a

composition of botulinum toxin types A and B formulated with gelatin); U.S. Pat. No. 5,512,547 (Johnson et al) entitled "Pharmaceutical Composition of Botulinum Neurotoxin and Method of Preparation" issued Apr. 30, 1996 and claims a pure botulinum type A formulation comprising albumin and trehalose, storage stable at 37 °C ; U.S. Pat. No. 5,756,468 (Johnson et al) issued May 26, 1998 ("Pharmaceutical Compositions of Botulinum Toxin or Botulinum Neurotoxin and Method of Preparation"), and claims a lyophilized botulinum toxin formulation comprising a thioalkyl, albumin and trehalose which can be stored between 25 °C and 42 °C; U.S. Pat. No. 5,696,077 (Johnson et al) entitled "Pharmaceutical Composition Containing Botulinum B Complex" issued Dec. 9, 1997 and claims a freeze dried, sodium chloride-free botulinum type B complex formation comprising a type B complex and a protein excipient; and U.S. patent application publication number 2003 0118598 (Hunt) discloses uses of various excipients such as a recombinant albumin, collagen or a starch to stabilize a botulinum toxin (all of these published U.S. patent applications or U.S. patents are hereby incorporated by reference in their entirety), all provide examples of various useful formulations/excipients that may be used to compound the bulk botulinum neurotoxin provided by our IAPF process and provide a pharmaceutical composition.

The botulinum toxin complex obtained can be eluted from an ion exchange column in a pH 7-8 buffer to disassociate the non toxin complex proteins from the botulinum toxin molecule, thereby providing (depending upon the type of *Clostridium botulinum* bacterium fermented) botulinum toxin type A neurotoxic component with an approximately 150 kDa molecular weight, and a specific potency of 1-2 X 10^8 LD_{50} U/mg or greater; or purified botulinum toxin type B with an approximately 156 kDa molecular weight and a specific potency of 1-2 X 10^8 LD_{50} U/mg or greater, or purified botulinum toxin type F with an approximately 155 kDa molecular weight and a specific potency of 1-2 X 10^7 LD_{50} U/mg or greater.

Our invention provides many benefits. Firstly, the two and three column processes of Example 2 eliminates the use of animal source reagents and media (e.g. casein hydrolysate and Columbia blood agar plates) thus markedly decreasing the theoretical risks of patient exposure to prion-like agents or other infectious agents. Secondly, the two and three column chromatographic processes (and associated systems and apparatus) of example 2 are highly reproducible, as

evidenced by excellent batch to batch consistency. This improvement translates to a more consistent clinical profile in patients who require repeated treatments with commercially available botulinum toxin containing compounds over several years. Analytical studies of drug substance (botulinum neurotoxin) from the herein disclosed IAPF processes (2 and 3 column) revealed a lower load of protein and nucleic acid impurities. This lower load of protein impurities translates into a lower risk of immunogenicity (antibody production). In addition, the improved purity of the IPAF process translates into a lower incidence of the non-specific symptoms commonly associated with biologic drugs (eg, nasopharyngitis, upper respiratory tract symptoms, musculoskeletal symptoms, headache, etc.). Furthermore, the improved downsized scale of this process decreases the risk of BoNT/A exposure in laboratory and manufacturing facility staff.

Exemplary advantages of the present invention include, for example:

1. Safety is improved since no component or substance derived from animal source (e.g. human or animal) is used in the process, use of DNase and RNase, Columbia blood agar plates, casein is eliminated (replaced, for example, by: charged filtration during the clarification/harvesting step and modern chromatography techniques; by seeding culture media directly with cells from a working cell bank, that is, cells previously selected and propagated/maintained in APF media; and culture bottle and fermentation media replaced with Soy Peptone Type II (SPTII) as a peptone source).

2. Between about 50 mg to about 200 mg of high quality botulinum toxin type A complex can be obtained per 10 L of fermentation medium.

3. The purified bulk toxin is obtained from a process which is robust, consistent, scalable, validatable, and cGMP compliant. Robust means the process is reproducible even upon an about ± 10% change in one or more of the process parameters. Validatable means the process reproducibly provides consistent yields of purified toxin. cGMP compliance means that the process can be easily converted to a manufacturing process that complies with FDA required current Good Manufacturing Practices.

4. The potency of the final purified botulinum toxin complex meets or exceeds the potency (e.g. as determined by the MLD50 assay) of purified botulinum toxin complex obtained from a Schantz or modified Schantz process.

5. Replacement of any precipitation steps with chromatographic steps to purify a bulk botulinum toxin complex, which improves the specificity of the purification process.

6. New improved process facilitates reduction of scale resulting in improved handling and achievement of an operational success rate of >95% (for example, reduced from typical volumes utilizing 110 L -120 L of fermentation media down to about 10 L to about 50 L, even down to about 2 L to about 30 L of fermentation media or an amount therebetween. Typical current production scale for bulk drug substance is 115 L of non-APF fermentation medium, and has, as one aspect of our invention, been reduced to 20 L of fermentation medium. This reduction in scale is made possible by optimizing the synthesis and cellular release of the BoNT/A complex as well as overall yield across the purification steps, resulting in similar quantity of final bulk botulinum toxin (drug substance) as obtained in prior processes requiring, for example 5X or even more fermentation volumes (e.g. 115 L). This reduced scale facilitates easier management of the fermentation working volume and thus minimizes the potential risk of operator exposure to the BoNT/A complex, an important operational and safety advantage.

7. Due to the potentially lethal nature of the BoNT/A complex, closed systems have been implemented throughout the manufacturing process as herein disclosed. Unlike prior art methods, no drug substance material produced in accordance with aspects of the present invention is exposed to the environment during transfer between unit operations; all operations are wholly contained.

8. The bulk botulinum toxin manufacturing process herein disclosed is simplified at all steps without sacrificing the identity, quality, purity, or potency of the drug substance during manufacture. A number of steps utilized in a non-APF process have been eliminated in the redesigned IAPF process, thereby reducing production time from, for example, 21 days to 6 days or less.

9. The storage condition of bulk botulinum toxin as a frozen solution greatly improves drug substance stability.

Various publications, patents and/or references have been cited herein, the contents of which, in their entireties, are incorporated herein by reference. Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member may be referred to and claimed

individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group may be included in, or deleted from, a group for reasons of convenience and/or patentability. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

Although the present invention has been described in detail with regard to certain preferred methods, other embodiments, versions, and modifications within the scope of the present invention are possible. Accordingly, the spirit and scope of the following claims should not be limited to particular descriptions of the embodiments set forth above.

We claim:

1. A substantially animal product free (APF) process utilizing chromatography for obtaining a biologically active botulinum neurotoxin, the process comprising the following steps:

(a) providing a substantially APF fermentation medium;

(b) fermenting *Clostridium botulinum* bacteria in the fermentation medium, and;

(c) recovering the biologically active botulinum neurotoxin from the fermentation medium by contacting the fermentation medium with an anion exchange chromatography media followed by contacting an eluent from the anion exchange chromatography medium with a cation exchange chromatography, thereby obtaining the biologically active botulinum neurotoxin from the substantially APF chromatography process.

2. The process of claim 1, wherein the botulinum neurotoxin obtained comprises one part or less residual nucleic acid per million of the botulinum neurotoxin obtained.

3. The process of claim 1, wherein the process is carried out in one week or less.

4. A biologically active botulinum neurotoxin obtained by the process of claim 1.

5. A substantially APF chromatographic process for obtaining a biologically active botulinum neurotoxin type A complex, the process comprising the following sequential steps:

(a) culturing *Clostridium botulinum* bacteria in a substantially APF culture medium;

(b) fermenting *Clostridium botulinum* bacteria from the culture medium in about 2 L to about 75 L of a substantially APF fermentation medium, wherein at least one of the culture medium and the fermentation medium includes a vegetable protein;

(c) harvesting the fermentation medium by removing cellular debris present in the fermentation medium;

(d) concentrating the harvested fermentation medium by filtration;

(e) diluting the concentrated fermentation medium by adding a buffer;

(f) a first contacting step in which the diluted harvested fermentation medium is contacted with an anion exchange media so that the biologically active botulinum neurotoxin becomes captured with the anion exchange media;

(g) eluting the captured botulinum neurotoxin from the anion exchange media to thereby obtain a first eluent;

(h) a second contacting step in which the first eluent is contacted with a cation exchange media to remove impurities from the first eluent, to thereby obtain a second eluent;

(i) processing the second eluent by diafiltration; and

(j) filtering the processed second eluent, thereby obtaining biologically active botulinum neurotoxin type A complex using a substantially APF chromatographic process, wherein the botulinum neurotoxin type A complex obtained has a potency of 2.0 x 10^7 to about 6.0 x 10^7 units/mg of botulinum neurotoxin type A complex.

6. The process of claim 5, wherein the fermentation medium comprises no more than about 5% w/v of a vegetable-derived protein product, no more than about 2% w/v of a yeast extract and no more than about 2% w/v glucose, and wherein the pH level of the fermentation medium is from about pH 6.5 to about pH 8.0 at the commencement of the fermenting step.

7. The process of claim 5, wherein the culturing step is carried out until the optical density of the culture medium at about 540 nm is between about 0.8 AU and about 4.5 AU.

8. The process of claim 5, wherein the fermenting step is carried out for about 60 to 80 hours and until an optical density of the fermentation medium at about 890 nm decreases to between about 0.05 AU to about 0.7 AU.

9. The process of claim 5, wherein the culturing step is initiated by introducing a *Clostridium botulinum* APF working cell bank content to the culture medium, wherein the working cell bank comprises at least about 1 x 10^4 to 5 x 10^7 colony-forming units of *Clostridium botulinum* per milliliter of the working cell bank, and wherein the

Clostridium botulinum bacterium in the working cell bank have a substantially uniform morphology.

10. The process of claim 5, wherein the botulinum neurotoxin obtained comprises one ng or less than one ng of residual nucleic acid for each mg of the botulinum neurotoxin obtained.

11. The process of claim 5, wherein the process is carried out in one week or less.

12. An APF chromatographic process for obtaining a biologically active botulinum neurotoxin, comprising the following sequential steps of:

(a) adding *Clostridium botulinum* bacteria from an APF working cell bank to an APF culture medium;

(b) culturing the *Clostridium botulinum* bacteria in the culture medium;

(c) fermenting the *Clostridium botulinum* bacteria from step (b) in an APF fermentation medium until *Clostridium botulinum* cell lysis occurs;

(d) harvesting the fermentation culture to provide a harvested fermentation medium;

(e) subjecting the harvested fermentation medium to concentration by filtration;

(f) diluting the filtered fermentation medium by addition of a buffer to obtain a diluted fermentation medium;

(g) a first contacting step in which the diluted fermentation medium is contacted with a capture chromatography, wherein the capture chromatography media is an anion exchange media;

(h) a second contacting step wherein an eluent from the first contacting step is contacted with a polishing chromatography media, wherein the polishing chromatography media is a cation exchange media, and

(i) filtering eluent from the second contacting step, thereby obtaining biologically active botulinum neurotoxin by the improved APF process, wherein the botulinum neurotoxin obtained comprises one ng or less than one ng of residual nucleic acid for each mg of the botulinum neurotoxin obtained and the process is carried out in one week or less.

13. A substantially animal product free (APF) chromatographic system for obtaining a biologically active botulinum neurotoxin, the system comprising:

(a) a substantially APF fermentation medium;

(b) *Clostridium botulinum* bacteria for fermenting in the fermentation medium;

(c) an anion exchange chromatography medium for recovering biologically active botulinum neurotoxin from the fermentation medium, and;

(d) a cation exchange chromatography medium for recovering further biologically active botulinum neurotoxin from an eluent from the anion exchange chromatography medium, thereby obtaining biologically active botulinum neurotoxin from a substantially APF chromatography process.

14. The system of claim 13, further comprising a first apparatus for anaerobically culturing the *Clostridium botulinum* bacteria in a substantially APF culture medium.

15. The system of claim 14, further comprising a second apparatus for anaerobically fermenting the *Clostridium botulinum* bacteria in the substantially APF fermentation medium, wherein the *Clostridium botulinum* bacteria are obtained from the first apparatus.

16. The system of claim 15, further comprising a harvesting apparatus for removing cellular debris from the fermentation medium obtained from the second apparatus, to thereby provide a harvested fermentation medium.

17. The system of claim 16, further comprising a concentration and diluting apparatus to concentrate then subsequently dilute the harvested fermentation medium.

18. The system of claim 13, further comprising hydrophobic interaction medium for recovering further purified biologically active botulinum neurotoxin from an eluent from the cation exchange chromatography medium.

19. The system of claim 13, further comprising a filtration apparatus for reducing bioburden in the obtained biologically active botulinum neurotoxin.

20. The system of claim 18, further comprising a filtration apparatus for reducing bioburden in the obtained biologically active botulinum neurotoxin.

21. The system of claim 13, further comprising an anaerobic chamber having an integrated high efficiency particulate air filter within its workspace, for culturing *Clostridium botulinum* bacteria in a substantially APF culture medium.

22. The system of claim 13, wherein the botulinum neurotoxin obtained has a potency of at least about 2.0×10^7 units/mg of botulinum neurotoxin and the botulinum neurotoxin obtained comprises one ng or less than one ng of residual nucleic acid for each mg of the botulinum neurotoxin obtained.

23. The system of claim 13, wherein the a substantially APF fermentation medium is provided in an amount of from about 2 L to about 75 L.

24. The system of claim 14, wherein from about 2 L to about 75 L of the substantially APF fermentation medium is utilized and from about 200 mL to about 1 L of substantially APF culture medium is utilized.

25. A substantially APF chromatographic system for obtaining a biologically active botulinum neurotoxin, the system comprising:

a first apparatus for culturing *Clostridium botulinum* bacteria, the first apparatus capable of containing a substantially APF culture medium;

a second apparatus for fermenting *Clostridium botulinum* bacteria which have been cultured in the first apparatus, the second apparatus capable of containing a substantially APF fermentation medium;

a third apparatus for harvesting the fermentation medium;

a fourth apparatus for concentrating the harvested fermentation medium and diluting the filtered fermentation medium;

a fifth apparatus for carrying out a first purification of the botulinum neurotoxin from the harvested medium, wherein the fifth apparatus comprises an anion

exchange chromatography media, thereby obtaining a first purified botulinum neurotoxin; and

a sixth apparatus for carrying out a second purification of the first purified botulinum neurotoxin wherein the sixth apparatus comprises a cation exchange chromatography media, thereby obtaining a second purified botulinum neurotoxin, wherein the botulinum neurotoxin obtained has a potency of at least about 2.0×10^7 units/mg of botulinum neurotoxin, the botulinum neurotoxin obtained comprises one ng or less than one ng of residual nucleic acid for each mg of the botulinum neurotoxin obtained and the process is carried out in one week or less.

26. The system of claim 25, further comprising a seventh apparatus for carrying out a further purification of the botulinum neurotoxin obtained from the sixth apparatus, wherein the seventh apparatus comprises a hydrophobic interaction media, thereby obtaining a third purified botulinum neurotoxin.

27. The system of claim 26, further comprising a eighth apparatus comprising a filtration membrane for filtering eluent from the either sixth or seventh apparatus.

28. A biologically active botulinum neurotoxin obtained utilizing the system of claim 25.

29. A substantially APF chromatography system for obtaining a biologically active botulinum neurotoxin, the system comprising:

a first apparatus for anaerobic culturing *Clostridium botulinum* bacteria, the first apparatus capable of containing from about 200 mL to about 1 L of a substantially APF culture medium;

a second apparatus for anaerobic fermentation of *Clostridium botulinum* bacteria which has been cultured in the first apparatus, the second apparatus capable of containing from about 2 L to about 75 L of a substantially APF fermentation medium and including at least one disposable probe selected from the group consisting of a reduction-oxidation probe, a pH probe and a turbidity probe;

a third apparatus for harvesting the fermentation medium;

a fourth apparatus for concentrating the harvested fermentation medium and diluting the filtered fermentation medium;

a fifth apparatus for carrying out a first purification of botulinum neurotoxin obtained from the harvested fermentation medium, the fifth apparatus comprising an anion exchange chromatography media, thereby obtaining a first purified botulinum neurotoxin;

a sixth apparatus for carrying out a second purification of the botulinum neurotoxin, the sixth apparatus comprising a cation exchange chromatography media, thereby obtaining a second purified botulinum neurotoxin;

a seventh apparatus carrying out a third purification of botulinum neurotoxin, the seventh apparatus comprising hydrophobic interaction media, thereby obtaining a third purified botulinum neurotoxin; and

a eighth apparatus for filtering the third purified botulinum neurotoxin, the eighth apparatus comprising a filtration membrane, wherein the botulinum neurotoxin obtained has a potency of about 2.0×10^7 to about 6.0×10^7 units/mg of botulinum neurotoxin, the botulinum neurotoxin obtained comprises about one ng or less than about one ng of residual nucleic acid for each mg of the botulinum neurotoxin obtained and the process is carried out in one week or less.

30. The system of claim 29, further comprising a modular atmosphere controlled system that includes an anaerobic chamber capable of containing the first apparatus, wherein the an anaerobic chamber has an integrated high efficiency particulate air filter within the anaerobic chamber.

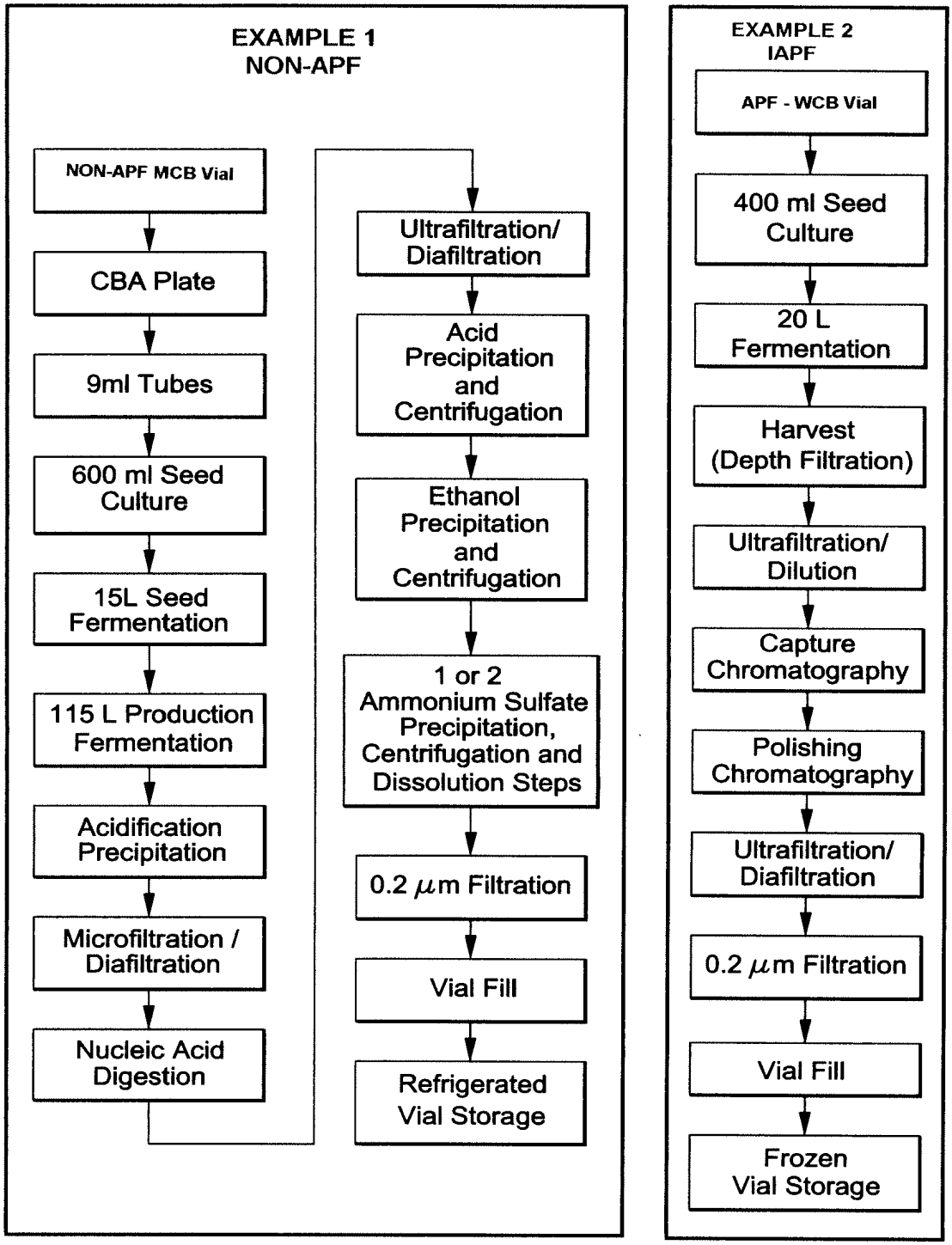

EXAMPLE 1
NON-APF
NON-APF MCB Vial
CBA Plate
9ml Tubes
600 ml Seed Culture
15L Seed Fermentation
115 L Production Fermentation
Acidification Precipitation
Microfiltration / Diafiltration
Nucleic Acid Digestion
Ultrafiltration/ Diafiltration
Acid Precipitation and Centrifugation
Ethanol Precipitation and Centrifugation
1 or 2 Ammonium Sulfate Precipitation, Centrifugation and Dissolution Steps
0.2 μm Filtration
Vial Fill
Refrigerated Vial Storage
A
EXAMPLE 2
IAPF
APF - WCB Vial
400 ml Seed Culture
20 L Fermentation
Harvest (Depth Filtration)
Ultrafiltration/ Dilution
Capture Chromatography
Polishing Chromatography
Ultrafiltration/ Diafiltration
0.2 μm Filtration
Vial Fill
Frozen Vial Storage
B

FIG. 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/041745

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/33 C12N1/20 C12P21/02 A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N A61K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2006/228780 A1 (LUO MINGJIANG [US] ET AL) 12 October 2006 (2006-10-12) cited in the application the whole document	1-4,13, 18,20
X	WO 2006/096164 A1 (ALLERGAN INC [US]; WANG PING [US]; DONOVAN STEPHEN [US]) 14 September 2006 (2006-09-14) the whole document	1-4,13, 18,20
X	GIMENEZ J. ET AL.: "SIMPLIFIED PURIFICATION METHOD FOR CLOSTRIDIUM BOTULINUM TYPE E TOXIN" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 53, no. 12, 1 January 1987 (1987-01-01), pages 2827-2830, XP002343950 the whole document	1-4,13, 18,20

☐ Further documents are listed in the continuation of Box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
19 August 2010	10/11/2010

Name and mailing address of the ISA/	Authorized officer
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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2010/041745

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-4, 13(completely); 18, 20(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-4, 13(completely); 18, 20(partially)

Process for preparing biologically active botulinum neurotoxin characterized by using an APF fermentation medium to culture Clostridium bacteria and recovering the botulinum neurotoxon by using an anion exchange chromatography followed by contacting the eluent with a cation exchange chromatography, the botulinum neurotoxin obtained by said process and systems appropriate for carrying out the process.

---

2. claims: 5-11, 14-17, 22-24(completely); 18-21, 25-30(partially)

Process for preparing botulinum neurotoxin type A complex characterized by culturing Clostridium bacteria in an APF medium which included vegetable proteins, containing filtrations steps for concentration purpose, subsequently diluting the concentrated fermentation medium, purifying the neurotoxin via anion exchange chromatography, subsequently purifying the eluent with an cation exchange chromatography, processing the second eluent by diafiltration and finally filtering the processed second eluent and systems appropriate for carrying out said process

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3. claims: 12(completely); 18-21, 25-30(partially)

Process to prepare botulinum neurotoxin containing steps (a) to (i) as defined in claim 12 and systems appropriate for carrying out said process.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2010/041745

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2006228780 A1	12-10-2006	US 2005238669 A1	27-10-2005
		US 2009124790 A1	14-05-2009
		US 2009123497 A1	14-05-2009
WO 2006096164 A1	14-09-2006	AU 2005327457 A1	05-10-2006
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		BR PI0508299 A	31-07-2007
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		WO 2006096163 A1	14-09-2006

Form PCT/ISA/210 (patent family annex) (April 2005)

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08/287,350 8 August 1994 (08.08.94) US

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(81) Designated States: AU, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

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(54) Title: PURIFICATION AND PHARMACEUTICAL COMPOSITIONS CONTAINING TYPE G BOTULINUM NEUROTOXIN

(57) Abstract

The isolation and purification of type G botulinum neurotoxin and complexes thereof is disclosed. Compositions containing the type G neurotoxin and the preparation of a type G toxoid are also disclosed.

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PURIFICATION AND PHARMACEUTICAL COMPOSITIONS CONTAINING TYPE G BOTULINUM NEUROTOXIN

Field of the Invention

The present invention relates to botulinum toxin. More particularly, it relates to a method of purifying type G botulinum neurotoxin and novel pharmaceutical compositions containing type G botulinum neurotoxin.

Background of the Invention

The most serious form of bacterial food poisoning is botulism which is caused by neurotoxins produced by *Clostridium botulinum*. The toxins are absorbed from the intestinal tract and transported via the circulatory system to motor nerve synapses where their action blocks normal neural transmissions. There are seven serotypes of botulinum toxin with similar toxic activity but differing antigenically. They are type A, B, C, D, E, F and G. Type A is the predominant toxin in cases of botulism in the United States, and type B toxin is most prevalent in Europe. The symptoms for the disease caused by the various serotypes are about the same, but the mechanism of action is different. It is possible to protect humans by a pentavalent immunization against types A to E, but because of unavailability of the purified toxin, specific antitoxins are not available for type G.

Crystalline botulinum toxin type A prepared in 1979 by E. J. Schantz of the Food Research Institute/Department of Food Microbiology and Toxicology, University of Wisconsin,

Madison, Wisconsin, USA, has been used medicinally in the treatment of hyperactive muscle movement disorders, such as strabismus and other involuntary muscle disorders. Treatment involves injection of nanogram quantities (1 ng is equal to 30 mouse 50% lethal doses [30 U]) of the toxin directly into the hyperactive muscles. The toxin inhibits the release of acetylcholine from the motor neuron across the synaptic junction, causing a decrease in the hyperactivity of the injected muscles.

Botulinum toxin type A has become one of the most effective treatments for various types of dystonias, or involuntary muscle contractions, for many thousands of people. A major drawback to the use of botulinum toxin type A in the treatment of hyperactive muscle disorders in the development antibodies to the type A toxin resulting is ineffective treatment with the toxin, particularly for patients treated for spasmodic torticollis which requires larger and sometimes more frequent doses of the toxin by injection. The type A toxin is recognized by a patient's immune systems as a foreign protein which stimulates antibody production. The fact that antibodies to the type A toxin have been formed in patients has been verified by the U.S. Centers for Disease Control and other medical units.

Although the commercially available type A preparation is very useful, there is need to have a pharmaceutical composition of a botulinum toxin of alternative serotype which can be used in patients who have developed antibodies to the type A toxin, and the many individuals who have

antibodies to types A-E, including military soldiers and researchers who have been immunized with pentavalent toxoid.

There also is a need to have a purified type G botulinum neurotoxin which can be used to prepare a toxoid to protect against type G botulism as might occur naturally or in biological warfare.

Brief Summary of the Invention

It is an object of the present invention to disclose novel pharmaceutical compositions that contain type G botulinum neurotoxin and its protein complexes and which can be used in patients who have developed antibodies to the type A toxin.

It is a further object to disclose a novel method of purifying the type G neurotoxin from a natural source so that it can be used to prepare such pharmaceutical compositions and a toxoid that can be used to immunize patients against the type G botulism.

We have discovered a method of purifying type G botulinum neurotoxin from natural sources to obtain type G neurotoxin of high purity which can be used to prepare a toxoid or for medicinal use. Since type G botulinum neurotoxin has a different mechanism of action than type A, it could be used in patients who have developed antibodies to the type A toxin or in individuals immunized with A-E pentavalent toxoid.

The novel method of purifying the type G botulinum neurotoxin basically comprises producing C. *botulinum* type G

botulinum toxin in a liquid medium culture, precipitating the neurotoxin with acid, and then purifying it by ion exchange chromatography, affinity chromatography and gel filtration chromatography to obtain the purified type G neurotoxin or type G neurotoxin complexed with nontoxic binding proteins. The pure type G neurotoxin is isolated from the complex by treating the complex by further chromatographic steps including affinity chromatography and size exclusion chromatography depending on the preparation. The toxin is activated by treatment with an endoproteinase.

The novel pharmaceutical compositions of the present invention contain about 10^5 to 5 x 10^7 mouse LD_{50}'s (U) per mg of protein of active botulinum neurotoxin type G.

At the time of use, the compositions can be diluted to the desired concentration by aseptically adding physiological saline or buffers to obtain a solution suitable for injection. The desired amount of the solution (e.g. 0.1 ml) is then injected using a suitable needle, such as an electromyographic needle, into the proper or hyperactive muscle.

Description of the Preferred Embodiment

The preferred embodiment of the compositions of the present invention is a product containing type G botulinum neurotoxin or its protein complex containing 10^5 to 5 x 10^7 U of activity dependent upon the degree of purification. The toxin may be stabilized in organic or inorganic buffers of appropriate pH and ionic strength. The toxin can also be

stabilized by the addition of exogenous proteins and other excipients.

The preferred method of preparing the compositions of the present invention comprises dissolving the purified, isolated botulinum type G toxin with a solubilizing amount of sodium phosphate buffer or other appropriate buffer (pH 6.2 to 7.4) and ultrafiltering the solution to maintain sterility. It could be dried by lyophilization for dispensing to physicians.

Type G botulinum complexes and purified neurotoxin are produced in liquid medium culture. The neurotoxin component has a molecular weight of about 140-150 kD as determined by SDS-PAGE. Its activity is increased by treatment with trypsin and Endoproteinase Lys-C. High-quality type G toxin produced from *Clostridium botulinum* strain 89 has a specific LD_{50} per mg. toxicity in mice of 1 to 5 x 10^7 U/mg, an A_{260}/A_{278} ratio of less than 0.60, and a distinct pattern of banding on gel electrophoresis. The neurotoxin also can be isolated as complexes of 300-500 kD (10^5 to 10^7 U/mg) which are more stable than the purified neurotoxin and may have even greater utility in medicine.

For lyophilization, aliquots (usually 0.1 ml) of the toxin solutions can be pipetted into 0.5 dram (ca. 1.85-ml), screw- cap glass vials with rubber-lined closures. The solutions in glass vials may then be frozen in a bath of liquid nitrogen at -200°C or by placing them in a freezer at -20°C or -70°C. The loosely capped vials then can be placed into a 150 ml lyophilization flask that had been precooled in

liquid nitrogen. The lyophilization flask is partially immersed in liquid nitrogen and connected to a lyophilizer (Virtis Freezemobile 12, Virtis Co., Inc. Gardiner, N.Y. USA). The liquid nitrogen is maintained in contact with the lyophilization flask until the pressure drops to approximately 30 millitorrs (ca. 4.0 kPa). The liquid nitrogen jacket is then removed, the flask and contents are allowed to come to room temperature (ca. 3 to 4 h), and the drying procedure is continued for 12 to 18 h. The pressure is maintained at or below 20 millitorrs for the remainder of the cycle. Condensor temperature on the lyophilizer is kept constant at ca. -60°C. After the drying cycle is completed, the lyophilized toxin vials are removed from the flask, tightly capped, and held at room temperature for up to three days until the mouse assay. The lyophilized preparations can be reconstituted in 1.0 ml of distilled water or 0.85% saline as a diluent.

MATERIALS AND METHODS

Toxin production

C. botulinum Type G strain 89 (available from Centers for Disease Control, Atlanta, Georgia USA) was initially obtained from a soil sample from Mendoza, Argentina. Long term storage is in cooked meat medium (Difco). Working stocks were stored in a medium (TPGY) containing 5% trypticase-peptone (BBL), 0.5% Bactopeptone (Difco), 2% yeast extract (Difco), 0.4% glucose (Mallinckrodt), and 0.2% L-cysteine HCl (Sigma) adjusted to pH 7.3. An 8% final

concentration of sterile glycerol was added to 16 to 40 hour cultures as a cryoprotectant. Cultures were stored at -60 to -80°C.

Toxin production medium was 2.0% trypticase-peptone (BBL), 1.0% proteose-peptone (Difco), 0.5% yeast extract (Difco), and 0.5% glucose (Mallinckrodt) adjusted to pH 7.3 (2). When large volumes were used, a 14% glucose solution was autoclaved for 20 minutes and added to the large volume which was autoclaved for one hour.

After thawing, a working stock culture was mixed and 0.1-0.2 ml inoculated into a 10 ml tube of TPGY and incubated at 30°C. After 16-40 hours incubation, 10 ml of TPGY were inoculated into 100 ml of toxin production medium. After 16 hour incubation, 100 ml were inoculated into 1400 ml of toxin production medium. Finally after 16 hours incubation, 1400 ml were inoculated into 14 liters of toxin production medium in 20 liter carboys. The carboys were incubated six days at 30°C. A 10% CO_2 + 90% N_2 gas mixture filtered through a 0.2 um filter (Gilman) was bubbled through a gas dispersion tube into the toxin production medium contained in a 20 liter carboy for 3-4 hours prior to inoculation and during the entire six day incubation. Exhaust from carboy was bubbled through gas dispersion tubes into two side arm flasks connected in series containing 5.25% hypochlorite.

Toxin purification

All buffers except acetate were made by titrations of equimolar solutions of basic and acidic components. Acetate

buffer was made by addition of NaOH solution to the desired molarity of acetic acid. All chromatography was done at room temperature.

The toxin was precipitated by adding 0.2g/liter RNA (Sigma) and lowering the pH to 3.4 with 3 M HCl. The addition of RNA which is not essential improved the yield. The acidified culture was stored 16 to 72 hours at 4°C. The precipitate was collected by siphoning off liquid and centrifugation of the precipitate. The pellet was extracted in 0.2 M phosphate + 0.5 M NaCl buffer, pH 7.0 for two hours at room temperature. The extract was centrifuged and the clarified supernatant fluid saved as the first extract. The solution was again acidified to pH 3.4 with HCl. The second pellet was reextracted in the same buffer for one hour at room temperature and then centrifuged. The clarified supernatant fluid was combined with first extract fluid and made to 60% saturation with $(NH_4)_2SO_4$ (399/100 ml) before being stored at 4°C.

The precipitate of the extraction was collected by centrifugation and resuspended in 0.05 M phosphate buffer pH 6.0. The toxin solution was treated with 0.05 mg/ml RNase (Sigma type 1A) at 37°C for three hours. This step removes the RNA associated with the toxin complex. The toxin solution was precipitated with 60% $(NH_4)_2SO_4$ and stored at 4°C.

DEAE-Sephadex chromatography. The precipitate of RNase treated material was collected by centrifugation, resuspended in 40 ml of 0.05 M sodium citrate buffer, pH 5.5. The sample

was dialyzed for three hours at room temperature against one liter of the citrate buffer. Buffer was changed three times. The toxin solution was clarified by centrifugation and loaded on DEAE-Sephadex A-50 gel (Sigma) that had been equilibrated and washed with citrate buffer. The toxin sample did not bind while nucleic acids did under these conditions and the toxin was washed out of the column with the citrate buffer. The toxin came off in the void volume in a large protein peak. Fractions with A_{260}/A_{278} ratio of 0.6 or less were pooled and precipitated with 60% $(NH_4)_2SO_4$.

SP-Sephadex chromatography. The precipitated toxin pool was collected by centrifugation and resuspended in 0.02 M acetate buffer, pH 6.0. The sample was dialyzed against three changes of the acetate buffer overnight at 4°C. During this dialysis a precipitate formed. It was removed by centrifugation. The toxin was loaded on a SP-Sephadex C-50 column equilibrated and washed with the acetate buffer. After sample application, the column was washed with a column volume of buffer and then eluted with NaCl gradient of 0 to 0.5 M NaCl. The toxin was split between the protein peaks coming off the column. The first peak unretarded by the column contained 40-60% of the recovered toxin. The second peak eluted with 0.25 M NaCl also contained 40-60% of the recovered protein. The toxic pools were not combined but purified further separately. They differ in their affinity for the column for unknown reasons, but this may be related to the presence of sugars.

PAPTG affinity chromatography. The toxin that was eluted with NaCl was further purified although the other fraction pool can also be purified with different recoveries of purified neurotoxin. It was dialyzed at 4°C against 0.02 M acetate, pH 6.0, to remove NaCl. The toxin sample was centrifuged to remove any precipitate that formed. The toxin was loaded onto PAPTG affinity gel, that was prepared as described previously (11) except that 0.02 M acetate (pH 6.0) was used in place of 0.025 M phosphate buffer, and mixed with gentle mixing for two hours at room temperature. The gel was then washed on a splintered glass filter with 100 ml of 0.02 M acetate pH 6.0. The gel was washed with 100 ml of 0.1 M tris buffer, pH 7.9. The column was then washed with 15 ml of the tris buffer +0.05 M NaCl. The toxin was eluted with the tris buffer +0.5 M NaCl. The toxin at this stage is relatively pure and this stage contained a specific toxicity of 1 - 4 x 10^7 mouse LD_{50} per mg. protein. It can be slightly more purified by size exclusion chromatography using an appropriate gel, e.g. Sephadex 200.

SDS-PAGE. The results of the purification process were analyzed by SDS-PAGE using the Phastsystem (Pharmacia) with 10-15% acrylamide gradient slab gels (sample load per well was 2 to 4 ug protein/4 ul). Gels were run and stained with Coomassie blue according to the Phastsystem manual with the exception that sample buffer did not contain reducing agent. Four percent 2-mercaptoethanol was added to samples when S-S bond reduction was desired. The electrophoretic analysis showed purified neurotoxin of about 144,000 in molecular

weight. The toxin complexes contained 1 to 5 additional bands and had molecular weights of 300-500 kD.

Electrophoresis and electroblotting. Gels for blotting were made and electrophoresis and electroblotting were preformed as described previously (7).

Amino acid sequence. The toxin was partially sequenced using pulsed-liquid phase (model 477-A) Applied Biosystems protein sequencers. The sequence confirmed its uniqueness compared to other botulinum neurotoxins.

Protein assays. Protein was determined by the BIO-RAD method (BIO-RAD Lab., Richmond, California, USA) with standards of serum albumin. Protein was estimated spectrophotometrically assuming an A_{278} of 1.65 for a 1.0 mg/ml solution.

Activation of Toxin. Crude samples were activated with .1% trypsin (Difco 1/250) as described previously (2). Purified samples were activated with 1 part trypsin (Type XIII-TPCK treated; Sigma) to 40 parts protein in .1 M phosphate buffer pH 6.0 for 15 minutes at 37°C. The reaction was stopped by addition of soybean trypsin inhibitor at twice the trypsin weight. Endoproteinase Lys-C (Sigma) was also used to activate purified toxin solutions (7,9). Two concentrations were tried, 0.1 unit/mg protein and 5 units/mg protein. Activation was done at pH 6.0 to 7.9 for one hour at 37°C. The reaction was stopped by addition of TLCK (Sigma) at ten times the enzyme weight. The toxin was activated 10 to 100 fold. Endoproteinase Arg-C (Sigma) was

also tried at 5 units/mg protein under same conditions as Lys-C, but did not activate the toxin.

Toxicity assays. Toxicity was determined in mice of 18-25 grams. A standard curve was plotted of the log of intraperitoneal (IP) LD_{50} versus the log of minutes to death intravenously (3). Type G toxin, eluted from the SP-Sephadex C-50 column in the NaCl gradient, having specific toxicity of 6×10^4 LD_{50}/mg and 4×10^6 LD_{50}/ml after activation with TPCK treated trypsin was used to develop the curve. IP toxicity was determined by injecting (0.5 ml) of two fold serial diluted samples into separate groups of five mice. Dilutions were in .034 M phosphate buffer + 0.2% gelatin, pH 6.3. Deaths were recorded for four days and LD_{50} determined as previously described (15). IV injections (0.1 ml) were made into groups of three mice and an average time to death determined for each concentration.

Molecular weight. Toxic fractions off SP-Sephadex C-50 and PAPTG chromatography were gel filtered on a Superose 6 column (Pharmacia).

Samples were dissolved in 1 ml of 0.075 M phosphate buffer + 0.5 M NaCl, pH 7.9. The column was run at pH 7.9 to prevent simple charge interactions that may occur at an acidic pH.

The column was calibrated with thyroglobulin (molecular weight 669,000), ferritin (440,000), catalase (232,000), and aldolase (158,000) from the high molecular weight calibration kit (Pharmacia)(1).

RESULTS

Toxin production. The strain, methods and medium used in these experiments produced 3 to 4 x 10_4 LD^{50}/ml in the culture fluid.

Purification. The addition of RNA resulted in all the toxin consistently precipitating and did not interfere with the purification. A sample purified by precipitation with acid only gave the same results on SDS-PAGE, but the yield was 75% lower.

The toxin loaded to SP-Sephadex C-50 was split into two toxic peaks. The first peak that came through the column unretarded consisted of the toxin molecule and other proteins of similar weight. This toxin peak represented 22% of total toxicity loaded. The toxin in this peak was purified to a bimolecular complex on the PAPTG affinity column (Fig. 4). The bimolecular complex could not be broken without dissociation of the toxin molecule and loss of activity.

The second band on SP-Sephadex eluted with 0.25 M NaCl. It contained 35% of total toxicity loaded. This toxin molecule eluted from the PAPTG gel after a sequence of washes contained only two minor contaminates (33,000 and 15,000 molecular weight) and had a specific activity of greater than 4 x 10^7 LD_{50}/mg protein. The toxin off the PAPTG column was stable in the elution buffer for up to seven days. This was the longest period tested.

The two minor contaminants were removed by applying the sample to a second SP-Sephadex C-50 column under the same conditions used in the earlier run. However, there was some

dissociation and a reduction in activity (50 to 75%) upon dialysis of the sample. In this second run, the toxin did not bind and the low molecular weight contaminants did. The resulting isolated type G botulinum neurotoxin was approximately 95% pure.

Amino acid sequence. The partial amino acid sequences obtained (16 for the light chain and 13 for the heavy chain) showed greatest homology to type B toxin with 11 out of 16 AA being the same in the light chain and 6 out of 13 being the same in the heavy chain. This homology, only about 60%, confirms that the G toxin is unique among the botulinum neurotoxins. Type A toxin matched 8 of 16 in the light chain and 6 of 13 in the heavy chain. The partial sequences obtained by amino acid sequencing of the G toxin are in 100% agreement for the light chain and 73% agreement for the heavy chain with those obtained by the DNA sequence (10). Further amino acid sequencing was blocked by an unknown structure. The light chain sequence was used to make a probe to identify the location of the DNA sequence.

Activation. Trypsin and .1U Endoproteinase Lys-C give the best results activating crude material. However samples off the SP column and PAPTG column were best activated with 5 U of Endoproteinase Lys-C/mg protein generally giving 20 to 100 fold activation. These results were the same for pH 6.0, 7.0, 7.9. Endoproteinase Arg-C had little to no effect at 1 and 5 U/mg protein at pH 7.0. Activation of samples off the SP-Sephadex and the PAPTG column with trypsin resulted in the nicking of a 10,000 to 15,000 molecular weight peptide off of

the heavy chain. Activation with 0.1 U Endoproteinase Lys-C resulted in only partial nicking (Gel) while 5 U resulted in all toxin being nicked. This material had the best activity and produced a heavy chain (100,000 molecular weight) and a light chain (50,000) similar in size to Type A or B botulinum toxin chains on SDS-PAGE under reducing conditions.

Toxicity. The regression equation for type G toxin on IV injection was y = 14.052 - 4.5658x, where y equals the log IP LD_{50} and x equals the log of time to death IV in minutes.

Molecular weight determination. The unbound material off SP-Sephadex formed one major peak during gel filtration with a molecular weight of 375,000.

The pooled material off the DEAE-Sephadex A-50 formed a precipitate upon cooling to 4°C. Removal of the precipitate as it formed during the purification was accomplished by centrifugation. The precipitated protein corresponds to the cryoprotein characterized by J. Gimenez (6). Prior to removal of the cryoprotein, trypsin and 0.1 Unit of Endoproteinase Lys-C gave the best activation results in terms of toxicity. However, after removal of the cryoprotein and purification on the SP-Sephadex column, 5 Units Endoproteinase Lys-C gave the best activation results in toxicity and by SDS-PAGE.

Type G toxin associates with different proteins to form complexes. The first type which passes unbound through the SP-Sephadex column consists of a bimolecular complex similar in size to type E toxin complex but does not dissociate at higher pH values (> pH 8.0). The second has a molecular

weight of greater than 550,000. Gel filtration at acidic and alkaline pH indicated that the large complex association was pH dependent. A portion of the toxin molecule present in this complex was purified to a molecular weight of 144,000.

The purified toxin had a specific toxicity of greater than 4 x 10^7 LD_{50}/mg protein which, in conjunction with SDS-PAGE, indicates that the toxin is highly purified. Affinity chromatography and the pH-dependent association of the large protein complex were used to bind the toxin and dissociate it. At pH 7.9 the complex dissociated and the toxin was eluted by a higher NaCl concentration than the contaminants. The total activation of the toxin by Endoproteinase Lys-C was also responsible for the high specific toxicity.

Example 1

A pharmaceutical composition suitable for injection was prepared and contained 100 U type G toxin and 0.5 mg stabilizing protein, pH 6.4 to 6.6, in 1.0 ml volume.

Example 2

Botulinum toxin G paralyzing ability was tested in a rabbit ptosis model which showed that it had paralytic activity. These results showed that it can be used in humans for treating hyperactive muscle disease and that it can be used where type A is ineffective. Antitoxins to botulinum serotypes A-F do not neutralize type G botulinum toxin activity.

Example 3

A safe and effective amount of the composition of Example 1 is administered to a patient in which types A-F botulinum toxin are no longer effective to treat hyperactive muscle disease and the hyperactive muscle disease condition is improved.

Example 4

A toxoid for type G botulinum toxin is prepared by treating the active type G toxin with a dilute solution of formalin (0.1-0.5%) for several days at room temperature. On injection good titers of antibodies are formed in rabbits, horses, monkeys and humans.

While the exact dose of the purified type G botulinum neurotoxin to be administered varies with the size of the patient and the condition to be treated the dosage will normally range from about 1 U to about 300 U.

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Claims

1. Type G botulinum neurotoxin and toxin complexes purified from *Clostridium botulinum* having a purity of about 60-95%.

2. A type G botulinum neurotoxin complex of claim 1 having a molecular weight of about 144,000 as determined by SDS-PAGE, said neurotoxin having a specific toxicity greater than 4 x 10^7 LD_{50}/mg protein.

3. A type G botulinum neurotoxin complex of claim 1 having a molecular weight of about 300 to about 500 kD and an activity of 10^5 to 10^7 u/mg.

4. A method of purifying natural type G botulinum neurotoxin which comprises:

(a) culturing C. *botulinum* type G in a suitable medium to obtain greater than 10^4 U per ml of culture broth, and

(b) purifying the toxin by adjusting the pH of the broth to about 3.4 to precipitate the toxin, and purifying the precipitated toxin by ion exchange purification and affinity and size exclusion chromatography.

5. A pharmaceutical composition containing type G botulinum neurotoxin (60-95% pure) 10-40 U/ng of protein in a formulation suitable for lyophilization, storage and reconstitution.

6. A toxoid for type G botulinum toxin which is prepared by inactivation of type G toxin.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 95/07932

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/33, A61K 39/08, C12P 1/04
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, A61K, C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALINDEX (BIOCHEM), EPODOC, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Applied and Environmental Microbiology, Volume 42, No 6, December 1981, G.E. LEWIS, Jr. et al., "Detection of Clostridium botulinum Type G Toxin by Enzyme-Linked Immunosorbent Assay" page 1018 - page 1022	1,4,6
X	Zentralblatt fur Bakteriologie, Mikrobiologie und Hygiene, Volume A268, 1988, M. NUKINA et al., "Purification of Clostridium botulinum Type G Progenitor Toxin" page 220 - page 227	1,3
A	EP, A2, 0593176 (WISCONSIN ALUMNI RESEARCH FOUNDATION), 20 April 1994 (20.04.94), claims 1-5	5,6

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search	Date of mailing of the international search report
26 Sept 1995	21. 11. 95
Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer INGA-KARIN PETERSSON

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 95/07932

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO, A1, 9400481 (ASSOCIATED SYNAPSE BIOLOGICS), 6 January 1994 (06.01.94), claim 1	5

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INTERNATIONAL SEARCH REPORT

Information on patent family members

28/08/95

International application No.
PCT/US 95/07932

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0593176	20/04/94	NONE	
WO-A1- 9400481	06/01/94	NONE	

Hugel Ex. 1006 - Page 289 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference GN-55281-PCT	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. **PCT/IB2017/057476**	International filing date *(day/month/year)* **29 November 2017 (29.11.2017)**	(Earliest) Priority Date *(day/month/year)* 04 October 2016 (04.10.2016)
Applicant **MEDYTOX INC.**		

This International search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of **3** sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language**, the international search was carried out on the basis of :
      ☒ the international application in the language in which it was filed
      ☐ a translation of the international application into _____ , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.
2. ☐ **Certain claims were found unsearchable** (See Box No. II)
3. ☐ **Unity of invention is lacking** (See Box No. III)
4. With regard to the **title,**
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established by this Authority to read as follows:
5. With regard to the **abstract,**
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
6. With regard to the drawings,
   a. the figure of the **drawings** to be published with the abstract is Figure No. **1**
      ☒ as suggested by the applicant.
      ☐ as selected by this Authority, because the applicant failed to suggest a figure.
      ☐ as selected by this Authority, because this figure better characterizes the invention.
   b. ☐ none of the figures is to be published with the abstract.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2017/057476

A. CLASSIFICATION OF SUBJECT MATTER

C07K 1/18(2006.01)i, C07K 14/33(2006.01)i, B01D 15/36(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K 1/18; C07K 14/33; C12P 21/04; C12P 1/00; C07H 21/00; C12N 1/20; B01D 15/36

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: botulinum toxin complex, anion exchange chromatography, cation exchange chromatography

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 9206409 B2 (ALLERGAN, INC.) 08 December 2015 See columns 2-3, 6-9, 31, and 38-39.	1-9
A	EISELE, KARL-HEINZ et al., `Studies on the dissociation of botulinum neurotoxin type A complexes`, Toxicon, 2011, Vol.57, No.4, pp.555-565 See abstract; and pages 556-558.	1-9
A	US 2011-0171226 A1 (JOHNSON, ERIC A. et al.) 14 July 2011 See the whole document.	1-9
A	KR 10-2003-0060150 A (MEDYTOX INC.) 16 July 2003 See the whole document.	1-9
A	HASEGAWA, KIMIKO et al., `Characterization of toxin complex produced by a unique strain of Clostridium botulinum serotype D 4947`, The Protein Journal, 2004, Vol.23, No.6, pp.371-378 See the whole document.	1-9

☐ Further documents are listed in the continuation of Box C.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 2018 (22.02.2018)	**22 February 2018 (22.02.2018)**

Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
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Authorized officer
KIM, Sun Hee
Telephone No. +82-42-481-5405

Form PCT/ISA/210 (second sheet) (January 2015)

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 9206409 B2	08/12/2015	AU 2010-273601 A1	20/01/2011
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KR 10-2003-0060150 A	16/07/2003	None	

Electronic Acknowledgement Receipt

EFS ID:	37635887
Application Number:	16338561
International Application Number:	
Confirmation Number:	3639
Title of Invention:	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
First Named Inventor/Applicant Name:	Hyun Ho JUNG
Customer Number:	137713
Filer:	Judith Unjin Kim/Jessica LaBossiere
Filer Authorized By:	Judith Unjin Kim
Attorney Docket Number:	2479-0007US01
Receipt Date:	01-NOV-2019
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Time Stamp:	16:31:30
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	2479-0007US01_NatlStageTransmittal.pdf	252961 d07f3b9d6b0c3f142e29a6affce91242afc22b6d	no	4

Warnings:

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2	Oath or Declaration filed	2479-0007US01_ExecutedDeclaration.pdf	1194776 70888923d3b5c16c8eda934dee6c957c4769ad36	no	16
Warnings:					
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3	Request for Corrected Filing Receipt	2479-0007US01_CorrectedOFR.pdf	90664 091fe33ec8715caafdfa66f66d5861747e464a95	no	3
Warnings:					
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4	Power of Attorney	2479-0007US01_POATransmittal.pdf	2838068 fd51160cbade236a4428a218f6fb7a6009e3bb58	no	3
Warnings:					
Information:					
5	Information Disclosure Statement (IDS) Form (SB08)	2479-0007US01_IDS_20191101.pdf	1054885 3ab5cc3b59032d71de4b5d2544e1069b88df19c9	no	5
Warnings:					
Information:					
6	Foreign Reference	FP1_KR-10-2012-0105417-A.pdf	694159 2535513370da32e7b29766cb47bd7f624fad9a22	no	39
Warnings:					
Information:					
7	Foreign Reference	FP2_KR-10-2003-0060150-A_wAbstract.pdf	507845 5cfc10c54e2e4a7cc77c5b14d9ade6e5a4c1cebc	no	5
Warnings:					
Information:					
8	Foreign Reference	FP3_WO-2011-008713-A1.pdf	4931076 0b7f325cbeca7294c392957e6448d30283b59187	no	81
Warnings:					
Information:					

9	Foreign Reference	FP4_WO-96-05222-A1.pdf	1071084 f93a9ff94cc44e342df2db7c8ccdbd85337cfd48	no	26
Warnings:					
Information:					
10	Non Patent Literature	NPL1_Binz-1990.pdf	3785855 b3b6c6a67472e1b4a9686021a153d72539518207	no	6
Warnings:					
Information:					
11	Non Patent Literature	NPL2_Eisele-2011.pdf	1135994 6807c3956592666c7e53b0a1a64ad8639dd8888f	no	11
Warnings:					
Information:					
12	Non Patent Literature	NPL3_Hasagawa-2004.pdf	189813 7ed7b5bbc66341b28fcfb50a53980ed739ef8e1f	no	8
Warnings:					
Information:					
13	Non Patent Literature	NPL4_ISR-2018.pdf	155354 c93d7158e1a22bf173a6aa2c367b88351181e9fd	no	3
Warnings:					
Information:					
14	Non Patent Literature	NPL5_Mantecucco-1993.pdf	532464 74177688087d8d433855759923ace8049337c738	no	4
Warnings:					
Information:					
15	Non Patent Literature	NPL6_Park-1990.pdf	338297 928f676bffd71480f7852399b8e87039c251b732	no	5
Warnings:					
Information:					

16	Non Patent Literature	NPL7_Poulain-1988.pdf	1012569 9f69785a05db40dcee207585 4420f680c69e 520d	no	5
Warnings:					
Information:					
17	Non Patent Literature	NPL8_Schantz-1992.pdf	4852922 a3993009d9412a83a50119322ac92e62bc8 5ab69	no	20
Warnings:					
Information:					
18	Non Patent Literature	NPL9_Simpson-1986.pdf	1050921 473ea2aa52e3bc4df488c51b78d0364839f6 0d4d	no	27
Warnings:					
Information:					
19	Non Patent Literature	NPL10_Singh-1996.pdf	4297882 fe15987886d19dc5e962325c177d2fe1b14f c7ca	no	22
Warnings:					
Information:					
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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A SUBMISSION UNDER 35 U.S.C. 371

Attorney Docket No.	U.S. Application No. (if known, see 37 CFR 1.5)
2479-0007US01	16/338,561

International Application No.	International Filing Date	Priority Date Claimed
PCT/IB2017/057476	November 29, 2017	October 4, 2016

Title of Invention
METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

First Named Inventor
Hyun Ho JUNG

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). NOTE: The express request under 35 U.S.C. 371(f) will not be effective unless the requirements under 35 U.S.C. 371(c)(1), (2), and (4) for payment of the basic national fee, copy of the International Application and English translation thereof (if required), and the oath or declaration of the inventor(s) have been received.
2. ☐ A copy of the International Application (35 U.S.C. 371(c)(2)) is attached hereto (not required if the International Application was previously communicated by the International Bureau or was filed in the United States Receiving Office (RO/US)).
3. An English language translation of the International Application (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
4. An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4))
   a. ☒ is attached.
   b. ☐ was previously filed in the international phase under PCT Rule 4.17(iv).

Items 5 to 8 below concern amendments made in the international phase.

PCT Article 19 and 34 amendments

5. ☐ Amendments to the claims under PCT Article 19 are attached (not required if communicated by the International Bureau) (35 U.S.C. 371(c)(3)).
6. ☐ English translation of the PCT Article 19 amendment is attached (35 U.S.C. 371(c)(3)).
7. ☐ English translation of annexes (Article 19 and/or 34 amendments only) of the International Preliminary Examination Report is attached (35 U.S.C. 371(c)(5)).

Cancellation of amendments made in the international phase

8a. ☐ Do not enter the amendment made in the international phase under PCT Article 19.

8b. ☐ Do not enter the amendment made in the international phase under PCT Article 34.

NOTE: A proper amendment made in English under Article 19 or 34 will be entered in the U.S. national phase application absent a clear instruction from applicant not to enter the amendment(s).

The following items 9 to 17 concern a document(s) or information included.

9. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
10. ☐ A preliminary amendment.
11. ☐ An Application Data Sheet under 37 CFR 1.76.
12. ☐ A substitute specification. NOTE: A substitute specification cannot include claims. See 37 CFR 1.125(b).
13. ☒ A power of attorney and/or change of address letter.
14. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13*ter*.3 and 37 CFR 1.821-1.825 (not required if sequence listing in text format was indicated on the PCT Request as part of the International Application and the sequence listing was published as part of the international application).
15. ☐ Assignment papers *(cover sheet and document(s))*. Name of Assignee: _____
16. ☐ 37 CFR 3.73(c) Statement *(when there is an Assignee)*.

This collection of information is required by 37 CFR 1.414 and 1.491-1.492. The information is required to obtain or retain a benefit by the public, which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 15 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

U.S. APPLN. No. (if known – see 37 CFR 1.5)	INTERNATIONAL APPLICATION No.	ATTORNEY DOCKET No.
16/338,561	PCT/IB2017/057476	2479-0007US01

17. [X] Other items or information:

Form PTO/SB/08a;
Documents FP1-FP4 and NPL1-NPL11

The following fees have been submitted.	CALCULATIONS
18. [ ] Basic national fee (37 CFR 1.492(a)) **$300**	$
19. [ ] Examination fee (37 CFR 1.492(c)) • If the written opinion prepared by ISA/US or the international preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4)..... **$0** • All other situations **$760**	$
20. [ ] Search fee (37 CFR 1.492(b)) • If the written opinion prepared by ISA/US or the international preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4)..... **$0** • Search fee (37 CFR 1.445(a)(2)) has been paid on the international application to the USPTO as an International Searching Authority ..... **$140** • International Search Report prepared by an ISA other than the US and provided to the Office or previously communicated to the US by the IB..... **$520** • All other situations **$660**	$
TOTAL OF 18, 19, and 20 =	$

[ ] Additional fee for specification and drawings filed in paper over 100 sheets (excluding sequence listing in compliance with 37 CFR 1.821(c) or (e) in an electronic medium or computer program listing in an electronic medium) (37 CFR 1.492(j)).

Fee for each additional 50 sheets of paper or fraction thereof **$400**

Total Sheets	Extra Sheets	Number of each additional 50 or fraction thereof (round **up** to a whole number)	RATE	
- 100 =	/ 50 =		x **$400**	$

Surcharge for furnishing any of the search fee, examination fee, or the oath or declaration after the date of commencement of the national stage (37 CFR 1.492(h))..... **$140**	$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	- 20 =		x **$100**	$
Independent claims	- 3 =		x **$460**	$
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			**+ $820**	$

Fee for submission of Sequence Listing text file of 300 MB to 800 MB (37 CFR 1.21(o)(1))..... **$1,000**	$
Fee for submission of Sequence Listing text file of more than 800 MB (37 CFR 1.21(o)(2))..... **$10,000**	$
Processing fee for furnishing the English translation later than 30 months from the earliest claimed priority date (37 CFR 1.492(i))..... **$140 +**	$
TOTAL OF ABOVE CALCULATIONS =	$
[ ] **Applicant asserts small entity status.** See 37 CFR 1.27. Fees above are reduced by ½.	
[ ] **Applicant certifies micro entity status.** See 37 CFR 1.29. Fees above are reduced by ¾. Applicant must attach form PTO/SB/15A or B or equivalent.	
TOTAL NATIONAL FEE =	$
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31)..... **$50.00** per property **+**	$
TOTAL FEES ENCLOSED =	$

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees.

c. ☒ The Director is hereby authorized to charge additional fees which may be required, or credit any overpayment, to Deposit Account No. 50-5875 as follows:

i. ☐ any required fee.

ii. ☒ any required fee except for excess claims fees required under 37 CFR 1.492(d) and (e) and multiple dependent claim fee required under 37 CFR 1.492(f).

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038. The PTO-2038 should only be mailed or faxed to the USPTO. However, when paying the basic national fee, the PTO-2038 may NOT be faxed to the USPTO.

ADVISORY: If filing by EFS-Web, do **NOT** attach the PTO-2038 form as a PDF along with your EFS-Web submission. Please be advised that this is **not** recommended and by doing so your **credit card information may be displayed via PAIR**. To protect your information, it is recommended to pay fees online by using the electronic payment method.

NOTE: Where an appropriate time limit under 37 CFR 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the International Application to pending status.

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013, and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.

NOTE 1: By providing this statement under 37 CFR 1.55 or 1.78, **this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA**.

NOTE 2: A U.S. national stage application may not claim priority to the international application of which it is the national phase. The filing date of a U.S. national stage application is the international filing date. See 35 U.S.C. 363.

Correspondence Address

☒ The address associated with Customer Number: 137713 **OR** ☐ Correspondence address below

Name					
Address					
City		State		Zip Code	
Country		Telephone			
Email					

Signature	/Judith U. Kim/	Date	11-01-2019
Name (Print/Type)	Judith U. Kim	Registration No. (Attorney/Agent)	40,679

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/AIA/01 (06-12)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number PCT/IB2017/057476 filed on November 29, 2017.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Hyun Ho JUNG Date (Optional): _____

Signature: _____

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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LEGAL NAME OF INVENTOR

Inventor: Gi Hyeok YANG Date (Optional): _____

Signature: _____

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

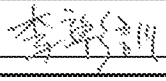
I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

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LEGAL NAME OF INVENTOR

Inventor: Chang Hoon RHEE Date (Optional): _____

Signature: 

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number PCT/IB2017/057476 filed on November 29, 2017.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

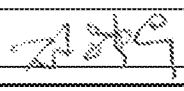
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LEGAL NAME OF INVENTOR

Inventor: Hack Woo KIM Date (Optional): _____

Signature: 

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LEGAL NAME OF INVENTOR

Inventor: Gi Hyuck RYU Date (Optional): _____

Signature: _____

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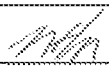
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Inventor: Hyung Pyo HONG Date (Optional): _____

Signature: 

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Inventor: Jung Hyun SONG Date (Optional): _____

Signature: JHSONG

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8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/AIA/01 (06-12)
Approved for use through 11/30/2020. OMB 0651-0032
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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number PCT/IB2017/057476 filed on November 29, 2017.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Joon Chan KONG Date (Optional): _____

Signature: _____

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/338,561	04/01/2019	Hyun Ho JUNG	2479-0007US01

CONFIRMATION NO. 3639

POA ACCEPTANCE LETTER

137713
Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

OC000000112416348

Date Mailed: 11/06/2019

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 11/01/2019.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/cvorachack/

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/338,561	04/01/2019	Hyun Ho JUNG	2479-0007US01

CONFIRMATION NO. 3639

POWER OF ATTORNEY NOTICE

137713
Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

OC000000112416322

Date Mailed: 11/06/2019

NOTICE REGARDING CHANGE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 11/01/2019.

- The Power of Attorney to you in this application has been revoked by the applicant. Future correspondence will be mailed to the new address of record(37 CFR 1.33).

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/cvorachack/

Hugel Ex. 1006 - Page 319 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
16/338,561	04/01/2019	1778	1720	2479-0007US01	9	1

CONFIRMATION NO. 3639

CORRECTED FILING RECEIPT

137713
Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

OC000000112416448

Date Mailed: 11/06/2019

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

Inventor(s)

Hyun Ho JUNG, Seoul, KOREA, REPUBLIC OF;
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Jung Hyun SONG, Gunsan-si, KOREA, REPUBLIC OF;
Joon Chan KONG, Cheongju-si, KOREA, REPUBLIC OF;

Applicant(s)

MEDYTOX INC., Cheongju-si, KOREA, REPUBLIC OF

Power of Attorney: The patent practitioners associated with Customer Number 137713

Domestic Priority data as claimed by applicant

This application is a 371 of PCT/IB2017/057476 11/29/2017

Foreign Applications (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.)
REPUBLIC OF KOREA 10-2016-0127540 10/04/2016

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 09/30/2019
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/338,561**
Projected Publication Date: 01/09/2020
Non-Publication Request: No
Early Publication Request: No
Title

METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

Preliminary Class

210

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative,

this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/338,561	04/01/2019	Hyun Ho JUNG	2479-0007US01

CONFIRMATION NO. 3639

137713
Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

PUBLICATION NOTICE

OC000000113830927

Title:METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

Publication No.US-2020-0009473-A1
Publication Date:01/09/2020

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/338,561	04/01/2019	Hyun Ho JUNG	2479-0007US01	3639

137713 7590 05/18/2020

Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

EXAMINER	
HUANG, RYAN	

ART UNIT	PAPER NUMBER
1777	

NOTIFICATION DATE	DELIVERY MODE
05/18/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

eofficeaction@appcoll.com
patents@potomaclaw.com

Office Action Summary	Application No. 16/338,561	Applicant(s) JUNG et al.	
	Examiner RYAN B HUANG	Art Unit 1777	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 01 April 2019.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-9 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-9 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 01 April 2019 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☑ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☑ All b)☐ Some** c)☐ None of the:

1.☑ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date 01 November 2019.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Summary

This is a non-final Office action for the application filed 01 April 2019. Claims 1-9 are pending.

Notice of Pre-AIA or AIA Status

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Priority

Applicant's claim for the benefit of a prior-filed application (371 of PCT/IB2017/057476, filed 29 November 2017) under 35 U.S.C. 119(e) or under 35 U.S.C. 120, 121, 365(c), or 386(c) is acknowledged.

Acknowledgment is made of applicant's claim for foreign priority (KR10-2016-0127540, filed 04 October 2016) under 35 U.S.C. 119 (a)-(d).

Claim Objections

Claim 1 is objected to because of the following informalities:

- Regarding the phrase "contacting the solution, which is not bound to the anion exchange chromatography medium, with a cation exchange chromatography medium", the use of the commas delimiting the phrase "which is not bound…" renders the claim confusing and indefinite i.e., it is unclear which "solution" is being referenced (see the 35 USC 112(b) rejection of Claim 1); and
- "contacting the solution containing the toxin separated from the cation exchange chromatography medium[[,]] with the anion exchange chromatography medium…".

Appropriate correction is required.

Claim Rejections - 35 USC § 112

The following is a quotation of 35 U.S.C. 112(b):

(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

1. Claims 1-9 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

Regarding Claim 1, there is insufficient antecedent basis for "the solution" in the limitation "contacting the solution, which is not bound to the anion exchange chromatography medium". Please distinguish this "solution" from the prior introduced solution of "a botulinum toxin-containing solution". Claims 2-9 are also rejected due to their dependence on Claim 1.

Regarding Claim 3, there is insufficient antecedent basis for "the botulinum toxin… solution" in lines 1-2. Claims 4-7 are also rejected due to their dependence on Claim 3.

Regarding Claim 3, it is unclear as to which "the solution" are being referenced in lines 3-4 and line 5 because there are at least two "solutions" introduced in Claim 1. Claims 4-7 are also rejected due to their dependence on Claim 3.

Regarding Claim 8, there is insufficient antecedent basis for "the anion exchange chromatography".

Regarding Claim 9, there is insufficient antecedent basis for "the cation exchange chromatography".

Claim Rejections - 35 USC § 103

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103 are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims the examiner presumes that the subject matter of the various claims was commonly owned as of the effective filing date of the claimed invention(s) absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and effective filing dates of each claim that was not commonly owned as of the effective filing date of the later invention in order for the examiner to consider the applicability of 35 U.S.C. 102(b)(2)(C) for any potential 35 U.S.C. 102(a)(2) prior art against the later invention.

2. Claims 1-7 is/are rejected under 35 U.S.C. 103 as being unpatentable over TON et al. (US Patent 9,206,409) with evidentiary support from THERMOSCI-AEX (Product Information Sheet: POROS Anion Exchange Resins) and THERMOSCI-CEX (Product Information Sheet: POROS Cation Exchange Resins).

Regarding Claims 1 and 2, TON teaches a purification method for obtaining botulinum neurotoxin type A from a mixture of botulinum toxins using a chromatographic process (i.e., a method of isolating a botulinum toxin type A macro complex from a botulinum toxin-containing solution; c6/28-56; c6/60-67). Briefly, a harvested fermentation medium is loaded onto an anion exchange column, subsequently onto a cation exchange column, and finally eluted from the cation exchange column for further downstream purification (i.e., contacting the botulinum toxin-containing solution with an anion exchange chromatography medium; contacting the solution which is not bound to the anion exchange chromatography medium with a cation exchange chromatography medium; and separating the botulinum toxin type A macro complex from the cation exchange chromatography medium; c31/30-37). TON discloses that during the anion exchange step, botulinum neurotoxin type A complex was eluted, whereas more negatively charged components and other host cell proteins remained bound to the anion exchange column (i.e., the solution which is not bound to the anion exchange chromatography medium; c31/47-51). Further, TON teaches that botulinum neurotoxin type A is known to be soluble at pH 4-6.8 (c2/66-c3/3), i.e., one of ordinary skill in the art would find it advantageous to maintain the harvested fermentation medium containing botulinum neurotoxin type A at a pH that would maintain its solubility in buffer (i.e., at pH 4-6.8). TON discloses a number of buffers can be used for the purification of botulinum neurotoxin type A, including 50 mM sodium acetate and 170 mM, 250 mM, or 1 M sodium chloride, H 4.8 and 50 mM sodium acetate at pH 4.0 (c38/63-c39/8), which all have pH values below the 6.06

isoelectric point of botulinum neurotoxin type A and therefore, reads on the claimed limitations of contacting the botulinum toxin-containing solution with an anion exchange chromatography medium at a pH lower than an isoelectric point of botulinum toxin; contacting the solution which is not bound to the anion exchange chromatography medium with a cation exchange chromatography medium at a pH lower than the PI of botulinum toxin; and wherein the pH lower than the PI of botulinum toxin is pH 3.5 to 6.0.

Regarding Claims 3-7, as applied to the rejection of Claim 1, TON further teaches that harvested fermentation medium containing botulinum neurotoxin is diluted with a pH 6.5 buffer (c30/41-43). While TON is deficient in explicitly disclosing adjusting the pH of the botulinum toxin-containing solution to a pH lower than the PI of botulinum toxin before the contacting of the solution with the anion echange chromatography medium, TON does disclose that botulinum neurotoxin type A is known to be soluble at pH 4-6.8 (c2/66-c3/3), i.e., one of ordinary skill in the art would find it advantageous to maintain the harvested fermentation medium containing botulinum neurotoxin type A at a pH that would maintain its solubility in buffer (i.e., at pH 4-6.8). TON discloses a number of buffers can be used for the purification of botulinum neurotoxin type A, including 50 mM sodium acetate and 170 mM, 250 mM, or 1 M sodium chloride, H 4.8 and 50 mM sodium acetate at pH 4.0 (i.e., wherein the adjusting comprises mixing the botulinum toxin-containing solution with a buffer having a pH lower than the PI of botulinum toxin; wherein the buffer is a phosphate buffer, a citrate buffer, or an acetate buffer; wherein the buffer has pH of 3.5 to 6.0; wherein the buffer comprises a 10 mM to 100 mM phosphate buffer, a 10 mM to 100 mM citrate buffer, or a 10 mM to 100 mM acetate buffer; c38/63-c39/8). Thus, such a claimed method step of adjusting the pH would have been inherently practiced by one of ordinary skill in the art at the time of the filing of the invention.

3. Claims 8 and 9 is/are rejected under 35 U.S.C. 103 as being unpatentable over TON et al. (US Patent 9,206,409) with evidentiary support from THERMOSCI-AEX (Product Information Sheet: POROS Anion Exchange Resins) and THERMOSCI-CEX (Product Information Sheet: POROS Cation Exchange Resins).

Regarding Claims 8 and 9, as applied to the rejection of Claim 1, TON further discloses a POROS 50HQ anion exchange resin, i.e., a quaternary ammonium polyethylenimine resin (i.e., wherein the anion exchange chromatography is DEAE or Q anion exchange chromatography; c31/52-53; THERMOSCI-AEX, Table 1) and a POROS 20HS cation exchange resin, i.e., a sulfopropyl resin (i.e., wherein the cation exchange chromatography is CM, S, or SP cation exchange chromatography; c32/15-16; THERMOSCI-CEX, Table 1).

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RYAN B HUANG whose telephone number is (571)270-0327. The examiner can normally be reached on 9 am-5 pm EST.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Vickie Kim can be reached on (571)272-0579. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Ryan B Huang/
Primary Examiner, Art Unit 1777

Notice of References Cited	Application/Control No. 16/338,561	Applicant(s)/Patent Under Reexamination JUNG et al.	
	Examiner RYAN B HUANG	Art Unit 1777	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-9206409-B2	12-2015	Ton; Jennifer L.	C07K14/33	1/1
	B					
	C					
	D					
	E					
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	POROS Anion Exchange Resins (ThermoFisher Scientific Product Information Sheet; 2018). (Year: 2018)
	V	POROS Strong Cation Exchange Resins (ThermoFisher Scientific Product Information Sheet; 2018). (Year: 2018)
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/338,561	JUNG et al.
	Examiner	Art Unit
	RYAN B HUANG	1777

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner
B01D15/36$ and A61K38/4893	05/12/2020	RBH
B01D15/36$	05/12/2020	RBH
B01D15/362 and B01D15/363	05/12/2020	RBH

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
Applicants search on EAST and PALM	05/12/2020	RBH
botulinum toxin generic search	05/12/2020	RBH
AEX flow-through, CEX bind-elute search	05/12/2020	RBH

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/Ryan B Huang/ Primary Examiner, Art Unit 1777	

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/338,561	JUNG et al.
	Examiner	Art Unit
	RYAN B HUANG	1777

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	05/12/2020								
	1	✓								
	2	✓								
	3	✓								
	4	✓								
	5	✓								
	6	✓								
	7	✓								
	8	✓								
	9	✓								

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	46	botulinum same isoelectric adj point	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/05/12 15:30
L2	148	botulinum and (flow ajd through flow-through) with anion adj exchange and cation adj exchange	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/05/12 16:02
L3	113	botulinum and (flow ajd through flow-through) with anion adj exchange same cation adj exchange	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/05/12 16:03
S1	106	((("JUNG") near3 ("Hyun") near3 ("Ho")) OR (("YANG") near3 ("Gi") near3 ("Hyeok")) OR (("RHEE") near3 ("Chang") near3 ("Hoon")) OR (("KIM") near3 ("Hack") near3 ("Woo")) OR (("RYU") near3 ("Gi") near3 ("Hyuck")) OR (("HONG") near3 ("Hyung") near3 ("Pyo")) OR (("SONG") near3 ("Jung") near3 ("Hyun")) OR (("KONG") near3 ("Joon") near3 ("Chan"))).INV.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/05/12 11:49
S2	25	botulinum and S1	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/05/12 11:49
S3	0	B01D15/36$.cpc. and A61K38/4893.cpc.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/05/12 11:53
S4	3952	B01D15/36$.cpc.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/05/12 11:53
S5	0	B01D15/36$.cpc. and A61K38/4893.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/05/12 11:53

S6	11085	B01D15/36$.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/05/12 11:53
S7	2024	B01D15/362.cpc. and B01D15/363.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/05/12 11:54
S8	83	botulinum and S7	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/05/12 11:56
S9	31	botulinum and S7	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/05/12 11:58

EAST Search History (Interference)

<This search history is empty>

5/12/2020 4:49:05 PM
C:\Users\rhuang\Documents\EAST\Workspaces\16338561-Botox.wsp

Bibliographic Data

Application No: 16/338,561

Foreign Priority claimed: (●) Yes ( ) No

35 USC 119 (a-d) conditions met: (☑) Yes ( ) No | ( ) Met After Allowance

Verified and Acknowledged: /Ryan B Huang/ | Examiner's Signature | Initials

Title: METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
04/01/2019	210	1777	2479-0007US01
RULE			

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CONTINUING DATA

This application is a 371 of PCT/IB2017/057476 11/29/2017

FOREIGN APPLICATIONS

KOREA, REPUBLIC OF 10-2016-0127540 10/04/2016

IF REQUIRED, FOREIGN LICENSE GRANTED**

09/30/2019

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UNITED STATES

FILING FEE RECEIVED

$1,580

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16338561
Filing Date	2017-11-29
First Named Inventor	Hyun Ho JUNG
Art Unit	1778
Examiner Name	
Attorney Docket Number	2479-0007US01

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	5696077	A	1997-12-09	Johnson et al.	
	2	5846929	A	1998-12-08	Johnson et al.	
	3	9206409	B2	2015-12-08	Ton et al.	

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	1	20110171226	A1	2011-07-14	Johnson et al.	

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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	1020120105417	KR	A	2012-09-25	ALLERGAN, INC.	Corresponds to WO 2011/008713 A1	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /RBH/

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	First Named Inventor	Hyun Ho JUNG
	Art Unit	1778
	Examiner Name	
	Attorney Docket Number	2479-0007US01

	2	20030060150	KR	A	2003-07-16	Medy-Tox Inc.	English language abstract attached.	
	3	2011008713	WO	A1	2011-01-20	ALLERGAN, INC.	Corresponds to KR 10-2012-0105417 A	
	4	9605222	WO	A1	1996-02-22	Wisconsin Alumni Research Foundation		

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NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1	BINZ et al., "The Complete Sequence of Botulinum Neurotoxin Type A and Comparison with Other Clostridial Neurotoxins," The Journal of Biological Chemistry, 265:9153-9158 (1990), American Society of Biological Chemists; Rockefeller Institute for Medical Research; American Society for Biochemistry and Molecular Biology, American Society for Biochemistry and Molecular Biology.	
	2	EISELE et al., "Studies on the dissociation of botulinum neurotoxin type A complexes," Toxicon, 47:555-565 (2011), International Society on Toxinology, Elsevier.	
	3	HASEGAWA et al., "Characterization of Toxin Complex Produced by a Unique Strain of Clostridium botulinum Serotype D 4947," The Protein Journal, 23:371-378 (2004), Kluwer Academic Publishers-Plenum Publisher.	
	4	International Search Report dated February 22, 2018 for International Application No. PCT/IB2017/057476, ISA/KR, Korean Intellectual Property Office, Republic of Korea.	
	5	MANTECUCCO et al., "Tetanus and botulism neurotoxins: a new group of zinc proteases," Trends in Biochemical Sciences, 18:324-327 (1993), Elsevier Inc.	
	6	PARK et al., "Binding of Clostridium botulinum type B toxin to rat brain synaptosome," FEMS Microbiology Letters, 72:243-247 (1990), Federation of European Microbiological Societies.	

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	Attorney Docket Number	2479-0007US01

	7	POULAIN et al., "Neurotransmitter release is blocked intracellularly by botulinum neurotoxin, and this requires uptake of both toxin polypeptides by a process mediated by the larger chain," Proceedings of the National Academy of Sciences of the United States of America, 85:4090-4094 (1988).	
	8	SCHANTZ et al., "Properties and Use of Botulinum Toxin and Other Microbial Nerotoxins in Medicine," Microbiological Reviews, 56:80-99 (1992), American Society for Microbiology Journals.	
	9	SIMPSON, "Molecular Pharmacology of Botulinum Toxin and Tetanus Toxin," Annual Review of Pharmacology and Toxicology, 26:427-453 (1986).	
	10	SINGH et al., "Critical Aspects of Bacterial Protein Toxins," Advances in Experimental Medicine and Biology, pp. 63-84 (1996), Plenum Press, New York.	
	11	SUGIYAMA, "Clostridium botulinum Neurotoxin," Microbiological Reviews, 44:419-448 (1980), American Society for Microbiology (ASM)	

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EXAMINER SIGNATURE

Examiner Signature	/Ryan B Huang/	Date Considered	05/12/2020

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Art Unit	1778
Examiner Name	
Attorney Docket Number	2479-0007US01

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Judith U. Kim/	Date (YYYY-MM-DD)	2019-11-01
Name/Print	Judith U. Kim	Registration Number	40679

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

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4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Examiner Name	Ryan HUANG
Attorney Docket Number	2479-0007US01

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

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	2	2011050072	WO	A1	2011-04-28	REVANCE THERAPEUTICS, INC., et al.		
	3	2015016462	WO	A1	2015-02-05	DAEWOON CO., LTD.		

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16338561
	Filing Date	2017-11-29
	First Named Inventor	Hyun Ho JUNG
	Art Unit	1777
	Examiner Name	Ryan HUANG
	Attorney Docket Number	2479-0007US01

	4	2017095062	WO	A1	2017-06-08	DAEWOONG CO., LTD.		

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	1	DASGUPTA et al., "Cation-exchange chromatography of Clostridium botulinum type a toxin on amberlite IRC-50 resin at pH 5.55", Biochimica Et Biophysica ACTA - PROTEIN STRUCTURE, 1968, Vol. 168(3), pages 522-531, ELSEVIER SCIENCE BV, AMSTERDAM, NL	
	2	DASGUPTA et al., "Purification of Clostridium botulinum type a toxin", Biochimica Et Biophysica ACTA - PROTEIN STRUCTURE, 1970, Vol. 214(2), pages 343-349, ELSEVIER SCIENCE BV, AMSTERDAM, NL.	
	3	Extended European Search Report dated May 4, 2020, issued in European Application No. 17857967.8	
	4	SIMPSON et al., "Isolation and Characterization of the Botulinum Neurotoxins", METHODS IN ENZYMOLOGY, 1988, Vol. 165, pages 76-85, ELSEVIER, ACADEMIC PRESS, US.	

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	Art Unit	1777
	Examiner Name	Ryan HUANG
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Name/Print	Judith U. Kim	Registration Number	40679

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Zur Erklärung der Zweibuchstaben-Codes, und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

(54) Title: THERAPEUTIC AGENT COMPRISING A BOTULINUM NEUROTOXIN

(54) Bezeichnung: THERAPEUTIKUM MIT EINEM BOTULINUM-NEUROTOXIN

(57) Abstract: The invention relates to a pharmaceutical preparation containing one of the botulinum neurotoxins of *Clostridium botulinum* of types A, B, C, D, E, F or G or a mixture of two or more of these neurotoxins. The inventive preparation is characterized in that the neurotoxin or the mixture of neurotoxins does not contain the complexing proteins which, together with the neurotoxins, naturally form the botulinum neurotoxin complexes.

(57) Zusammenfassung: Pharmazeutische Zubereitung, enthaltend eines der Botulinum-Neurotoxine von *Clostridium botulinum* der Typen A, B, C, D, E, F oder G oder ein Gemisch von zwei oder mehr dieser Neurotoxine, dadurch gekennzeichnet, dass das Neurotoxin bzw. das Gemisch der Neurotoxine frei ist von den komplexierenden Proteinen, die natürlicherweise zusammen mit den Neurotoxinen die Botulinum-Neurotoxin-Komplexe bilden.

WO 00/74703 A2

Hugel Ex. 1006
Hugel Inc v. Medytox Inc.
IPR2023-00604 - U.S. Patent No. 11,331,598

5

Therapeutikum mit einem Botulinum-Neurotoxin

Die vorliegende Erfindung betrifft pharmazeutische Zubereitungen, die ein Botulinum-Neurotoxin von *Clostridium botulinum* enthalten, wobei das Neurotoxin frei ist von den komplexierenden Proteinen, die natürlicherweise in dem Komplex vorliegen. Die unmittelbare Folge davon ist die der vorliegenden Erfindung zu Grunde liegende Erkenntnis, dass das freie Neurotoxin im Gegensatz zum Komplex im Patienten zu keiner oder nur zu einer deutlich verminderten Induktion neutralisierender Antikörper führt. Die vorliegende Erfindung betrifft weiterhin die Verwendung von Botulinum-Neurotoxinen von *Clostridium botulinum* zur Herstellung eines Medikaments zur Behandlung von Erkrankungen des Nervensystems. Ein anderer Aspekt der vorliegenden Erfindung betrifft die Verwendung der Botulinum-Neurotoxine von *Clostridium botulinum* für die kosmetische Behandlung.

Clostridium botulinum Toxinkomplex Typ A (M_r 900.000) wird seit mehreren Jahren zur Therapie verschiedener Dystonien eingesetzt. Derzeit sind zwei verschiedene, diesen Komplex enthaltende Präparate für die Behandlung des *Blepharospasmus*, hämifacialer Spasmen und des *Torticollis spasmodicus* zugelassen: BOTOX® und DYSPORT®. Die Therapie weiterer Erkrankungen des Nervensystems (z. B. Spastizitäten, Migräne, Lumbalgie, Cervicalsyndrom, Hypersalivation) wird derzeit klinisch geprüft. Die Präparate werden außerdem bei kosmetischen Indikationen wie Hyperhidrose und ausgeprägter Faltenbildung eingesetzt. Auch die übrigen *Clostridium botulinum* Toxinkomplexe (der Typen B, C, D, E, F, G) eignen sich für diese Therapien. Allerdings gibt es derzeit noch kein zugelassenes Produkt auf dem Markt, das Einsender Toxine vom Typ B-G enthält.

Botulinumtoxin-Komplexe setzen sich aus einem Gemisch clostridieller Proteine zusammen. Dies sind Hämagglutinine mit unterschiedlichen Molekularmassen, ein nicht-toxisches nicht-hämagglutinierendes Protein (M_r ca. 120.000) und ein Neurotoxin (M_r ca. 150.000). Sie bilden einen säurestabilen Komplex, der für die orale Toxizität bei Lebensmittelintoxikationen verantwortlich ist. Im Gegensatz zum reinen Neurotoxin widersteht der Komplex dem agressiven

Milieu im Magen-Darm-Trakt und ermöglicht die enterale Resorption des Neurotoxins, welches über den Blutkreislauf oder das Lymphsystem die Zielzellen erreicht und dort eine Blockade der Transmitterfreisetzung auslöst. Daraufhin kommt es zur Paralyse der quergestreiften und glatten Muskulatur und zum Versiegen verschiedener vegetativer Funktionen. Vergiftete Patienten sterben an einer Insuffizienz der respiratorischen Muskulatur. Da das reine Neurotoxin im Magen-Darm-Trakt abgebaut und somit nicht enteral resorbiert wird, ist es nach einer Ingestion nicht giftig. Parenteral appliziert unterscheiden sich die therapeutischen Wirkungen des Neurotoxins und des Komplexes nicht denn der Komplex zerfällt im Gewebe in seine Bestandteile, und nur das Neurotoxin wird in die Zielzellen aufgenommen.

Zur therapeutischen Anwendung wird der Komplex nach dem heutigen Stand der Technik direkt in dystone oder spastische Muskel injiziert, wo das Neurotoxin bei physiologischem pH aus dem Komplex freigesetzt wird und die erwünschte pharmakologische Wirkung hervorruft. Obwohl der Komplex nur in äußerst geringen Dosen appliziert wird (1-25 ng, je nach Indikation und Größe des betroffenen Muskels), kommt es nach wiederholten Injektionen bei einer beträchtlichen Anzahl der Patienten zur Bildung von spezifischen, neutralisierenden Antikörpern, die auch gegen das Neurotoxin gerichtet sind. Unmittelbare Folge ist, dass Antikörper-positive Patienten auf den Komplex nicht mehr ansprechen. Sie könnten aber mit anderen Toxintypen behandelt werden, von denen allerdings keiner zur Therapie zugelassen ist. Wenn der Patient alle Toxin-Typen durchprobiert und Antikörper gegen sie gebildet hat, ist die weitere Applikation eines Botulinumtoxin-Komplexes (egal welchen Typs) nicht mehr hilfreich. Dabei ist zu berücksichtigen, dass jede Gabe des Komplexes zu einer Erhöhung des Antikörper-Titers beiträgt bis zu dem Punkt, an dem eine weitere Applikation des Komplexes keinen Sinn mehr macht, weil kein Effekt mehr erzielt wird. Bis der Antikörper-Titer nennenswert gesunken ist, vergehen oft Jahre, so dass diese Patienten über lange Zeiträume nicht (mit Botulinum-Neurotoxin) behandelt werden (können).

Die Bildung von spezifischen Antikörpern wird durch zwei Faktoren begünstigt. Zum einen bleibt das Neurotoxin, fixiert im Komplex, über einen längeren Zeitraum im Gewebe liegen und kann ins Gewebe wandernde Immunzellen zur Antikörperbildung aktivieren. Die lange Verweildauer führt jedoch zu keiner gesteigerten Aufnahme in die Zielzellen, denn vergiftete Zielzellen können kein Toxin mehr aufnehmen. Das langsam aus dem Komplex herausdissoziierende Neurotoxin ist also nur noch immunologisch wirksam. Zum anderen verstärken die im Komplex enthaltenen Proteine eine Immunantwort. Hämagglutinine sind

Lektine, also Proteine, die sich durch eine hohe Affinität zu bestimmten Zuckern auszeichnen. Aufgrund ihrer Bindung an Zuckerstrukturen wirken Lektine immunstimulierend. So konnte gezeigt werden, daß die Lektine Concanavalin A, Phytohämagglutinin und Pokeweed Mitogen die T- und B-Lymphozyten aktivieren. Die Hämagglutinine der Botulinumtoxin-Komplexe, die ebenfalls an membranständige Zucker binden, können also in ähnlicher Weise als Immunadjuvanzien fungieren und zur Antikörperbildung und damit zum Therapieversagen beitragen.

Deswegen stellte sich für die Erfinder der vorliegenden Erfindung die Aufgabe, einen alternativen Behandlungsweg für oben genannte Erkrankungen und Störungen zu entwickeln. Insbesondere wollten die Erfinder einen geeigneten Wirkstoff vorschlagen, mit dem Patienten, die bereits neutralisierende Antikörper gebildet haben, therapiert werden können.

Als Lösung der oben gestellten Aufgabe, als Alternative zu den beiden kommerziellen Präparaten aus Botulinumtoxin-Komplex des Typs A, BOTOX® und DYSPORT® und auch als Alternative zu den im Stand der Technik beschriebenen Komplexen der übrigen Typen (B, C, D, E, F, G), wurde ein neues Arzneimittel entwickelt, welches nur reines Neurotoxin (Typ A bzw. B, C, D, E, F, G) enthält und frei von Hämagglutininen und anderen körperfremden Proteinen ist. Wegen seiner geringeren Molekularmasse diffundiert es schneller zu den Zielzellen, in die es aufgenommen wird, bevor Immunzellen, von Hämagglutininen angelockt, aktiviert werden. In Antigenitätsstudien fanden wir, daß das reine Neurotoxin aller Typen - im Unterschied zu kommerziellen Präparaten des Typs A und den Komplexen der Typen B bis G keine oder allenfalls eine sehr geringe Bildung von Antikörpern auslöst. Bei therapeutischem Einsatz dieser neu entwickelten Arzneimittel (reines Neurotoxin der Typen A, B, C, D, E, F, G) kommt es auch nach wiederholten Applikationen zu keinem Antikörper-bedingten Therapieversagen. Ferner konnte gezeigt werden, daß sich die reinen Neurotoxine wegen ihrer sofortigen Bioverfügbarkeit auch weiterhin zur Therapie von Patienten eignen, die nach Applikation eines Botulinumtoxin-Komplexes, z.B. nach Behandlung mit BOTOX® oder DYSPORT®, einen Antikörpertiter gegen den entsprechenden Typ entwickelt haben (sog. secondary non-responders), also einer weiteren Behandlung mit BOTOX® oder DYSPORT® nicht mehr zugänglich sind, da die Verabreichung der kommerziellen Toxine keine Linderung der Beschwerden mehr bringt.

Das erfindungsgemäß bereitgestellte Arzneimittel eignet sich als Therapeutikum besonders bei Patienten, die einen Antikörper-Titer gegen ein Botulinum-Toxin, insbesondere gegen das des

Typs A, aufweisen. Besonders geeignet ist das erfindungsgemäße Pharmazeutikum (reines Neurotoxin oder Gemisch von mehreren reinen Neurotoxinen) bei solchen Patienten, die einen Antikörper-Titer von nicht größer als 50, bevorzugt nicht größer als 30, mehr bevorzugt nicht größer als 20, besonders bevorzugt nicht mehr als 10, und ganz besonders bevorzugt nicht mehr als 5 mU/ml aufweisen. Dabei ist 1 mU Antikörper diejenige Menge an Antikörper, die 10 U Toxin neutralisiert.

Andererseits kann das erfindungsgemäße Arzneimittel besonders vorteilhafterweise bei solchen Personen eingesetzt werden, die noch nie zuvor, oder schon lange Jahre nicht mehr, mit Botulinum-Neurotoxin behandelt worden sind, da deren Antikörper-Titer von Anfang an niedrig oder gleich Null ist. Das Vorteilhafte an der vorliegenden Erfindung besteht dann darin, dass der Titer dieser Patienten durch die Behandlung mit dem reinen Toxin gemäß der vorliegenden Erfindung nicht, oder allenfalls sehr unwesentlich, erhöht wird. Mit anderen Worten, das erfindungsgemäße Therapeutikum kann über lange Zeiträume verabreicht werden, ohne dass es seine Wirkung einbüßt.

Die Induktion von Antikörpern bei der Therapie mit einem *C. botulinum* Toxin wird also dadurch verhindert, dass statt der hochmolekularen toxischen Komplexe ein reines Neurotoxin appliziert wird. Das von den Komplexproteinen vollständig getrennte Neurotoxin ist sofort bioverfügbar und kann unmittelbar an die Nervenendigungen der motorischen Endplatten binden.

Ein Aspekt der vorliegenden Erfindung betrifft somit eine pharmazeutische Zubereitung, die wenigstens eines der Botulinum-Neurotoxine von *Clostridium botulinum* der Typen A, B, C, D, E, F oder G (oder ein Gemisch von zwei oder mehr dieser Neurotoxine) enthält, wobei alle Neurotoxine frei sind von den komplexierenden Proteinen, die natürlicherweise in dem Komplex vorliegen.

Gemäß einer bevorzugten Ausführungsform handelt es sich um eine pharmazeutische Zubereitung, die dadurch gekennzeichnet ist, dass das Neurotoxin bzw. das Gemisch der Neurotoxine im Patienten zu keiner oder, im Vergleich zu den Komplexen, nur zu einer verminderten Induktion neutralisierender Antikörper führt.

Eine weitere bevorzugte Ausführungsform sieht eine pharmazeutische Zubereitung vor, die als Neurotoxin bzw. als Gemisch der Neurotoxine ein natürliches Neurotoxin bzw. ein Gemisch natürlicher Neurotoxine enthält.

Eine weitere bevorzugte Ausführungsform sieht eine pharmazeutische Zubereitung vor, die als Neurotoxin bzw. als Gemisch der Neurotoxine ein rekombinantes Neurotoxin bzw. ein Gemisch rekombinanter Neurotoxine enthält.

Eine weiterhin bevorzugte Ausführungsform der erfindungsgemäßen pharmazeutischen Zubereitung sieht eine Zubereitung vor, die als Neurotoxin das Neurotoxin von *Clostridium botulinum* Typ A oder B bzw. als Gemisch der Neurotoxine ein Gemisch der Neurotoxine von *Clostridium botulinum* Typ A und B aufweist.

Ein weiterer Aspekt der vorliegenden Erfindung bezieht sich auf die Verwendung der Botulinum-Neurotoxine von *Clostridium botulinum* der Typen A, B, C, D, E, F oder G oder eines Gemisches von zwei oder mehr dieser Neurotoxine zur Herstellung eines Medikaments zur Behandlung von Erkrankungen des Nervensystems bzw. von Dystonien. Bei den Erkrankungen des Nervensystems bzw. den Dystonien handelt es sich gemäß einer bevorzugten Ausführungsform um Torticollis spasmodicus und Blepharospasmus, um Spastizitäten wie Spitzfuß, hämifasciale Spasmen, Migräne, Lumbalgie, Cervicalsyndrom oder Hypersalivation.

Wiederum ein anderer Aspekt der vorliegenden Erfindung betrifft die Verwendung der Botulinum-Neurotoxine von *Clostridium botulinum* der Typen A, B, C, D, E, F oder G oder eines Gemisches von zwei oder mehr dieser Neurotoxine für die kosmetische Behandlung, wobei eine kosmetische Behandlung zur Behandlung von Hyperhidrose und Faltenbildung, insbesondere im Gesichtsbereich, besonders bevorzugt ist.

Ganz besonders bevorzugt im Sinne der vorliegenden Erfindung ist die Verwendung eines der Neurotoxine allein oder im Gemisch zur Herstellung eines Medikamentes zur Behandlung der oben genannten Nervenerkrankungen bei solchen Personen (vorzugsweise Mensch, aber auch Tier), die bereits neutralisierende Antikörper gegen einen Botulinum-Neurotoxin-Komplex, insbesondere gegen den Komplex von *Clostridium botulinum* Typ A oder B, oder gegen mehrere Komplexe, insbesondere gegen die Komplexe von *Clostridium botulinum* Typ A und B, aufweisen (sogenannte secondary non-responders).

Die Neurotoxine, ihre Gemische bzw. die erfindungsgemäßen pharmazeutischen Zubereitungen können als wässrige Lösung, insbesondere als wässrige Injektionslösung, aber auch als lyophilisierte Produkte vorliegen.

Die an sich bekannten reinen Neurotoxine der Typen A-G wurden hergestellt nach den Protokollen, die in den in der Literaturliste aufgeführten Veröffentlichungen enthalten sind. Beispielhaft ist die Aufreinigung von zwei Neurotoxinen (Typ A und B) in den nachfolgenden Beispielen beschrieben.

Beispiel 1: Isolierung des reinen Neurotoxins

Das reine Neurotoxin aus *Clostridium botulinum* Typ A wird nach einem Verfahren gewonnen, das sich an das Verfahren von DasGupta & Sathyamoorthy anlehnt. *Clostridium botulinum* Typ A wird in einem 20-l-Fermenter in einem Medium kultiviert, das aus 2% Proteose Peptone, 1% Yeast-Extrakt, 1% Glukose und 0.05% Natriumthioglycolat besteht. Nach 72 Stunden Wachstum wird das Toxin durch Zugabe von 3N H_2SO_4 (End-pH = 3.5) gefällt. Die präzipitierte und zentrifugierte Biomasse wird mit 0.2 M Natriumphosphatpuffer pH 6.0 extrahiert.

Nach Abtrennung der Nukleinsäuren durch Fällung mit Protaminsulfat wird das Toxin durch Zugabe von Ammoniumsulfat gefällt. Das solubilisierte und gegen 50 mM Natriumphosphat pH 6.0 dialysierte Präzipitat wird auf einer DEAE-Sephadex-Säule beim gleichen pH-Wert gebunden und mit 150 mM NaCl abgelöst. Anschließend erfolgt eine Chromatographie über eine QAE-Sephadex-Säule, die mit einem 50 mM Tris/HCl-Puffer pH 7.9 äquilibriert wurde. Das Toxin wird über einen NaCl-Gradienten eluiert. Im letzten Schritt wird das Toxin über SP-Sephadex bei pH 7.0 chromatographiert. Das gebundene Toxin wird dabei mittels eines NaCl-Gradienten (0 – 300 mM) von der Säule abgelöst. Das gereinigte Toxin wird in einer SDS-Polyacrylamidgelelektrophorese (SDS-PAGE) analysiert und weist eine Reinheit von 95 ± 5 % auf. Die biologische Aktivität wird im Maus LD_{50}-Assay bestimmt: einer LD_{50}-Einheit entsprechen 4.8 pg Protein.

Beispiel 2: Herstellung eines Botulinum-Neurotoxin-haltigen Fertigarzneimittels

Mit dem gereinigten Neurotoxin aus Beispiel 1 wird eine Lösung hergestellt, die 200 Maus-LD_{50}-Einheiten, 10 mg Saccharose und 2 mg humanes Serumalbumin pro ml enthält. 0.5 ml der

Lösung werden in Vials abgefüllt und gefriergetrocknet. Die Lyophilisate werden mit physiologischer Kochsalzlösung rekonstituiert und die biologische Aktivität bestimmt. Die Vials enthalten 100 ± 30 LD_{50}-Einheiten.

Beispiel 3: Isolierung von reinem Neurotoxin B

Clostridium botulinum Typ B wird im gleichen Medium und unter gleichen Bedingungen wie Typ A kultiviert und bis zur Ammoniumsulfatfällung aufgearbeitet. Anschließend erfolgt wiederum eine DEAE-Sephadex-Chromatographie bei pH 6.0. Die mit 150 mM NaCl von der Säule eluierten Fraktionen werden vereinigt und gegen Natriumphosphat pH 7.0 dialysiert, woran sich eine Chromatographie über QAE-Sephadex anschließt. Die toxinhaltigen Fraktionen werden weiter über eine DEAE-Sephadex-Chromatographie bei pH 8.5 (50 mM Tris/HCl pH 8.5) chromatographiert.

Schließlich erhält man das hochreine Botulinumtoxin Typ B über eine Chromatographie an Hydroxylapatit äquilibriert mit 10 mM Na-Phosphat pH 8.0. Das gebundene homogene Toxin wird mit 80 mM Na-Phosphat pH 8.0 eluiert und anschließend die biologische Aktivität im LD_{50}-Assay bestimmt ($2 - 4 \times 10^7$ LD_{50}-Einheiten/mg Protein).

Beispiel 4: Nachweis von Antikörpern

20 Kaninchen wurden 25 U BOTOX® über einen Zeitraum von 12 Wochen im Abstand von 14 Tagen (5 Injektionen) intracutan injiziert. Nach 3 Wochen und dann im Abstand von 14 Tagen wurde Serum gewonnen.

Antikörper gegen *Clostridium botulinum* Neurotoxin A wurden mit einem Enzymimmunoassay nachgewiesen, indem das homogene Neurotoxin auf einer Mikrotiterplatte immobilisiert war. An das Neurotoxin bindende Antikörper wurden mittels eines zweiten, enzymmarkierten Antikörpers quantifiziert.

Das Ergebnis ist in Tabelle 1 dargestellt. Bereits 5 Wochen nach der ersten Applikation konnten bei 5 Kaninchen Antikörper nachgewiesen werden. Nach 11 Wochen enthielten Seren von 17 Kaninchen, also 85% der eingesetzten Tiere, Antikörper gegen das Neurotoxin Im biologischen Aktivitätstest wurde gezeigt, daß 12 der 17 Seren neutralisierende Antikörper enthielten (Tabelle 2).

Tab. 1 Bestimmung von Serumproben (1:100 verdünnt) aus Kaninchen, die mit BOTOX® behandelt wurden mit einem Enzym-Immunoassay. $OD_{490nm} > 0.1$ sind angegeben. Alle OD-Werte sind korrigiert mit den OD-Werten der Präimmunseren (OD ca. 0,150).

Kaninchen Nr.	3. Woche	5. Woche	7. Woche	9. Woche	11. Woche
1	-	-	-	0,11	0,36
2	-	-	-	2,36	2,23
3	-	-	0,57	1,43	1,44
4	-	-	0,68	1,68	0,93
5	-	0,97	3,52	3,49	3,44
6	-	-	1,34	2,32	2,70
7	-	-	2,13	3,09	3,00
8 *	-	0,53	1,47	2,75	2,75
9	-	-	0,43	2,44	2,85
10	-	-	2,99	3,15	2,73
11	-	0,10	2,42	2,45	1,93
12	-	-	-	1,13	1,95
13	-	-	-	-	1,89
14	-	-	-	-	-
15	-	-	-	-	-
16	-	-	-	-	-
17	-	2,93	3,62	3,72	3,44
18	-	-	1,18	2,28	2,62
19	-	-	0,43	0,43	0,81
20	-	1,65	3,20	2,97	2,88

* Die Werte wurden nicht korrigiert, da kein Präimmunserum vorhanden war

"-" bedeutet Optische Dichte (OD_{490}) < 0,1

Tab. 2 Neutralisation durch Seren von Kaninchen, die mit BOTOX® (Woche 11 nach der ersten Immunisierung) im Maus-Hemidiaphragma-Assay (Nachweisgrenze: 0,35 mU/ml Antikörper)

Kaninchen	Neutralisation mU/ml
1	2,0
2	n. d.
3	n. d.
4	> 10
5	> 100
6	n. d.
7	> 10
8	> 10
9	n. d.
10	n. d.
11	n. d.
12	> 10
13	n. d.
14	n. d.
15	< 0,35
16	0,4
17	> 10
18	> 10
19	2,0
20	> 10

n. d. = nicht bestimmt

Beispiel 5: Antigenitätsstest mit Marktprodukt und reinem Neurotoxin

Nachdem gezeigt wurde, daß der Komplex aus Neurotoxin und Hämagglutininen und dem nicht-toxischen, nicht-hämagglutinierenden Protein die Bildung neutralisierender Antikörper auslöst, wurde die immunogene Wirkung des reinen Neurotoxins (Typ A) geprüft. Dazu wurden 8 Kaninchen mit dem Toxinkomplex und 12 Kaninchen mit dem reinen Toxin behandelt. Nach dem oben beschriebenen Verfahren (siehe Beispiel 1) wurden 25 U des jeweiligen Präparates intracutan appliziert. Die Menge an Neurotoxin, gemessen als Gewicht, war in beiden Präparaten

gleich (200 pg/Dosis), wie in einem ELISA nachgewiesen wurde. BOTOX® enthielt zusätzlich noch Komplexproteine (ca. 800 pg/Dosis).

Vier der acht mit BOTOX® behandelten Tiere zeigten im ELISA einen Antikörpertiter, während bei den 12 mit reinem Neurotoxin behandelten Tieren keine Antikörper gegen das reine Neurotoxin nachzuweisen waren. Das Ergebnis wurde im biologischen Aktivitätstest bestätigt. Alle vier Kaninchenseren enthielten neutralisierende Antikörpertiter, die eine Toxinwirkung verhinderten (Tabelle 3).

Tab. 3 Neutralisation durch Seren (1:3 verdünnt) von Kaninchen, die mit BOTOX® (Woche 11 nach der ersten Immunisierung) im Maus-Hemidiaphragma-Assay (Nachweisgrenze: 1 mU/ml Antikörper)

Kaninchen	Neutralisation mU/ml
1	12 mU
2	> 30 mU
3	4.5 mU
8	> 30 mU

Beispiel 6: (Vergleichsbeispiel)
In diesem Experiment wurde die Antikörperbildung durch BOTOX® mit der durch DYSPORT ® verglichen. Hierzu wurden jeweils zehn Kaninchen entweder mit BOTOX® (Gruppe 1), mit DYSPORT ® (Gruppe 2) oder mit dem reinen Neurotoxin (Gruppe 3) nach beschriebenem Schema behandelt.

Während in Gruppe 1 und 2 mehr als 50% der Tiere einen neutralisierenden Antikörpertiter bildeten, waren die Seren aus den Tieren der Gruppe 3 frei von Antikörpern.

Beispiel 7: Klinischer Test
Ein Patient (Alter 45 Jahre), der über einen Zeitraum von 5 Jahren wegen eines *Torticollis spasmodicus* mit BOTOX® behandelt wurde, hatte eine Antikörpertiter von 3 mU/ml Serum

entwickelt. Weder BOTOX® noch DYSPORT® waren bei diesem Patienten therapeutisch wirksam. Ein Therapieversuch mit dem reinen Botulinum-Neurotoxin in einer Dosis von 145 U, welche äquivalent der zuletzt injizierten Dosis von BOTOX® war, führte innerhalb von 72 Stunden zur Lockerung des Muskels, zur Normalisierung der Kopfhaltung und zum Verschwinden des Muskelschmerzes. Unerwünschte Wirkungen traten nicht auf.

Beispiel 8: Klinischer Test

Ein Patient (Alter 52 Jahre) wurde 3 Jahre wegen Cerebralparesee mit BOTOX® behandelt. Er hatte einen Antikörpertiter von 1 mU/ml Serum entwickelt, die Therapie mußte deshalb abgebrochen werden. Die Injektion von 200 U reinen Neurotoxins ermöglichte eine erfolgreiche Therapie.

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Patentansprüche

1. Pharmazeutische Zubereitung, enthaltend eines der Botulinum-Neurotoxine von *Clostridium botulinum* der Typen A, B, C, D, E, F oder G oder ein Gemisch von zwei oder mehr dieser Neurotoxine, dadurch gekennzeichnet, dass das Neurotoxin bzw. das Gemisch der Neurotoxine frei ist von den komplexierenden Proteinen, die natürlicherweise zusammen mit den Neurotoxinen die Botulinum-Neurotoxin-Komplexe bilden.

2. Pharmazeutische Zubereitung nach Anspruch 1, dadurch gekennzeichnet, dass das Neurotoxin bzw. das Gemisch der Neurotoxine im Patienten zu keiner oder, im Vergleich zu den Komplexen, nur zu einer verminderten Induktion neutralisierender Antikörper führt.

3. Pharmazeutische Zubereitung nach Anspruch 1 oder 2, dadurch gekennzeichnet, dass das Neurotoxin bzw. das Gemisch der Neurotoxine ein natürliches Neurotoxin bzw. ein Gemisch natürlicher Neurotoxine ist oder enthält.

4. Pharmazeutische Zubereitung nach Anspruch 1 oder 2, dadurch gekennzeichnet, dass das Neurotoxin bzw. das Gemisch der Neurotoxine ein rekombinantes Neurotoxin bzw. ein Gemisch rekombinanter Neurotoxine ist oder enthält.

5. Pharmazeutische Zubereitung nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass das Neurotoxin das Neurotoxin von *Clostridium botulinum* Typ A oder B bzw. das Gemisch der Neurotoxine ein Gemisch der Neurotoxine von *Clostridium botulinum* Typ A und B ist oder enthält.

6. Verwendung der Botulinum-Neurotoxine von *Clostridium botulinum* der Typen A, B, C, D, E, F oder G oder eines Gemisches von zwei oder mehr dieser Neurotoxine zur

Herstellung eines Medikaments zur Behandlung von Erkrankungen des Nervensystems bzw. Dystonien.

7. Verwendung nach Anspruch 6, dadurch gekennzeichnet, dass es sich bei den Erkrankungen des Nervensystem bzw. den Dystonien um Torticollis spasmodicus und Blepharospasmus, Spastizitäten wie Spitzfuß, hämifasciale Spasmen, Migräne, Lumbalgie, Cervicalsyndrom oder Hypersalivation handelt.

8. Verwendung der Botulinum-Neurotoxine von *Clostridium botulinum* der Typen A, B, C, D, E, F oder G oder eines Gemisches von zwei oder mehr dieser Neurotoxine für die kosmetische Behandlung.

9. Verwendung nach Anspruch 8, dadurch gekennzeichnet, dass die kosmetische Behandlung zur Behandlung von Hyperhidrose und Faltenbildung, insbesondere im Gesichtsbereich, erfolgt.

10. Verwendung nach einem der Ansprüche 6 bis 9, dadurch gekennzeichnet, dass die mit dem Neurotoxin von *Clostridium botulinum* bzw. dem Gemisch der Neurotoxine zu behandelnde Person ein Mensch oder Tier ist, der/das bereits neutralisierende Antikörper gegen einen Botulinum-Neurotoxin-Komplex, insbesondere gegen den Komplex von *Clostridium botulinum* Typ A oder B, oder gegen mehrere Komplexe, insbesondere gegen die Komplexe von *Clostridium botulinum* Typ A und B, aufweist.

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

(19) Weltorganisation für geistiges Eigentum
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(74) Anwälte: **BETTENHAUSEN, Berthold** usw.; Dehmel & Bettenhausen, Müllerstrasse 1, D-80469 München (DE).

(81) Bestimmungsstaaten *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Bestimmungsstaaten *(regional)*: ARIPO-Patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI-Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Veröffentlicht:
— *Mit internationalem Recherchenbericht.*
— *Vor Ablauf der für Änderungen der Ansprüche geltenden Frist; Veröffentlichung wird wiederholt, falls Änderungen eintreffen.*

(88) Veröffentlichungsdatum des internationalen Recherchenberichts: 26. April 2001

Zur Erklärung der Zweibuchstaben-Codes, und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

(54) Title: THERAPEUTIC AGENT COMPRISING A BOTULINUM NEUROTOXIN

(54) Bezeichnung: THERAPEUTIKUM MIT EINEM BOTULINUM-NEUROTOXIN

(57) Abstract: The invention relates to a pharmaceutical preparation containing one of the botulinum neurotoxins of *Clostridium botulinum* of types A, B, C, D, E, F or G or a mixture of two or more of these neurotoxins. The inventive preparation is characterized in that the neurotoxin or the mixture of neurotoxins does not contain the complexing proteins which, together with the neurotoxins, naturally form the botulinum neurotoxin complexes.

(57) Zusammenfassung: Pharmazeutische Zubereitung, enthaltend eines der Botulinum-Neurotoxine von *Clostridium botulinum* der Typen A, B, C, D, E, F oder G oder ein Gemisch von zwei oder mehr dieser Neurotoxine, dadurch gekennzeichnet, dass das Neurotoxin bzw. das Gemisch der Neurotoxine frei ist von den komplexierenden Proteinen, die natürlicherweise zusammen mit den Neurotoxinen die Botulinum-Neurotoxin-Komplexe bilden.

WO 00/74703 A3

Hugel Ex. 1006 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

INTERNATIONAL SEARCH REPORT

Inter...ional Application No
PCT/DE 00/01777

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/16 A61K7/00 A61P25/00 //C07K14/33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, MEDLINE, CHEM ABS Data, EMBASE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 5 512 547 A (JOHNSON ERIC A ET AL) 30 April 1996 (1996-04-30) the whole document especially column 7 lines 29-39	1-3,5-7, 10 1,3-7,10
Y	WO 96 39167 A (PEARCE L BRUCE) 12 December 1996 (1996-12-12) page 7, line 8 - line 19 page 8, line 12 - line 17 page 11, line 17 -page 12, line 6 page 13, line 1 - line 7 claims 1-5,13-24; examples 2-4	1,3-7,10
Y	WO 94 28923 A (ALLERGAN INC) 22 December 1994 (1994-12-22) the whole document	1,3,5-7, 10

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[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 February 2001 (13.02.01)	16 February 2001 (16.02.01)

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Stein, A

Form PCT/ISA/210 (second sheet) (July 1992)

BNSDOCID: Hugel Ex. 1006, Page 364 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

INTERNATIONAL SEARCH REPORT

Inter...ional Application No
PCT/DE 00/01777

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GOESCHEL HILKE ET AL: "Botulinum A toxin therapy: Neutralizing and nonneutralizing antibodies-therapeutic consequences." EXPERIMENTAL NEUROLOGY, vol. 147, no. 1, 1997, pages 96-102, XP002158434 ISSN: 0014-4886 the whole document especially page 102 column 1 lines 13-27	1-3,5-10
A	EDITORIAL: "botulinum toxin" THE LANCET, vol. 340, no. 19/26, December 1992 (1992-12), pages 1508-1509, XP002158435 the whole document	1-10
A	KEEN M ET AL: "Botulinum Toxin A for Hyperkinetic Facial Lines: Results of a Double-Blind, Placebo-Controlled Study" PLASTIC AND RECONSTRUCTIVE SURGERY, vol. 94, no. 1, July 1994 (1994-07), pages 94-99, XP000978742 the whole document	8,9
A	NAUMANN M ET AL: "Depletion of neutralising antibodies resensitizes a secondary non-responder to botulinum A neurotoxin." JOURNAL OF NEUROLOGY NEUROSURGERY AND PSYCHIATRY, vol. 65, no. 6, December 1998 (1998-12), pages 924-927, XP000979025 ISSN: 0022-3050 the whole document	10
P,A	US 5 939 070 A (GOODNOUGH MICHAEL C ET AL) 17 August 1999 (1999-08-17) see the whole document, in particular column 11 lines 8-29	1-10
E	DE 198 56 897 A (BIOTECON GES FUER BIOTECHNOLOG) 15 June 2000 (2000-06-15) the whole document	1-7

1

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DOCID: <WO 0078811 A1> Hugel Ex. 1006 - Page 365 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

INTERNATIONAL SEARCH REPORT

International application No
PCT/DE 00/01777

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although the claims 8-10 relate to a method for treatment of the human/animal body, the search was carried out and was based on the cited effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/DE 00/01777

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Form PCT/ISA/210 (patent family annex) (July 1992)

INTERNATIONALER RECHERCHENBERICHT

Interr. ales Aktenzeichen
PCT/DE 00/01777

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
IPK 7 A61K38/16 A61K7/00 A61P25/00 //C07K14/33

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)
IPK 7 A61K C07K C12N

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)
BIOSIS, EPO-Internal, MEDLINE, CHEM ABS Data, EMBASE, SCISEARCH

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X Y	US 5 512 547 A (JOHNSON ERIC A ET AL) 30. April 1996 (1996-04-30) siehe die ganze dokument besonder spalte 7 zeilen 29-39	1-3,5-7, 10 1,3-7,10

Y	WO 96 39167 A (PEARCE L BRUCE) 12. Dezember 1996 (1996-12-12) Seite 7, Zeile 8 - Zeile 19 Seite 8, Zeile 12 - Zeile 17 Seite 11, Zeile 17 -Seite 12, Zeile 6 Seite 13, Zeile 1 - Zeile 7 Ansprüche 1-5,13-24; Beispiele 2-4	1,3-7,10

Y	WO 94 28923 A (ALLERGAN INC) 22. Dezember 1994 (1994-12-22) das ganze Dokument	1,3,5-7, 10

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[X] Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

[X] Siehe Anhang Patentfamilie

* Besondere Kategorien von angegebenen Veröffentlichungen :
"A" Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist
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"X" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden
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Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts
13. Februar 2001	16. 02. 2001

Name und Postanschrift der Internationalen Recherchenbehörde	Bevollmächtigter Bediensteter
Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Stein, A

Formblatt PCT/ISA/210 (Blatt 2) (Juli 1992)

BNSDOCID Hugel Ex. 1006 Page 368 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	GOESCHEL HILKE ET AL: "Botulinum A toxin therapy: Neutralizing and nonneutralizing antibodies-therapeutic consequences." EXPERIMENTAL NEUROLOGY, Bd. 147, Nr. 1, 1997, Seiten 96-102, XP002158434 ISSN: 0014-4886 siehe die ganze dokument besonder seite 102 spalte 1 zeilen 13-27	1-3,5-10
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A	NAUMANN M ET AL: "Depletion of neutralising antibodies resensitizes a secondary non-responder to botulinum A neurotoxin." JOURNAL OF NEUROLOGY NEUROSURGERY AND PSYCHIATRY, Bd. 65, Nr. 6, Dezember 1998 (1998-12), Seiten 924-927, XP000979025 ISSN: 0022-3050 das ganze Dokument	10
P,A	US 5 939 070 A (GOODNOUGH MICHAEL C ET AL) 17. August 1999 (1999-08-17) das ganze Dokument insbesondere Spalte 11 Zeilen 8-29	1-10
E	DE 198 56 897 A (BIOTECON GES FUER BIOTECHNOLOG) 15. Juni 2000 (2000-06-15) das ganze Dokument	1-7

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BNSDOCID: <WP... Hugel Ex. 1006 Page 369 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/DE 00/01777

Feld I Bemerkungen zu den Ansprüchen, die sich als nicht recherchierbar erwiesen haben (Fortsetzung von Punkt 2 auf Blatt 1)

Gemäß Artikel 17(2)a) wurde aus folgenden Gründen für bestimmte Ansprüche kein Recherchenbericht erstellt:

1. [X] Ansprüche Nr.
weil sie sich auf Gegenstände beziehen, zu deren Recherche die Behörde nicht verpflichtet ist, nämlich

Obwohl sich die Ansprüche 8-10 auf ein Verfahren zur Behandlung des menschlichen/tierischen Körpers beziehen, wurde die Recherche durchgeführt und gründete sich auf die angeführten Wirkungen der Verbindung/Zusammensetzung.

2. [ ] Ansprüche Nr.
weil sie sich auf Teile der internationalen Anmeldung beziehen, die den vorgeschriebenen Anforderungen so wenig entsprechen, daß eine sinnvolle internationale Recherche nicht durchgeführt werden kann, nämlich

3. [ ] Ansprüche Nr.
weil es sich dabei um abhängige Ansprüche handelt, die nicht entsprechend Satz 2 und 3 der Regel 6.4 a) abgefaßt sind.

Feld II Bemerkungen bei mangelnder Einheitlichkeit der Erfindung (Fortsetzung von Punkt 3 auf Blatt 1)

Die internationale Recherchenbehörde hat festgestellt, daß diese internationale Anmeldung mehrere Erfindungen enthält:

1. [ ] Da der Anmelder alle erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht auf alle recherchierbaren Ansprüche.

2. [ ] Da für alle recherchierbaren Ansprüche die Recherche ohne einen Arbeitsaufwand durchgeführt werden konnte, der eine zusätzliche Recherchengebühr gerechtfertigt hätte, hat die Behörde nicht zur Zahlung einer solchen Gebühr aufgefordert.

3. [ ] Da der Anmelder nur einige der erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht nur auf die Ansprüche, für die Gebühren entrichtet worden sind, nämlich auf die Ansprüche Nr.

4. [ ] Der Anmelder hat die erforderlichen zusätzlichen Recherchengebühren nicht rechtzeitig entrichtet. Der internationale Recherchenbericht beschränkt sich daher auf die in den Ansprüchen zuerst erwähnte Erfindung; diese ist in folgenden Ansprüchen erfaßt:

Bemerkungen hinsichtlich eines Widerspruchs

[ ] Die zusätzlichen Gebühren wurden vom Anmelder unter Widerspruch gezahlt.

[ ] Die Zahlung zusätzlicher Recherchengebühren erfolgte ohne Widerspruch.

INTERNATIONALER RECHERCHENBERICHT

Angaben zu Veröffentlichungen, die zur selben Patentfamilie gehören

Interr ales Aktenzeichen
PCT/DE 00/01777

Im Recherchenbericht angeführtes Patentdokument		Datum der Veröffentlichung	Mitglied(er) der Patentfamilie		Datum der Veröffentlichung
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(54) Title: METHODS AND SYSTEMS FOR PURIFYING NON-COMPLEXED BOTULINUM NEUROTOXIN

(57) Abstract: Methods and systems for chromatographically purifying a *botulinum* neurotoxin are provided. These methods and systems allow for efficient purification of a non-complexed form of the *botulinum* neurotoxin in high purity and yield that can be used as an active ingredient in pharmaceutical preparations.

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METHODS AND SYSTEMS FOR PURIFYING NON-COMPLEXED BOTULINUM NEUROTOXIN

[0001] This application claims the benefit of priority to U.S. Provisional Application No. 61/253,810, filed on October 21, 2009. The contents of this U.S. Provisional Application are hereby incorporated by reference in their entirety.

FIELD OF THE INVENTION

[0002] This invention relates generally to chromatographic methods and systems for purifying free *botulinum* neurotoxin from cell cultures to produce a high purity, high potency product.

BACKGROUND OF THE INVENTION

[0003] *Botulinum* toxin is a neurotoxic protein produced by the bacterium *Clostridium botulinum*, as well as other *Clostridial* species, such as *Clostridium butyricum*, and *Clostridium baraffi*. The toxin blocks neuromuscular transmission and causes a neuro-paralytic illness in humans and animals, known as botulism. *C. botulinum* and its spores commonly occur in soil and putrefying animal carcasses, and can grow in improperly sterilized or improperly sealed food containers, which are the cause of many botulism cases. Botulism symptoms can include difficulty walking, swallowing, and speaking, and can progress to paralysis of the respiratory muscles and finally death.

[0004] *Botulinum* toxin type A is the most lethal natural substance known to man. In addition to serotype A, six other generally immunologically distinct *botulinum* toxins have been characterized, namely *botulinum* toxin serotypes B, C_1, D, E, F, and G. The different serotypes can be distinguished by neutralization with type-specific antibodies and vary in severity of paralysis they evoke and the animal species they mostly affect. The molecular weight of the *botulinum* toxin protein molecule, for each of the known *botulinum* toxin serotypes, is about 150 kD, composed of an about 100 kD heavy chain joined to an about 50 kD light chain. Nonetheless, the *botulinum* toxins are released by *Clostridial* bacteria as complexes of the 150 kD toxin with one or more non-toxin proteins. For example, *botulinum* toxin type A exists as 900 kD, 500 kD and 300 kD complexes (approximate molecular weights).

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[0005] Despite the known toxic effects, *Botulinum* toxin type A is clinically used to treat a variety of indications, including, *e.g.*, neuromuscular disorders characterized by skeletal muscle hyperactivity. For example, BOTOX® is the trademark of a *botulinum* toxin type A complex available commercially from Allergan, Inc., of Irvine, Calif. *Botulinum* toxin type A finds use, for example, in the treatment of essential blepharospasm, strabismus, cervical dystonia, and glabellar line (facial) wrinkles. Other serotypes also have been used clinically. A *botulinum* toxin type B, for example, has been indicated for use in treating cervical dystonia. The *botulinum* toxins are believed to bind with high affinity to the presynaptic membrane of motor neurons, translocate into the neuron, and thereafter block the presynaptic release of acetylcholine.

[0006] The *botulinum* toxin for clinical use is typically isolated from cell culture and various purification approaches have been used. Historically, the toxin is purified in complexed form by a series of precipitation and tangential flow filtration steps. See, *e.g.*, Schantz E. J., *et al.*, Properties and use of *botulinum* toxin and other microbial neurotoxins in medicine, Microbiol Rev 1992 March 56(1):80-99. Such approaches have provided relatively low yields, however, typically less than about 10%. Other approaches have used size exclusion, ion exchange, and/or affinity chromatography. See, *e.g.*, Schmidt J. J., *et al.*, Purification of type E *botulinum* neurotoxin by high-performance ion exchange chromatography, Anal. Biochem. 1986 July; 156(1):213-219; Simpson L. L., *et al.*, Isolation and characterization of the *botulinum* neurotoxins, Harsman S, ed. Methods in Enzymology. Vol. 165, Microbial Toxins: Tools in Enzymology San Diego, Calif.: Academic Press; vol 165: pages 76-85 (1988); Kannan K., et al., Methods development for the biochemical assessment of Neurobloc (*botulinum* toxin type B), Mov Disord 2000; 15(Suppl 2):20 (2000); Wang Y.C., The preparation and quality of *botulinum* toxin type A for injection (BTXA) and its clinical use, Dermatol Las Faci Cosm Surg 2002; 58 (2002); and U.S. Pat. Appl. Publ. No. 2003/0008367.

[0007] Still other approaches have focused on just one of the toxin's heavy or light chains, rather than a complete and biologically active *botulinum* toxin protein. For example, one of the chains is individually synthesized by recombinant means. See, *e.g.*, Zhou L., et al., Expression and purification of the light chain of *botulinum* neurotoxin A: A single mutation abolishes its cleavage of SNAP-25 and neurotoxicity after reconstitution with the heavy chain, Biochemistry 1995; 34(46):15175-81

BNSDOCID: <WO 2011050072A1_I_> Hugel Ex. 1006 Page 374 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

(1995); and Johnson S. K., et al., Scale-up of the fermentation and purification of the recombination heavy chain fragment C of *botulinum* neurotoxin serotype F, expressed in Pichia pastoris, Protein Expr and Purif 2003; 32:1-9 (2003). These approaches, however, require extra steps to reform a complete and biologically active *botulinum* toxin protein.

[0008] A more recent approach involves the use of hydrophobic interaction chromatography, mixed mode, and/or ion exchange chromatography to purify a *botulinum* toxin as a complex. See, *e.g.*, U.S. Pat. Nos. 7,452,697 and 7,354,740, which are hereby incorporated by reference.

[0009] Accordingly, there is a need in the art for improved purification methods for isolating complete *botulinum* toxin proteins in stable, biologically active, but non-complexed forms. It is therefore an object of the invention to provide compositions and methods addressing these and other needs.

[0010] The foregoing discussion is presented solely to provide a better understanding of the nature of the problems confronting the art and should not be construed in any way as an admission as to prior art nor should the citation of any reference herein be construed as an admission that such reference constitutes "prior art" to the instant application.

SUMMARY OF THE INVENTION

[0011] This invention relates to systems and methods for purifying a non-complexed *botulinum* toxin. In one embodiment, the method comprises purifying crude non-complexed *botulinum* toxin to obtain a purified non-complexed *botulinum* toxin. In this embodiment, the method comprises loading an anion exchange column with the crude non-complexed *botulinum* toxin to capture the non-complexed *botulinum* toxin on the anion exchange column; eluting the non-complexed *botulinum* toxin with buffer to give an eluent comprising the non-complexed *botulinum* toxin; loading a cation exchange column with the eluent from the anion exchange to column to permit capture of the non-complexed *botulinum* toxin; and eluting the non-complexed *botulinum* toxin with another buffer to give an eluent, thereby obtaining a purified non-complexed *botulinum* toxin.

[0012] In certain embodiments, the *botulinum* toxin complex is itself obtained by a number of chromatography steps. In some embodiments, a method for obtaining

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the *botulinum* toxin complex comprises obtaining a sample comprising a *botulinum* toxin complex; loading a hydrophobic interaction column with the sample to permit capture of the toxin, wherein the captured *botulinum* toxin comprises a complexed *botulinum* toxin; and eluting the complexed *botulinum* toxin. The non-complexed *botulinum* toxin is then dissociated from the complex and the non-complexed *botulinum* toxin is purified according to the method described above. In some embodiments, the sample is obtained by subjecting a fermentation culture comprising *botulinum* toxin to acid to obtain an acid precipitate, which may be subjected to additional pre-chromatography purification steps, non-limiting examples of which include tangential flow filtration to concentrate the insoluble material of the precipitate, nuclease digest, clarifying centrifugation and/or filtration.

[0013] In some embodiments, the sample is subjected to a nuclease digestion before loading on the hydrophobic interaction column. Preferably, the nuclease is derived in an animal-product-free process, and even more preferably the entire purification process is animal product free or at least substantially animal product free.

[0014] In some embodiments, the sample to be used in the chromatographic separations is preferably a supernatant or filtrate fraction.

[0015] The purified non-complexed *botulinum* toxin comprises at least one of *botulinum* toxin type A, B, C_1, D, F, F and G, and preferably a *botulinum* toxin type A having a molecular weight of about 150 kD. In some preferred embodiments, the purified non-complexed *botulinum* toxin is at least 98% pure; and/or has an activity of at least 200 LD_{50} units/ng. In some embodiments, the method produces a yield of at least about 2 mg/L fermentation culture. In other embodiments, the method produces a yield of about 1 to about 2 mg/L fermentation culture.

[0016] These and other aspects of the invention will be better understood by reference to the following detailed description of the invention.

BRIEF DESCRIPTION OF THE FIGURES

[0017] Figure 1 is a summary flow chart comparing one embodiment of a process according to the instant invention for directly purifying a non-complexed *botulinum* toxin (Figure 1A) with a process for purifying a complexed *botulinum* toxin (Figure 1B).

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DETAILED DESCRIPTION

[0018] This invention relates to systems and methods for purifying a non-complexed *botulinum* toxin. In certain embodiments, the method comprises purifying a crude non-complexed *botulinum* toxin by loading an anion exchange column with the crude non-complexed *botulinum* toxin to permit capture of the non-complexed *botulinum* toxin by the anion exchange column. Non-complexed *botulinum* toxin is then eluted with buffer to give an eluent comprising the non-complexed *botulinum* toxin. The eluent from the anion column is loaded on a cation exchange column to permit capture of the non-complexed *botulinum* toxin and the purified non-complexed *botulinum* toxin is eluted with buffer, thereby obtaining a purified non-complexed *botulinum* toxin.

[0019] In some embodiments, the invention provides for the purification of non-complexed botulinum toxin in a relatively small number of steps to produce a high yield, high purity, and high potency product. Processes and systems within the scope of the invention can be used to efficiently produce a stable but non-complexed botulinum toxin from fermentation cultures. In other embodiments, the method further comprises providing a sample comprising *botulinum* toxin complex and loading a hydrophobic interaction column with the sample so as to permit capture of the *botulinum* toxin complex by the hydrophobic interaction column. The *botulinum* toxin complex is then eluted from the column with buffer. Crude non-complexed *botulinum* toxin is dissociated from the *botulinum* toxin complex to obtain a mixture comprising the crude non-complexed *botulinum* toxin. In this embodiment, the mixture comprising crude non-complexed *botulinum* toxin is purified to obtain pure or substantially pure *botulinum* toxin according to the method described above.

[0020] One aspect of this invention is the recognition that a pharmaceutical composition comprising non-complexed *botulinum* toxin as an active ingredient can provide greater purity compared to one comprising a complexed form. Non-toxin proteins typically associated with a *botulinum* toxin complex can account for about 90% by weight of the complex. Thus, providing a *botulinum* toxin as a complex necessarily includes at least about 90% by weight of impurities. In other words, at least about 80 to about 90% by weight of the pharmaceutical composition will include cell-derived impurities that are not part of the active molecule nor necessary for its biological activity. Such impurities, however, represent cell-derived materials that

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when administered to a patient may increase the risk of unwanted immunological reactions to the drug; may increase the risk of unwanted side effects; and/or may increase the risk of transmission of pathogenic agents. In contrast, the high purity of a non-complexed product, obtainable by methods and systems described herein, reduces the amount of host cell impurities that may remain in the pharmaceutical composition, thereby reducing the attendant risks of unwanted reactions and/or transmission. Accordingly, processes and systems described herein can provide a *botulinum* toxin in a form more readily suited to the preparation of safer, purer pharmaceutical compositions.

[0021] Moreover, unlike complexed forms, free *botulinum* toxin prepared in accordance with the method described herein does not need to be stabilized for storage in blood-derived products. *Botulinum* toxin type A complex, for example, is typically stabilized in an excipient comprising albumin, which is derived from human blood. For example, BOTOX® consists of a purified *botulinum* toxin type A complex, human serum albumin, and sodium chloride packaged in vacuum-dried form. The same is true for Dysport and Xeomin. While screenings reduce likelihood of contamination with pathogenic agents, use of human blood in pharmaceutical preparations generally increases the risk of unwanted transmission of certain pathogenic agents, *e.g.*, agents which are not or cannot yet be screened out. In contrast, free *botulinum* toxin prepared according to the instant invention can be stably stored, as taught herein, in ammonium sulfate. Further, in some preferred embodiments, methods and systems of the instant invention are substantially, essentially, or entirely animal product free, as discussed herein. The ability to also stably store the toxin product substantially, essentially, or entirely animal-product free, further reduces potential risks associated with animal-derived products. Accordingly, processes and systems described herein provide a *botulinum* toxin in a form particularly suited to pharmaceutical applications in terms of safety, *e.g.*, where the pharmaceutical composition may be prepared and stored substantially, essentially, or entirely animal-product free.

[0022] In certain preferred embodiments, the processes and systems described herein are scalable and/or cGMP compliant. Accordingly, methods and systems described herein may be used on a commercial, industrial scale, to produce non-complexed *botulinum* toxin for use, *e.g.*, in pharmaceutical compositions. A cGMP compliant process or system refers to one that can comply with the regulatory

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requirements for current good manufacturing practices, as required by the U.S. Code of Federal Regulations. In some preferred embodiments, the non-complexed *botulinum* toxin product is particularly suited to large scale production due to its ease of storage and usability, high activity, high purity, stability, and/or improved safety.

[0023] "*Botulinum* toxin" as used herein refers to a neurotoxin protein molecule that can be produced by a *Clostridial* bacterium, as well as recombinantly produced forms thereof. A recombinant *botulinum* toxin can have the light chain and/or heavy chain of the toxin protein synthesized via recombinant techniques, *e.g.*, by a recombinant *Clostridial* and/or non-*Clostridial* species. "*Botulinum* toxin" is used interchangeably herein with the related expressions "*botulinum* neurotoxin," "neurotoxin" or simply "toxin." "*Botulinum* toxin" encompasses any of the *botulinum* toxin serotypes A, B, C_1, D, E, F and G, and also encompasses both complexed and non-complexed forms.

[0024] By "complexed form" is meant a *botulinum* toxin complex comprising a botulinum toxin protein (*i.e.*, the toxin molecule with a molecular weight of about 150 kD) as well as at least one associated native non-toxin protein. Non-toxin proteins that make up the complexes typically include non-toxin hemagglutinin protein and non-toxin non-hemagglutinin protein. Thus complexed forms may comprise a *botulinum* toxin molecule (the neurotoxic component) and one or more non-toxin hemagglutinin proteins and/or one or more non-toxin non-hemagglutinin proteins. In certain embodiments, the molecular weight of the complex is greater than about 150 kD. For example, complexed forms of the *botulinum* toxin type A can have molecular weights of about 900 kD, about 500 kD or about 300 kD. Complexed forms of *botulinum* toxin types B and C_1 can have a molecular weight of 500 kD. Complexed forms of *botulinum* toxin type D can have a molecular weight of about 300 kD or about 500 kD. Finally, complexed forms of *botulinum* toxin types E and F can have a molecular weight of about 300 kD.

[0025] "Non-complexed" *botulinum* toxin refers to an isolated, or essentially or substantially isolated, *botulinum* toxin protein having a molecular weight of about 150 kD. That is, "non-complexed" forms exclude non-toxin proteins, such as non-toxin hemagglutinin and non-toxin non-hemagglutinin proteins, normally associated with the complexed form. "Non-complexed" *botulium* toxin is used interchangeably herein with "free" *botulinum* toxin. All the *botulinum* toxin serotypes made by native *Clostridium botulinum* bacteria are synthesized by the bacteria as inactive single chain

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proteins which are then cleaved or nicked by proteases to become neuroactive. The protein comprises an about 100 kD heavy chain joined by a disulfide bond to an about 50 kD light chain.

[0026] *Botulinum* toxin complexes can be dissociated into toxin and non-toxin proteins by various means, including, for example, raising the pH to about 7.0, treating the complex with red blood cells at a pH of about 7.3, and/or subjecting the complex to a separation process, such as column chromatography in a suitable buffer at a pH of about 7 to about 8.

[0027] The instant invention encompasses systems and methods that enable purification of a non-complexed *botulinum* toxin, without associated non-toxin proteins conventionally believed necessary during the purification process to maintain stability. In preferred embodiments, the methods and systems described herein facilitate purification of the free *botulinum* toxin without loss of stability. By "stability" or "stable" is meant that the *botulinum* toxin protein molecule retains both the about 100 kD heavy chain and the about 50 kD light chain, joined to each other by a disulfide bond, and in a conformation that allows for biological activity.

[0028] In some embodiments, a particular system within the scope of the present invention is operated in conjunction with a particular method within the scope of the present invention. A system within the scope of the present invention can comprise a plurality (preferably consecutive series) of chromatography columns for use with a corresponding plurality (preferably consecutive series) of chromatography steps. Further, a system within the scope of the instant invention may comprise a plurality (preferably a consecutive series) of non-chromatography devices, such as filtration and/or centrifugation apparatus, for use with a corresponding plurality (preferably consecutive series) of non-chromatography steps, *e.g.*, as pre-chromatography steps.

[0029] In preferred embodiments, a process within the scope of the present invention comprises obtaining a sample comprising *botulinum* toxin from a fermentation culture; subjecting it to a number of pre-chromatography purifications; and then passing it through a plurality of chromatography columns to obtain a highly purified, highly potent non-complexed *botulinum* toxin. Such a purified free *botulinum* toxin finds use in the preparation of pharmaceutical compositions comprising the free botulinum toxin as an active ingredient.

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[0030] The overall steps for both pre-chromatography and chromatography processes for some preferred embodiments of the instant invention are illustrated in Figure 1A. For comparison, Figure 1B shows a conventional method for obtaining purified botulinum toxin complex. Briefly, Figure 1B depicts a process involving depth filtration of a fermentation culture, followed by tangential flow filtration of the filtrate obtained (using 300kD ultramicrofiltration); followed by a clarifying centrifugation step. The pellet (insoluble fraction) resulting from the centrifugation step is then re-suspended in sodium chloride, and loaded onto a hydrophobic interaction or ion exchange column. The chromatographic purification step is repeated at least three times to give a final eluent containing the 900 kD *botulinum* toxin type A complex.

Fermentation and Acid Precipitation

[0031] As Figure 1A illustrates, the non-complexed *botulinum* toxin is generally purified from a fermentation culture. A "fermentation culture" as used herein refers to a culture or medium comprising cells, and/or components thereof, that are synthesizing and/or have synthesized at least one *botulinum* toxin. For example, *Clostridial* bacteria, such as *Clostridium botulinum*, may be cultured on agar plates in an environment conducive to bacterial growth, such as in a warm anaerobic atmosphere. The culture step typically allows *Clostridial* colonies with desirable morphology and other characteristics to be obtained. Selected cultured *Clostridial* colonies then can be fermented in a suitable medium as a fermentation culture. The cultured cells may include non-*Clostridial* species as the host cells, such as *E. coli* or yeast cells, that are rendered capable of biosynthesizing a *botulinum* toxin by recombinant technology. Suitable fermentation culture conditions can depend on the host cells used and are generally known in the art.

[0032] In preferred embodiments, fermentation may be allowed to progress to completion, such that cells are mature and have biosynthesized a *botulinum* toxin. Growth of *Clostridium botulinum* cultures is usually complete after about 24 to about 36 hours. After a certain additional period of time, the bacteria typically lyse and release into the medium the synthesized *botulinum* toxin complex in a complexed form. For example, during a fermentation of about 60 to about 96 hours, most *Clostridium botulinum* cells undergo lysis and release *botulinum* toxin type A complex.

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[0033] In some embodiments, the fermentation culture can comprise one or more animal products, such as animal proteins, used in conventional fermentation culture procedures. For example, *botulinum* toxin can be produced by anaerobic fermentation of *Clostridium botulinum* using a modified version of the well known Schantz process (see *e.g.* Schantz E. J., et al., Properties and use of *botulinum* toxin and other microbial neurotoxins in medicine, Microbiol Rev 1992 March; 56(1):80-99; Schantz E. J., et al., Preparation and characterization of *botulinum* toxin type A for human treatment, chapter 3 in Jankovic J, ed. Neurological Disease and Therapy. Therapy with *botulinum* toxin (1994), New York, Marcel Dekker; 1994, pages 41-49, and; Schantz E. J., et al., Use of crystalline type A *botulinum* toxin in medical research, in: Lewis G E Jr, ed. Biomedical Aspects of Botulism (1981) New York, Academic Press, pages 143-50, each incorporated herein by reference). Both the Schantz and the modified Schantz process for obtaining a *botulinum* toxin make use of animal products, including animal-derived-Bacto-Cooked Meat medium in the culture vial, and casein in the fermentation media. Additionally, the Schantz toxin purification makes use of DNase and RNase from bovine sources to hydrolyze nucleic acids present in the fermentation culture.

[0034] However, administration of a pharmaceutical containing an active ingredient that was purified using a process involving animal-derived products can subject a patient to a potential risk of receiving various pathogenic agents. For example, prions may be present in a pharmaceutical composition comprising contaminating animal-derived products, such as the prion responsible for Creutzfeldt-Jacob disease. As another example, there is a risk of transmitting a spongiform encephalopathy (TSE), such as a bovine spongiform encephalopathy (BSE) when animal products are used in the process of making a pharmaceutical composition. The use of a *botulinum* toxin obtained via processes free of animal products, however, reduces such risks. Therefore, in some preferred embodiments, the invention provides a process that is free of animal products, or essentially or substantially animal-product-free (APF). "Animal product free", "essentially animal product free", or "substantially animal product free" encompasses, respectively, "animal protein free", "essentially animal protein free", or "substantially animal protein free" and respectively means the absence, essential absence, or substantial absence, of products derived from animals, non-limiting examples of which include products derived from blood or pooled blood. "Animal" is used herein to refer to a mammal (such as a

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human), bird, reptile, amphibian, fish, insect, spider or other animal species, but excludes microorganisms, such as bacteria and yeasts.

[0035] An animal-product-free process (or a substantially or essentially animal product-free-process) refers to a process that is entirely, substantially, or essentially free of animal-derived products, reagents and proteins, such as immunoglobulins, other blood products, by-products, or digests; meat products, meat by-products, meat digests; and milk or dairy products, by-products or digests. Accordingly, an example of an animal-product free fermentation culture procedure is a fermentation process, such as bacterial culturing, which excludes blood, meat, and dairy products, by-products, and digests. An animal-product-free fermentation process for obtaining a non-complexed *botulinum* toxin reduces the possibility of contamination with viruses, prions or other undesirable agents, which can then accompany the toxin when administered to humans.

[0036] Animal-product-free fermentation procedures using *Clostridium* cultures are described, *e.g.*, in U.S. Pat. Nos. 7,452,697 and 7,354,740, hereby incorporated by reference. For example, the growth media for production of the *botulinum* toxin may comprise vegetable-based products, instead of animal-derived products, such as soy-based products and/or the debittered seed of *Lupinus campestris*. Soy-based fermentation media for use in an animal product free fermentation culture, for example, can comprise a soy-based product, a source of carbon such as glucose, salts such as NaCl and KCl, phosphate-containing ingredients such as Na_2HPO_4 and KH_2PO_4, divalent cations such as iron and magnesium, iron powder, amino acids such as L-cysteine and L-tyrosine, and the like. Preferably, the soy is hydrolyzed soy and the hydrolyzation has been conducted using enzymes not derived from animals. Sources of hydrolyzed soy include but are not limited to Hy-Soy (Quest International), Soy peptone (Gibco) Bac-soytone (Difco), AMISOY (Quest), NZ soy (Quest), NZ soy BL4, NZ soy BL7, SE50M (DMV International Nutritionals, Fraser, N.Y.), and SE50MK (DMV).

[0037] As Figure 1A illustrates, in certain embodiments a sample comprising *botulinum* toxin is obtained from a fermentation culture. For example, after a certain period of fermentation, in either animal product free or non-animal product free media, *botulinum* toxin complex is released into the medium and can be harvested by precipitation. For example, in some embodiments, as in the well-known Schantz process, the fermentation medium comprising the *botulinum* toxin may be subjected

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to acid precipitation to encourage the *botulinum* toxin complexes to associate with cell debris and form an acid precipitate. In some particularly preferred embodiments, about 3 M sulfuric acid solution may be added to the fermentation culture to form the acid precipitate. Preferably, the pH is reduced to about 3 to about 4, more preferably to about 3.2 to about 3.8, and even more preferably about 3.5. In some embodiments, the culture temperature is also reduced, *e.g.*, to about below 25°C, 24°C, 23°C, 22°C, 21°C, or 20°C. These conditions further enhance the association of *botulinum* toxin complexes with cell debris. The acid precipitate formed will comprise bound *botulinum* toxin complexes and can be used as the starting material in further purification steps, such as clarification steps; whereas the filtrate is discarded.

[0038] In contrast, the conventional process depicted in Figure 1B does not include an acid precipitation step. That is, while the purification procedure also begins with a fermentation culture comprising a *botulinum* toxin complex, the culture medium is subjected to depth filtration, and the filtrate, rather than the cell debris, is used in subsequent purification steps. In the Figure 1B process, the cell debris is discarded, rather than the filtrate, whereas, as illustrated in Figure 1A, the filtrate is discarded and the cell debris (acid precipitate) is used for further purification steps, *e.g.*, in the pre-chromatography purifications discussed below.

Pre-Chromatography Purifications

[0039] In some embodiments, the sample obtained from the fermentation medium is subjected to one or more pre-chromatography purifications. Pre-chromatography purifications can include at least one of tangential flow filtration, nuclease digest, and clarifying centrifugation and/or filtration. A non-limiting example of a process flow containing pre-chromatography purification contemplated by the invention is provided in Figure 1A. As noted above, in preferred embodiments, the pre-chromatography procedures are carried out on a precipitate (or insoluble fraction) of a fermentation culture comprising the *botulinum* toxin, rather than on the fermentation culture itself or on a filtrate derived therefrom, as in the process illustrated in Figure 1B. That is, in preferred embodiments of the invention, pre-chromatography (clarification) steps start with the acid precipitate (insoluble fraction).

[0040] In some embodiments, the sample (acid precipitate or insoluble fraction) comprising a *botulinum* toxin is subjected to tangential flow filtration. Tangential flow filtration is a process typically used to clarify, concentrate, and/or

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purify proteins. In contrast to normal flow filtration, where fluid moves directly towards a filter membrane under applied pressure, in tangential flow filtration, the fluid moves tangentially along, or parallel to, the surface of the membrane. Applied pressure serves to force a portion of the fluid through the filter membrane, to the filtrate side, while particulates and macromolecules too large to pass through membrane pores are retained. Unlike normal flow filtration, however, the retained components do not build up at the membrane surface but are swept along by the tangentially flowing fluid. In certain preferred embodiments, tangential flow filtration is used to concentrate the insoluble material (cell debris) with which the *botulinum* complex is associated, while permitting filtrate to pass through the membrane pores. (See, *e.g.*, Figure 1A.) Tangential flow filtration parameters, such as pore size, feed flow, applied pressure, and the like, may be selected by those of skill in the art to concentrate cell debris and to produce a more concentrated sample comprising the *botulinum* toxin complex. In some particularly preferred embodiments, for example, tangential flow filtration with filters having a pore size of about 0.1 μm may be used.

[0041] In some embodiments, the sample comprising a *botulinum* toxin is subjected to nuclease digestion. Nuclease digestion can facilitate removal of nucleic acid components with which the *Botulinum* toxin complexes tend to associate. In certain preferred embodiments, nuclease digestion follows tangential flow filtration and is carried out on the concentrated cell debris obtained therefrom. (See, *e.g.*, Figure 1A.) For example, the concentrated cell debris sample may have its pH adjusted to allow nuclease activity and may be incubated with one or more suitable nucleases, such as DNases and/or RNases that digest (hydrolyze) DNA and/or RNA, respectively. Depending on the nuclease enzyme used, suitable pH may be about 5 to about 7, preferably about 6. In some embodiments, benzamidine is used as a protease inhibitor to prevent proteolysis of the toxin during nuclease digestion step. The nuclease used may be derived from any suitable source, including animal sources and/or non-animal sources.

[0042] In more preferred embodiments, the nuclease is obtained from a non-animal source, to provide an animal-product-free nuclease and an animal-product-free process. Accordingly, the instant invention encompasses animal-product-free processes and systems (or substantially or essentially animal product free processes and systems) for purifying *botulinum* toxin which comprise use of a nuclease. An animal-product-free nuclease may be made recombinantly, *e.g.*, using recombinant

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bacteria, yeasts, or other suitable microorganisms, which have been transformed to express a DNase and/or RNase for use in a nuclease digestion step according to processes described herein. Nuclease digestion typically reduces the nucleic acid content of the sample, as the host cell nucleic acids are degraded and their removal is facilitated. For example, hydrolyzed nucleic acids and other low molecular weight impurities can be removed by further purification steps.

[0043] In certain embodiments, the sample comprising a *botulinum* toxin may be subjected to clarifying centrifugation and/or filtration. Clarifying centrifugation or filtration refers to centrifugation or filtration steps used to remove gross elements, such as whole and lysed cells and cell debris, from the sample, resulting in a measurably clearer sample. In certain embodiments, the centrifugation is performed at about 10,000xg to about 30,000xg, more preferably at about 15,000xg to about 20,000xg, and most preferably at about 17,700xg. Clarifying filtration will typically comprise normal flow filtration, also called "dead end" filtration, where fluid is moved directly toward a filter media under applied pressure, and particulates too large to pass through the filter pores accumulate at the surface or within the media itself, while smaller molecules pass through as the filtrate. In some particularly preferred embodiments, the sample is mixed with ammonium sulfate and normal flow filtration is performed using a filter with a pore size of about 0.1 to about 0.3 μm, and more preferably a pore size of about 0.2 μm. (See, *e.g.*, Figure 1A.) In certain particularly preferred embodiments, one or more clarifying step(s) follow the nuclease digestion step. In certain still more preferred embodiments, one or more clarifying step(s) immediately precede purification by chromatography.

[0044] Notably, in preferred embodiments, the clarified supernatant or filtrate provides the *botulinum* toxin-containing sample for use in further purification steps, such as the chromatography purification steps, rather than the insoluble fraction, which is discarded. This is in contrast with the process outlined in Figure 1B, where the *botulinum* toxin complex is contained in the insoluble fraction from pre-chromatography steps that do not involve acid precipitation, such as *e.g.*, as a centrifugation pellet, obtained from pre-chromatography centrifugation, and the supernatant is discarded.

[0045] Moreover, and again in contrast with the process outlined in Figure 1B, the pre-chromatography steps in some embodiments of the invention do not require a tangential flow filtration step of a filtrate obtained from fermentation culture. That is,

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the sample used for chromatography purification in some embodiments of the invention is not obtained by subjecting a soluble fraction of the fermentation culture to tangential flow filtration. Rather, in certain embodiments, the present invention uses insoluble material (such as an acid precipitate), eliminating any step where a fermentation culture filtrate is subjected to tangential flow filtration in an attempt to concentrate soluble *botulinum* toxin complexes. Thus, in preferred embodiments, the pre-chromatography steps of the invention eliminate the need for any such step, by instead using acid to precipitate the desired toxin complexes with other insoluble material (cell debris).

Chromatography Purification Steps

[0046] Figure 1A also illustrates chromatographic purification steps according to certain embodiments of the instant invention. According to one embodiment of the invention, chromatographic methods for purifying a non-complexed *botulinum* toxin comprise passing a sample comprising *botulinum* toxin through a plurality of chromatography columns to obtain a highly purified, highly potent, non-complexed form of the neurotoxin.

[0047] In certain embodiments, a complexed *botulinum* toxin is separated from other cellular components using a hydrophobic interaction column (see *e.g.*, Figure 1A). This column captures the *botulinum* toxin in complexed form, while allowing impurities to flow through the column. The column used may be any hydrophobic interaction column known in the art suitable for such purpose, such as Butyl Sepharose Fast Flow column or Phenyl Sepharose HP, commercially available from GE Healthcare Life Sciences. In some embodiments, the method further comprises conditioning the sample for hydrophobic interaction chromatography before loading onto the column. For example, for use in the Phenyl Sepharose HP column, the sample may be combined with a 0.5M ammonium sulfate solution at pH 6, and 50 mM phosphate before loading. Other columns, buffers and pH conditions that may be used include columns such as Phenyl Sepharose Fast Flow high substitution, Phenyl Sepharose Fast Flow low substitution, Butyl Sepharose, and Octyl Sepharose; buffers such as acetate, citrate, MES, histidine, piperazine, and malonate, each in the pH range of about 4.0 to about 7.0, more preferably about 4.5 to about 6.5, and even more preferably about 5.5. Other buffer and pH conditions may be determined to optimize yield from a particular column used, as known in the art, based on the teachings provided herein. Without wishing to be bound to theory, it is

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believed that separation involves binding of the toxin complex to resin at a pH below 7, to avoid dissociation at this step, while allowing many cell-derived impurities to flow through, such as, *e.g.*, smaller proteins, nucleic acids, and the like.

[0048] For eluting the captured (bound) toxin from the hydrophobic interaction column, a suitable buffer can be used, as known in the art. In some particularly preferred embodiments, a descending gradient of ammonium sulfate is used. The concentration range of the descending gradient may be from about 0.6 M to about 0.0 M, about 0.5 M to about 0.0 M, or about 0.4 M to about 0.0 M. Other eluting buffers that may be used include, for example descending gradients of sodium sulfate (Na_2SO_4); sodium chloride (NaCl); potassium chloride (KCl); ammonium acetate (NH_4OAc); and the like. Fraction(s) containing a product peak can be identified, as known in the art. The peak fraction is typically found, *e.g.*, when using ammonium sulfate, in a concentration range of about 0.4 M to about 0.0 M; more preferably about 0.3 M to about 0.0 M; and most preferably about 0.25 M to about 0.0 M ammonium sulfate, while the pH is kept at about 6 to maintain the complex. That is, the fraction(s) containing the eluted *botulinum* toxin complex can be identified and used in subsequent purification steps.

[0049] In preferred embodiments, the *botulinum* toxin complex obtained is caused to dissociate to give a non-complexed form. In certain preferred embodiments, the dissociation step is performed after the hydrophobic interaction chromatography step and/or before subsequent chromatography steps (*e.g.*, *see* Figure 1A). Accordingly, in some preferred embodiments, the instant invention encompasses methods and systems where the chromatographic target molecule differs from one chromatographic step to another. That is, in an initial chromatographic step, the target comprises a *botulinum* toxin complex, whereas in subsequent chromatographic steps, the target comprises the free *botulinum* toxin, dissociated from non-toxin proteins such as hemagglutinin and non-hemagglutinin proteins. In contrast, the process outlined in Figure 1B involves chromatography steps that are all designed to purify *botulinum* toxin complexes.

[0050] Dissociation of the *botulinum* toxin complex to produce the non-complexed *botulinum* toxin protein may be achieved in a number of ways, *e.g.*, as known in the art and/or described herein. For example, dissociation may be achieved by raising the pH to about 7.0; or, in embodiments in which animal protein free

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purification is not necessary, treating the complex with red blood cells at a pH of about 7.3.

[0051] In a preferred embodiment and to provide animal free toxin, the complex is subjected to a separation process based on adjustment pH of the complex in a suitable buffer Suitable buffers include, but are not limited to, cationic buffers, preferably cationic buffers that will not interact or will not substantially interact with the anion exchange column. Suitable cationic buffers include, *e.g.*, Tris, bis-Tris, triethanolamine, N-methyl diethanolamine. A pH of between about 7 to about 8.4; more preferably between about 7.4 to about 8.2; and most preferably a pH of about 7.8 is typically suitable for dissociating the complex to release the non-complexed botulinum toxin. In some particularly preferred embodiments, for example, the pH of the eluent of the hydrophobic interaction column is raised to about 7.5, about 7.8, or preferably to about 8.0. For example, in some embodiments, the eluent may be diluted into a Tris buffer having a pH of about 7.8 to cause the complex to dissociate into individual components, including the about 150 kD non-complexed *botulinum* toxin protein. The resulting mixture comprising dissociated components can then be subjected to one or more additional chromatography purification steps, such as ion exchange chromatography steps designed to capture and further purify the non-complexed toxin.

[0052] In certain embodiments according to the invention, the non-complexed *botulinum* toxin may be purified using one or more ion exchange chromatography steps, (*e.g.*, *see* Figure 1A). Ion exchange chromatography achieves fractionation based on electrostatic charge. The extent to which a given protein binds to the column matrix is a function of the protein's net charge, based on its individual amino acid composition and the charge of the column matrix. Cationic ion exchange columns have net positive charged matrix whereas anionic ion exchange columns have a net negative charged matrix. Bound proteins can be selectively eluted from the column using a solvent (the eluant) containing a charged substance, such as salt ions, which competes with the charged matrix support for binding to the charged proteins. Bound proteins can be thus fractionated on the basis of the strength of their charge. Alternatively, proteins may be eluted by adjusted the pH which may alter the net charge of the protein thereby altering its affinity to the charged matrix.

[0053] According to some preferred embodiments of the invention, the mixture comprising non-complexed *botulinum* toxin is loaded onto an anion exchange

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column (*e.g.*, *see* Figure 1A). Notably, this column captures the *botulinum* toxin in non-complexed form, such that the toxin protein and dissociated non-toxin proteins can be eluted in separate fractions. The column used may be any anion column known in the art suitable for separating charged proteins, non-limiting examples of which include Q Sepharose HP, Q Sepharose Fast Flow, or Q XL Sepharose, commercially available from GE Healthcare Life Sciences. In some particularly preferred embodiments, a Q XL Sepharose column is used. In some embodiments, the method further comprises conditioning the mixture comprising the non-complexed *botulinum* toxin for anion exchange chromatography before loading onto the column. For example, buffer and pH conditions may be determined to optimize yield from the particular column used, as known in the art, based on the teachings provided herein. For loading and use in the column, *e.g.*, suitable buffers include, but are not limited to, cationic buffers, preferably cationic buffers that will not interact or will not substantially interact with the anion exchange column. Suitable cationic buffers include, *e.g.*, Tris, bis-Tris, triethanolamine, N-methyl diethanolamine. For loading and equilibrating the column, a pH of between about 7.2 to about 8.6; more preferably between about 7.4 to about 8.2; and most preferably a pH of about 7.8 may be used..

[0054] For eluting the captured (bound) toxin and other dissociated components from the anion exchange column, a suitable buffer can be used, as known in the art. Examples of suitable buffers include, for example, sodium chloride (NaCl); and potassium chloride (KCl). In some particularly preferred embodiments, an ascending gradient of sodium chloride is used. For example, a sodium chloride buffer having a concentration range from about 0.0 M to about 0.4 M NaCl, more preferably from about 0.0 M to about 0.5 M NaCl, and even more preferably about 0.0 M to about 0.6 M NaCl may be used. Impurities separated in different fractions may include, *e.g.*, one or more non-toxin proteins of the dissociated complex, such as, the non-toxin hemagglutinin and/or non-toxin non-hemagglutinin proteins. Fraction(s) containing a product peak can be identified, as known in the art. The peak may occur, for example, at about 8 mSem to about 22 mSem at a pH between about 7.4 to about 8.4, and preferably at about 7.8, corresponding to about 0.08 M to about 0.18 M NaCl. Conversely, other impurities may elute at about 30 to about 45 mSem, corresponding to about 0.25 M to about 0.35 M NaCl.

[0055] The fraction(s) containing the eluted non-complexed *botulinum* toxin can be identified to provide an eluent comprising a non-complexed *botulinum* toxin.

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The peak may be identified by methods as known in the art, *e.g.*, using HPLC, western blot analysis, ELISA, non-reduced SDS-PAGE, and the like. SDS-PAGE under non-reducing conditions, for example, can identify the about 150 kDa toxin band, whereas other impurities will appear at bands corresponding to smaller molecules. This eluent comprising a non-complexed form may then be subjected to further chromatographic purification steps.

[0056] In one particularly preferred embodiment, toxin purity is assessed by SDS-PAGE. As the skilled artisan will appreciate, SDS-PAGE analysis can be conducted in the absence or presence of agents that cleave disulfide bonds present in the protein (i.e., non-reducing or reducing conditions, respectively). For example, with respect to botulinum toxin type A, the mature and active form of the botulinum toxin type A protein molecule is comprised of two polypeptide chains of 100 kD and 50 kD, respectively, which are held together by non-covalent interactions as well as a disulfide bond. When botulinum toxin type A produced by the inventive process is assayed using non-reducing conditions, the botulinum toxin type A protein molecules migrate as a single protein band of approximately 150 kD and the measured purity is typically greater than 98%. When the botulinum toxin type A protein amount loaded per gel lane is held to be within the dynamic range of the densitometer, then there are few, if any, detectable impurity bands resulting in a measured purity of 100%. When the type A botulinum toxin is overloaded such that the main toxin band is above the dynamic range of the densitometer, then some minor impurity bands may be detectable (as much as 1-2%).

[0057] However, when the SDS-PAGE analysis of botulinum toxin type A is conducted under reducing conditions, then the disulfide bond of the botulinum toxin is cleaved and the botulinum toxin type A protein migrates as two components having molecular weights of 100 kD and 50 kD, respectively,. When the botulinum toxin type A protein is loaded such that the main species are above the dynamic range of the densitometer and the SDS-page is run under reducing conditions then minor impurity species can be more easily detected. For instance, under these conditions there may be as much as 5% of the 150 kD species present due to incomplete proteolytic processing during the fermentation and recovery process. Under these conditions the inventive process yields a toxin product (comprised of the active, cleaved 100 kD and 50 kD polypeptide chains) that is typically greater than 90% of total protein and more likely greater than 95% of total protein. Thus, the reported measured purity of the

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toxin depends on the details of the SDS-PAGE method employed, as described herein. Furthermore, while the foregoing example concerns botulinum toxin type A, the skilled artisan will appreciate that the SDS-PAGE analysis described herein can be readily adapted to assess the purity of other serotypes of botulinum toxin.

[0058] In certain embodiments, the eluent from the anionic column comprising non-complexed *botulinum* toxin is loaded onto a cation exchange column (*see, e.g.* Figure 1A). Notably, this column also captures the *botulinum* toxin in non-complexed form, such that the toxin protein and dissociated non-toxin proteins can be eluted in separate fractions. The column used may be any cation column known in the art suitable for separating proteins, non-limiting examples of which include an SP Sepharose column, including SP Sepharose HP or SP Sephrose Fast Flow; a Mono S column; or a Source-S column, such as a Source-30S column, or preferably a Source-15S column, both commercially available from GE Healthcare Life Sciences. In some embodiments, the method further comprises conditioning the eluent from the anionic exchange columns comprising non-complexed *botulinum* toxin for cation exchange chromatography before loading onto the column. In some preferred embodiments, the pH is adjusted so that the pH of the eluent being loaded on the column allows for efficient binding of the free toxin to the column. For example, the pH can be maintained within a range of from about 4 to about 8, preferably from about 5 to about 7.5, more preferably from about 6 to about 7, and most preferably at about 7. Further, in some embodiments, the eluent from the anionic column can be treated to reduce conductivity before loading onto the cation exchange column, *e.g.*, using a sodium phosphate buffer, a non-limting example of which is a sodium phosphate buffer of about 20 mM NaH_2PO_4. For example, the eluent from the anionic column may contain as much as about 0.15 M NaCl, so that diluting in an about 20 mM NaH_2PO_4 buffer reduces conductivity. In some specific embodiments, conductivity is reduced from about 12 mSem to about 3.3 mSem. Dilution in buffer, dialysis or other methods known in the art also may be used to reduce the conductivity.

[0059] For loading and use in the column, *e.g.*, suitable buffers include, but are not limited to, anionic buffers, preferably anionic buffers that will not interact or will not substantially interact with the cationic exchange column. Suitable anionic buffers include, *e.g.*, as MES, HEPES, and the like, and preferably sodium phosphate buffer. For loading and equibrilating the column, a pH of between about 4 to about 8;

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preferably between about 5 to about 7.5; more preferably from about 6 to about 7; and most preferably a pH of about 6.8 to about 7 may be used.

[0060] For eluting the captured (bound) toxin from the cation exchange column separately from other dissociated non-toxin proteins and other impurities, a suitable buffer can be used, as known in the art. In some particularly preferred embodiments, an ascending gradient of sodium chloride is used. A suitable concentration range for the sodium chloride gradient may be from about 0.0 M to about 1 M NaCl. Other salts that may be used include, *e.g.*, potassium chloride, that may be used at a concentration gradient of about 0.0 M to about 0.5 M KCl. Fraction(s) containing a product peak can be identified, as known in the art. The peak may occur, for example from about 18 to about 25 mSem, corresponding to about 0.3 M to about 0.4 M NaCl, at a pH of about 6.7. That is, the fraction(s) containing the eluted non-complexed *botulinum* toxin can be identified to provide an eluent from the cationic column comprising non-complexed *botulinum* toxin. In particularly preferred embodiments, the eluent from the cationic column represents a non-complexed *botulinum* toxin of high purity, in high yield and having high activity. In contrast, the process outlined in Figure 1B provides a 900 kD *botulinum* toxin type A complex in the final eluent.

Purified Non-Complexed Botulinum Toxin Product

[0061] The methods and systems described herein are useful to provide a non-complexed *botulinum* toxin of high purity, in high yield, and having high activity. *See* Example 1 below. The product is also readily stabilized and conveniently used for the preparation of safe pharmaceutical compositions.

[0062] In some preferred embodiments, the purified non-complexed *botulinum* toxin is at least about 80% pure, preferably at least about 90% pure, more preferably at least about 95% pure, even more preferably at least about 98% pure, and most preferably at least about 99% pure, or even about 100% pure. "Purified non-complexed *botulinum* toxin" refers to a free *botulinum* toxin protein molecule that is isolated, or substantially isolated, from other proteins and impurities, which can otherwise accompany the non-complexed *botulinum* toxin as it is obtained from a culture or fermentation process. A purified non-complexed *botulinum* toxin that is, for example, "80% pure" refers to an isolated or substantially isolated non-complexed *botulinum* toxin protein wherein the toxin protein comprises 80% of total protein

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present as determined by or other suitable analytical methodology, non-limiting examples of which include SDS-PAGE, CE, and HPLC. For example, in some preferred embodiments, the cationic column eluent comprising the non-complexed *botulinum* toxin is at least about 99% pure, and contains less than about 1% of host cell proteins that are not the approximately 150 kD *botulinum* toxin originally present.

[0063] In some preferred embodiments, the purified non-complexed *botulinum* toxin has an activity of at least about 150 LD_{50} units/ng, preferably at least about 180 LD_{50} units/ng, more preferably at least about 200 LD_{50} units/ng, even more preferably at least about 210 LD_{50} units/ng, and most preferably at least about 220 LD_{50} units/ng. One unit of *botulinum* toxin is defined as the LD_{50} upon intraperitoneal injection into female Swiss Webster mice weighing about 18-20 grams each. In other words, one unit of *botulinum* toxin is the amount of *botulinum* toxin that kills 50% of a group of female Swiss Webster mice. "Activity" is used interchangeably herein with related expressions "biological activity", "potency" and "toxicity" to described the action of a *botulinum* toxin.

[0064] In preferred embodiments, the non-complexed *botulinum* toxins obtainable by processes and systems described herein demonstrate biological activity. That is, in preferred embodiments, the biological activity or toxicity of the product is not lost upon purification in accordance with preferred embodiments of the present invention, even though non-toxin proteins natively associated with the toxin protein are removed during purification. In even more preferred embodiments, the potency obtained using a given set of processes and parameters within the scope of the invention is consistent and/or reproducible. For example, the potency measurement can be made with less than about 40% variability, preferably less than about 35% variability, more preferably less than about 30% variability, even more preferably less than about 25% variability, and most preferably less than about 20% variability.

[0065] In some preferred embodiments, the purification process provides the non-complexed *botulinum* toxin in high yield. For example, the yield obtained from 30 L of a fermentation culture may be at least about 30 mg, preferably at least about 40 mg, more preferably at least about 70 mg, even more preferably at least about 80 mg, and most preferably at least about 90 mg, corresponding to a yield of at least about 1 mg/L, preferably at least about 1.3 mg/L, more preferably at least about 2.3 mg/L, even more preferably at least about 2.7 mg/L, and most preferably at least about 3 mg/L, respectively. In even more preferred embodiments, the yield obtained

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using a given set of processes and parameters within the scope of the invention is reproducible. For example, yield can be measured with less than about 40% variability, preferably less than about 35% variability, more preferably less than about 30% variability, even more preferably less than about 25% variability, and most preferably less than about 20% variability.

[0066] In some particularly preferred embodiments, the purified non-complexed *botulinum* toxin is stable during purification using the processes and systems described herein. It has been believed that removal of associated non-toxin proteins from a *botulinum* toxin complex, such as *botulinum* toxin type A complex, results in a markedly unstable *botulinum* toxin product. The instant invention, however, provides methods and systems that can stably isolate free *botulinum* toxin, without associated non-toxin proteins conventionally believed necessary during the purification process to maintain stability, as discussed above.

[0067] In some preferred embodiments, methods and systems described herein provide a non-complexed *botulinum* toxin that requires very few post-chromatography steps, *e.g.*, in terms of maintaining stability during storage, and in terms of applicability to pharmaceutical uses. For example, as known in the art, ammonium sulfate may be added to the free *botulinum* toxin to prepare an ammonium sulfate suspension for storage. The composition comprising free *botulinum* toxin and ammonium sulfate may be readily stored in a refrigerator and later can be readily retrieved for use in pharmaceutical applications. Indeed, the stability, high yield and purity, and high and consistent potency of the toxin obtainable by methods described herein facilitate pharmaceutical use of the purified product, as described in more detail below.

Uses of Purified Non-Complexed Botulinum Toxin

[0068] The non-complexed *botulinum* toxin purified according to this invention can be used in the preparation of pharmaceutical compositions comprising the toxin as an active ingredient for administration to any subject who would receive a benefit from such pharmaceutical compositions. In preferred embodiments, the subjects to be treated are mammals, preferably humans. "Pharmaceutical composition" as used herein refers to a formulation in which an active ingredient can be a *botulinum* toxin. The formulation will contain at least one additional ingredient and be suitable for diagnostic, therapeutic, and/or or cosmetic administration to a

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subject, such as a human patient. The pharmaceutical composition can be liquid or solid; and may be a single or multi-component system, for example a lyophilized composition reconstituted with a diluent such as saline.

[0069] Another aspect of the invention provides for administration of a purified botulinum toxin molecule to a patient. "Administration" as used herein refers to providing a pharmaceutical composition to a subject or patient. The pharmaceutical composition may be administered by, any method known in the art, including *e.g.*, intramuscular (i.m.), intradermal, intranasal, or subcutaneous administration, intrathecal administration, intracranial, intraperitoneal (i.p.) administration, or topical (transdermal) and implantation (*e.g.*, of a slow-release device) routes of administration. In certain preferred embodiments, the purified non-complexed botulinum toxin is administered topically or by injection in compositions as described in U.S. Patent Application Nos. 09/910,432; 10/793,138; 11/072,026; 11/073,307, 11/824,393, and 12/154,982, which are hereby incorporated by reference in their entirety.

[0070] In certain embodiments, compositions comprising non-complexed *botulinum* toxin in an ammonium sulfate suspension can be readily compounded into a pharmaceutical composition. For example, an ammonium sulfate suspension comprising non-complexed *botulinum* toxin protein can be centrifuged to recover the protein and the protein can be re-solubilized, diluted, and compounded with one or more pharmaceutically acceptable excipients. In certain embodiments, the pharmaceutical composition may comprise a non-complexed *botulinum* toxin as an active pharmaceutical ingredient, and may further comprise one or more buffers, carriers, stabilizers, preservatives and/or bulking agents. The pharmaceutical compositions may be lyophilized to powder for storage, and re-constituted for further use. Accordingly, processes and systems described herein can provide a *botulinum* toxin in a form particularly suited to pharmaceutical applications terms of ease of preparation.

[0071] The pharmaceutical composition may find use in therapeutic, diagnostic, research and/or cosmetic applications. For example, as discussed above, *botulinum* toxin type A is clinically used to treat neuromuscular disorders characterized by skeletal muscle hyperactivity, such as essential blepharospasm, strabismus, cervical dystonia, and glabellar line (facial) wrinkles. Moreover, in certain applications, non-complexed (about 150 kD) *botulinum* toxin is the preferred

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form for treating humans. See, *e.g.*, Kohl A., *et al.*, Comparison of the effect of botulinum toxin A (Botox®) with the highly-purified neurotoxin (NT 201) in the extensor digitorum brevis muscle test, Mov Disord 2000; 15(Suppl 3):165. Accordingly, certain *botulinum* toxin pharmaceutical compositions are preferably prepared using non-complexed *botulinum* toxin, as opposed to a *botulinum* toxin complex.

Examples

Example 1: Comparison of Inventive Process with a Modified Schantz Process

[0072] Purifications of non-complexed *botulinum* toxin type A using processes within the scope of the instant invention ("inventive process") were directly compared to purifications based on the traditional Schantz approach, further modified by the addition of chromatographic steps to provide the non-complexed form (Modified Schantz process"). Briefly, *Clostridium botulinum* bacteria were cultured and allowed to grow until fermentation was complete (usually about 72 to about 120 hours from inoculation to harvest). A volume of 30 L of the fermentation culture then was used in each of the following purification procedures.

[0073] The modified Schantz process used involved typical acidification of the fermentation culture to precipitate the toxin, followed by ultramicrofiltration (UF) and diafiltration (DF) to concentrate the raw toxin. DNase and RNase were added to the harvested toxin to digest (hydrolyze) nucleic acids, which were then removed by an additional UF step, using tangential flow filtration (300kD UF). The toxin was next extracted with phosphate buffer, followed by three sequential precipitation steps: cold ethanol precipitation; hydrochloric acid precipitation, and ammonium sulfate precipitation, where the supernatants each time were normally discarded. This procedure provided a 900 kD *botulinum* toxin type A complex, which was then subjected to additional chromatography steps to provide the free toxin. Specifically, the toxin complex was resolubilized and subjected to negative batch adsorption onto a DEAE resin. The eluent was then run on a gravity flow anion exchange column (DEAE-Sepharose), followed by a gravity flow cation exchange column (CM-Sepharose). Yield was determined, the length of time the process took was recorded (not counting the fermentation period), and the purified non-complexed *botulinum* toxin type A was measured for purity by SDS-PAGE analysis and assayed for

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potency, *e.g.*, by techniques known to those skilled in the art. The entire modified Schantz process was repeated for three different lots, lot numbers 1, 2 and 3, and the results recorded in Table 1 below.

[0074] The inventive process was used with three different lots, lot numbers 4, 5 and 6, in accordance with systems and methods described herein. Briefly, the fermentation culture was subjected to acid precipitation using 3M sulfuric acid to reduce pH to 3.5, at a temperature below 25°C. The acid precipitate was then subjected to 0.1 µm tangential flow filtration to concentrate cell mass. The pH then was adjusted to 6 and nucleases added to reduce host cell nucleic acid content, followed by clarification by centrifugation to remove cell debris and dead end filtration at 0.2 µm with added ammonium sulfate. The filtrate was then directly loaded onto the hydrophobic interaction column, Phenyl Sepharose HP (GE Life Sciences), eluted with a descending gradient of ammonium sulfate, and the product peak isolated. The eluent was then diluted into Tris buffer pH 7.8 to dissociate the toxin complex, which then was loaded onto the anion exchange column Q XL Sepharose (GE Lifesciences), eluted with an ascending gradient of sodium chloride, and again the product peak collected. This eluent was then diluted in a sodium phosphate buffer (to reduce conductivity) and loaded onto either the anion exchange column, Q XL Sepharose (for lots # 4 and 5), or the cation exchange column, Source-S (GE Life Sciences) (for lot #6), again eluted with an ascending gradient of sodium chloride, and a final product peak collected and stored. This process yielded non-complexed *botulinum* toxin type A. Yield was determined, the length of process time recorded (not counting the fermentation period), and the toxin measured for purity by SDS-PAGE analysis and assayed for potency, *e.g.*, by techniques known to those skilled in the art. Results also recorded Table 1 below.

Table 1

	Modified Schantz Process			Inventive Process		
Lot #	1	2	3	4	5	6
Process Time	10 days			4 days		
% Purity	99	n/a	97	98.6	95.3	100
Yield (30L scale)	11 mg	0 mg	4 mg	43 mg	99 mg	89 mg
Potency (LD50 Units/ng)	255	n/a	173	259	252	250

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[0075] As Table 1 indicates, there was a lot failure with respect to lot #2 in the modified Schantz process. The total lot failed giving zero yield. There was also a partial lot failure with respect to lot #3. There the failure occurred at the hydrochloric acid precipitation step, but some product was rescued from the normally discarded supernatant. The rescued product was reprocessed with a deviation step, accounting for the observed reduced yield compared with lot #1 (4 mg compared with 11 mg) and the observed reduced potency compared with lot #1 (173 LD50 units/ng compared with 255 LD50 units/ng).

[0076] With respect to the lots used with the inventive process, lot #4 showed a reduced yield, compared to lot #5 for example (43 mg compared with 99 mg) due to a chromatography system failure, involving high salt wash of a column. With the failure, there was premature elution of a portion of the toxin, resulting in the observed reduced yield, but also an observed higher purity (98.6 % purity compared with 95.3% purity).

[0077] Lot #6 represents the results of a highly preferred embodiment of the instant inventive processes and systems, where a cation exchange column was used in the third chromatography step. As Table 1 indicates, this embodiment resulted in improved purity compared with lot #5 for example (100% purity compared with 95.3% purity), while high yield (89 mg compared with 99 mg) and high potency (250 LD50 units/ng compared with 252 LD50 units/ng) were maintained.

[0078] As Table 1 also indicates, the total length of the purification can be shortened in preferred embodiments of the instant invention. For example, lot #6 was purified within only 4 days, compared to the 10 days it took to purify non-complexed *botulinum* toxin using the modified Schantz method that involved three additional chromatography steps after the conventional Schantz method.

[0079] The results indicate that the processes and systems taught herein can be used to prepare high yields of a non-complexed *botulinum* toxin, at high potency and purity, and suggests that methods and systems described herein can find use in large-scale efficient purification of a non-complexed *botulinum* toxin suitable for use, *e.g.*, as an active ingredient in pharmaceutical compositions.

[0080] All references including patent applications and publications cited herein are incorporated herein by reference in their entirety and for all purposes to the

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same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes. Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

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CLAIMS:

What is claimed is

1. A method for purifying a non-complexed *botulinum* toxin, the method comprising:

(i) providing a crude non-complexed *botulinum* toxin;

(ii) loading the crude non-complexed *botulinum* toxin on an anion exchange column so as to permit capture of the non-complexed *botulinum* toxin by the anion exchange column;

(iii) eluting the non-complexed *botulinum* toxin from the anion exchange column to give an eluent comprising the non-complexed *botulinum* toxin;

(iv) loading a cation exchange column with the eluent from the anion exchange column so as to permit capture of the non-complexed *botulinum* toxin by the cation exchange column; and

(v) eluting purified non-complexed *botulinum* toxin from the cation exchange column.

2. The method according to claim 1, wherein the crude non-complexed *botulinum* toxin is obtained by

obtaining a sample comprising *botulinum* toxin complex;

loading a hydrophobic interaction column with the sample so as to permit capture of the *botulinum* toxin complex by the hydrophobic interaction column;

eluting the *botulinum* toxin complex from the hydrophobic interaction chromatography column; and

dissociating the *botulinum* toxin complex to obtain a mixture comprising the crude non-complexed *botulinum* toxin.

3. The method according to claim 2, wherein the sample is a supernatant or filtrate comprising the *botulinum* toxin complex.

4. The method according to claim 2, wherein the sample is obtained by:

subjecting a fermentation culture comprising the *botulinum* toxin to acid precipitation to obtain an acid precipitate; and

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performing tangential flow filtration on the precipitate to concentrate precipitate.

5. The method according to claim 2, wherein the sample is obtained by subjecting an insoluble fraction of a fermentation culture to tangential flow filtration.

6. The method according to claim 2, wherein the sample is subjected to a nuclease digestion before loading on the hydrophobic interaction column.

7. The method according to claim 6, wherein the nuclease is derived from an animal product free process.

8. The method according to claim 1, wherein the method is substantially animal product free.

9. The method according to claim 1, wherein the purified non-complexed *botulinum* toxin comprises at least one of *botulinum* toxin type A, B, C_1, D, F, F and G.

10. The method according to claim 1, wherein the purified non-complexed *botulinum* toxin comprises a *botulinum* toxin type A.

11. The method according to claim 1, wherein the purified non-complexed *botulinum* toxin is at least 95% pure.

12. The method according to claim 1, wherein the purified non-complexed *botulinum* toxin has an activity of at least 200 LD_{50} units/ng.

13. The method according to claim 1, wherein the method produces a yield of at least about 2 mg/ L fermentation culture.

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14. The method according to claim 1 wherein the anionic column is selected from the group consisting of a Q Sepharose HP, Q Sepharose Fast Flow, and Q XL Sepharose column, and wherein the cationic column is selected from the group consisting of a SP Sepharose, SP Sepharose HP, SP Sephrose Fast Flow, Mono S, Source-S, Source-30S, and Source-15S column.

15. The method according to claim 1 wherein a buffer for loading the non-complexed *botulinum* toxin onto the anionic column is selected from the group consisting of Tris, bis-Tris, triethanolamine, and N-methyl diethanolamine.

16. The method according to claim 15 wherein the buffer is used at a pH from 7.4 to 8.2.

17. The method according to claim 1 wherein a buffer for loading the non-complexed *botulinum* toxin onto the cationic column is selected from the group consisting of sodium phosphate, MES, and HEPES.

18. The method according to claim 17 wherein the buffer is used at a pH from 6.0 to 7.0

19. The method according to claim 1 wherein pH of the anionic column is from 7.4 to 8.2.

20. The method according to claim 1 wherein pH of the cationic column is from 6.0 to 7.0.

21. The method according to claim 1 wherein a gradient for eluting the non-complexed *botulinum* toxin from the anionic column is selected from the group consisting of an ascending gradient of sodium chloride and an ascending gradient of potassium chloride.

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22. The method according to claim 21 wherein the gradient is used at a pH from 7.4 to 8.4.

23. The method according to claim 1 wherein a gradient for eluting the non-complexed *botulinum* toxin from the cationic column is selected from the group consisting of an ascending gradient of sodium chloride and an ascending gradient of potassium chloride.

24. The method according to claim 23 wherein the gradient is used at a pH from 6.0 to 7.0.

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Figure 1A

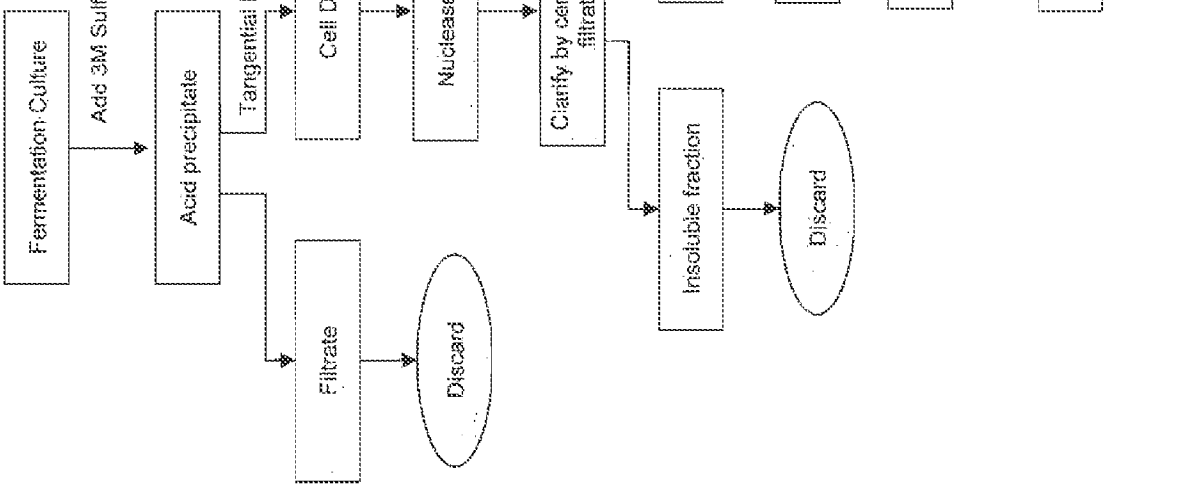


Figure 1B

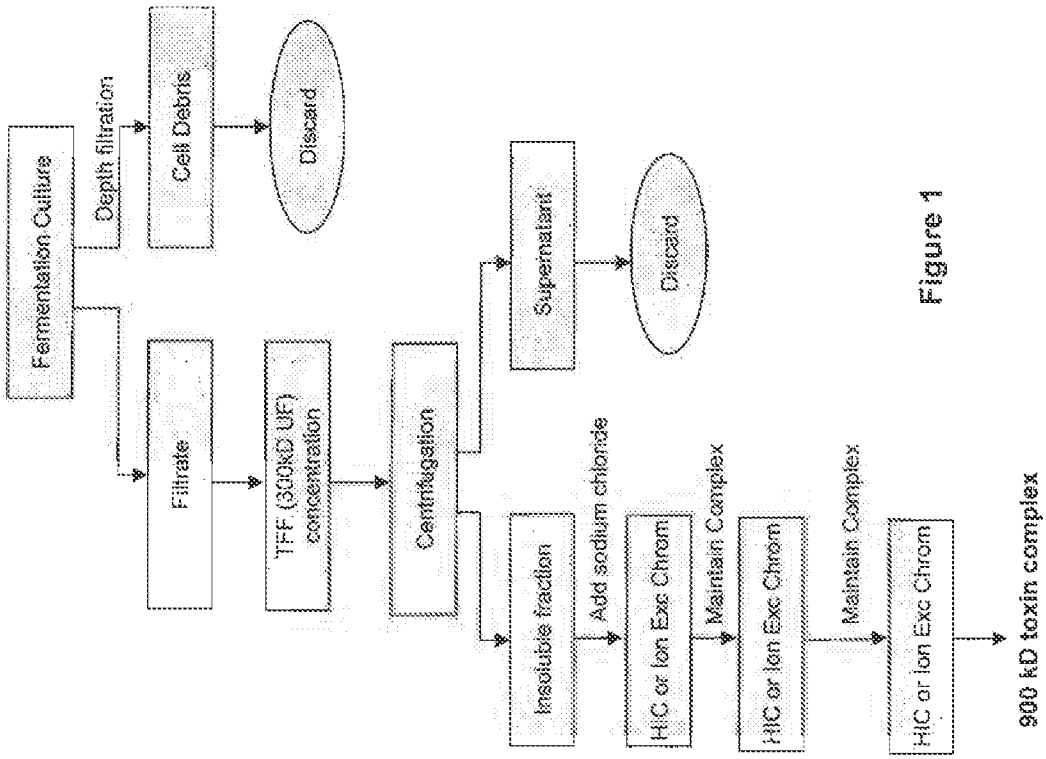


Figure 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/53389

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C02F 1/28; B01D 15/00 (2010.01)
USPC - 210/681, 210/663, 530/412
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8)-C02F 1/28; B01D 15/00 (2010.01)
USPC-210/681; 210/663; 530/412

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 210/683, 685, 660, 600; 530/350, 416, 415

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB); Google Patents, Google Scholar
botulinum toxin, purification, isolation, preparation, chromatography, ion exchange, cation exchange, anion exchange, hydrophobic interaction, filtration, ultrafiltration, tangential flow, tris, bis-tris, mes, phosphate, hepes, animal product free, nuclease

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2006/0228780 A1 (LUO et al.) 12 October 2006 (12.10.2006) (para [0042], [0070], [0093], [0094], [0194], [0200], [0253], [0257], [0262], [0293]-[0296], [0370]-[0376], [0415], [0442], [0461]-[0466], [0474], [0476], [0481], [0483], [0484])	1-5 and 8-24 --- 6, 7
Y	US 2004/0259197 A1 (Suppmann et al.) 23 December 2004 (23.12.2004) (abstract, paras [0029], [0031], [0033])	6, 7
Y	US 2005/0238669 A1 (XIANG et al.) 27 October 2005 (27.10.2005) (para [0058]-[0068], [0105]-[0115])	1-24
Y	US 2003/0008367 A1 (OGUMA) 09 January 2003 (09.01.2003) (para [0010], [0033])	1-24
Y	US 2007/0037257 A1 (SMITH et al.) 15 February 2007 (15.02.2007) (para [0016], [0122]-[0125])	1-24

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
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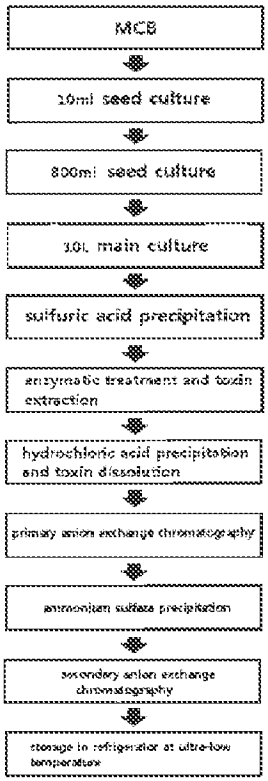
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(54) Title: METHOD FOR PRODUCTION OF BOTULINUM TOXIN



(57) Abstract: The present invention relates to a method for production of a botulinum toxin, and more particularly to a method for preparation of botulinum toxin, the method comprising the steps of: (a) treating a culture of a botulinum toxin-producing strain with acid to precipitate a botulinum toxin; (b) adding buffer to the precipitated botulinum toxin, followed by treatment with a protease inhibitor and nuclease, thereby extracting the botulinum toxin; (c) treating the extracted botulinum toxin with acid to precipitate the botulinum toxin and dissolving the precipitate in buffer; and (d) purifying the botulinum toxin by anion exchange chromatography. The use of the method of the invention toxin makes it possible to produce a high-purity botulinum toxin by a simple process, suggesting that the method is very economical and efficient. The botulinum toxin produced by the method of the invention has high purity compared to botulinum toxins produced by conventional methods, and thus has an increased ability to act in a local area. Thus, the systemic circulation of the botulinum toxin, which can result in side effects, is reduced to increase the safety. Accordingly, the botulinum toxin of the invention can be used for various purposes, including treatment of neuromuscular disorders, removal of wrinkles, and treatment of spastic hemiplegia and cerebral palsy.

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Description

Title of Invention: METHOD FOR PRODUCTION OF BOTULINUM TOXIN

Technical Field

[1] The present invention relates to a method for production of a botulinum toxin, and more particularly to a method for preparation of botulinum toxin, the method comprising the steps of: (a) treating a culture of a botulinum toxin-producing strain with acid to precipitate a botulinum toxin; (b) adding buffer to the precipitated botulinum toxin, followed by treatment with a protease inhibitor and nuclease, thereby extracting the botulinum toxin; (c) treating the extracted botulinum toxin with acid to precipitate the botulinum toxin and dissolving the precipitate in buffer; and (d) purifying the botulinum toxin by anion exchange chromatography.

[2]

Background Art

[3] A variety of Clostridium sp. strains that secrete neurotoxins have been discovered since 1890s, and the characterization of toxins that are secreted from these strains has been made for the past 70 years (Schant, E. J. *et al., Microbiol. Rev.*, 56:80, 1992).

[4] Neurotoxins derived from the *Clostridium sp.* strains, that is, botulinum toxins, are classified into seven types (types A to G) depending on their serological properties. Each of the toxins has a toxin protein having a size of about 150 KDa and naturally contains a complex of several non-toxic proteins. A medium complex (300 kDa) is composed of a toxin protein and a non-toxic non-hemagglutinin protein, and a large complex (450 kDa) and a very large complex (900 kDa) are composed of the medium complex bound to hemagglutinin (Sugiyama, H., *Microbiol. Rev.*, 44: 419, 1980). Such non-toxic hemagglutinin proteins are known to function to protect the toxin from low pH and various proteases in the intestines.

[5] The toxin is synthesized as a single polypeptide having a molecular weight of about 150 kDa in cells, and then cleaved at a position of 1/3 starting from the N-terminal end by the action of intracellular protease or treatment with an artificial enzyme such as trypsin into two units: a light chain (L; molecular weight: 50 kDa) and a heavy chain (H; molecular weight: 100 kDa). The cleaved toxin has greatly increased toxicity compared to the single polypeptide. The two units are linked to each other by a disulfide bond and have different functions. The heavy chain binds to a receptor of a target cell (Park. M.K., *et al., FEMS Microbiol. Lett.*, 72:243, 1990) and functions to interact with a biomembrane at low pH (pH 4) to form a channel (Mantecucco, C. *et al., TIBS.*, 18:324, 1993), and the light chain has pharmacological activity, and thus

imparts permeability to cells using a detergent or interferes with the secretion of a neurotransmitter when introduced into cells by, for example, electroporation (Poulain, B. *et al.*, *Proc. Natl. Acad. Sci. USA.*, 85:4090, 1988).

[6] The toxin inhibits the exocytosis of acetylcholine at the cholinergic presynapse of a neuromuscular junction to cause asthenia. It has been considered that treatment with a very small amount of the toxin exhibits toxicity, suggesting that the toxin has any enzymatic activity (Simpson, L. L. *et al.*, *Ann. Rev. Pharmacol. Toxicol.*, 26:427, 1986).

[7] According to a recent report, the toxin has metallopeptidase activity, and its substrate is composed of synaptobrevin, syntaxin, a synaptosomal associated protein of 25 KDa (SNAP25) or the like, which are the unit proteins of an exocytosis machinery complex. Each type of toxin uses one of the above-described three proteins as its substrate, and it is known that type B, D, F and G toxins cleave synaptobrevin at a specific site, type A and E toxins cleave SNAP25 at a specific site, and type C cleaves syntaxin at a specific site (Binz, T. *et al.*, *J. Biol. Chem.*, 265:9153, 1994).

[8] Particularly, type A botulinum toxin is known to be soluble in a dilute aqueous solution at a pH of 4.0-6.8. It is known that a stable non-toxic protein is separated from neurotoxin at a pH of about 7 or higher, and as a result, the toxicity is gradually lost. Particularly, it is known that the toxicity decreases as pH and temperature increase.

[9] The botulinum toxin is fatal to the human body even in small amounts and is easy to produce in large amounts. Thus, it constitutes four major bio-terror weapons together with Bacillus anthracis, Yersinia pestis and smallpox virus. However, it was found that, when type A botulinum toxin is injected at a dose that does not systematically affect the human body, it can paralyze local muscle in the injected site. Based on this characteristic, type A botulinum toxin can be used in a wide range of applications, including winkle removing agents, agents for treating spastic hemiplegia and cerebral palsy, etc. Thus, the demand for type A botulinum toxin has increased, and studies on methods of producing botulinum toxin so as to satisfy the demand have been actively conducted.

[10] A current typical commercial product is BOTOX® (a purified neurotoxin complex of type A botulinum toxin) that is commercially available from Allergan, Inc., USA. A 100-unit vial of BOTOX® is composed of about 5 ng of a purified neurotoxin complex of type A botulinum toxin, 0.5 mg of human serum albumin and 0.9 mg of sodium chloride and is reconstituted using sterile saline without a preservative (injection of 0.9% sodium chloride). Other commercial products include Dysport® (a complex of *Clostridium* botulinum type A toxin and hemagglutinin, which has lactose and human serum albumin in a pharmaceutical composition containing botulinum toxin and is reconstituted using 0.9% sodium chloride before use) that is commercially available

from Ipsen Ltd., UK, MyoBloc™ (an injectable solution (a pH of about 5.6) comprising botulinum type B toxin, human serum albumin, sodium succinate and sodium chloride) that is commercially available from Solstice Neurosciences, Inc. Conventional methods used to produce botulinum toxins include an acid precipitation method, a precipitation method by salt, and a chromatographic method.

[11] For example, Japanese Patent Laid-Open Publication No. 1994-192296 discloses a method of producing a crystalline botulinum type A toxin by culturing *Clostridium botulinum* bacteria, followed by acid precipitation, extraction, addition of nuclease, and crystallization. Further, US Patent No. 5696077 discloses a method of a crystalline botulinum type B toxin by culturing *Clostridium botulinum* bacteria, followed by acid precipitation, extraction, ion exchange chromatography, gel filtration chromatography and crystallization.

[12] Simpson et al. produced a botulinum type A toxin by purifying botulinum neurotoxin by gravity flow chromatography, followed by HPLC, capture using affinity resin, size exclusion chromatography, and ion (anion and cation) exchange chromatography including the use of two different ion exchange columns (*Method in Enzymology*, 165:76, 1988), and Wang et al. used precipitation and ion chromatography to purify a botulinum type A toxin (*Dermatol Las Faci Cosm Surg.*, 2002:58, 2002).

[13] Moreover, US Patent No. 6818409 discloses the use of ion exchange and lactose columns to purify a botulinum toxin, and US Patent No. 7452697 discloses a botulinum type A toxin by ion exchange chromatography and hydrophobic chromatography. Korean Patent Laid-Open Publication No. 2009-0091501 discloses a method of purifying a botulinum toxin by acid precipitation and anion exchange chromatography, and US Publication No. 2013-0156756 discloses a method of purifying a botulinum toxin by anion exchange chromatography and cation exchange chromatography.

[14] However, the conventional methods have problems in that the use of anion exchange chromatography adversely affects the gel banding pattern of botulinum toxins (US Patent No. 7452697) and in that these conventional methods are difficult to apply commercially, due to a long purification time. In addition, because *Clostridium botulinum* that is a botulinum toxin-producing strain is an anaerobic bacterium, there is a problem in that fermentation of the bacterium should be performed in an anaerobic system, and thus it is difficult to produce botulinum toxins in large amounts. In addition, there is a problem in that the active ingredient botulinum toxin purified by the above-described purification method is not clearly separated and identified, and thus contains impurities. Additionally, the conventional methods for production of botulinum toxins have a problem in that a filtration or dialysis process is necessarily performed to purify a high-purity botulinum toxin, suggesting that the purification process is complex and

difficult.

[15] Accordingly, the present inventors have made extensive efforts to solve the above-described problems occurring in the prior art, and as a result, have found that when a culture of a botulinum toxin-producing strain is treated with acid to form a botulinum toxin precipitate and the formed precipitate is purified by anion exchange chromatography, the steps of filtration, dialysis and ethanol precipitation can be omitted, and the process for production of the botulinum toxin is very easy, and a botulinum toxin having a purity of 98% or higher can be produced by this production method, thereby completing the present invention.

[16]

[17] Summary of Invention

[18] It is an object of the present invention to provide a method of producing a high-purity botulinum toxin by a simple process.

[19] To achieve the above object, the present invention provides a method for production of botulinum toxin, the method comprising the steps of: (a) treating a culture of a botulinum toxin-producing strain with acid to precipitate a botulinum toxin; (b) adding buffer to the precipitated botulinum toxin, followed by treatment with a protease inhibitor and nuclease, thereby extracting the botulinum toxin; (c) treating the extracted botulinum toxin with acid to precipitate the botulinum toxin and dissolving the precipitate in buffer; and (d) purifying the botulinum toxin by anion exchange chromatography.

[20]

Brief Description of Drawings

[21] FIG. 1 is a schematic view showing a process of producing a botulinum toxin according to the present invention.

[22] FIG. 2 shows the results of measuring the purity of a botulinum toxin after performing purification by primary anion exchange chromatography.

[23] FIG. 3 shows the results of measuring the purity of a botulinum toxin after performing purification by secondary anion exchange chromatography.

[24] FIG. 4 shows the results of measuring the increase or decrease in compound muscle action potential amplitude caused by treatment with a botulinum toxin produced by the method of the present invention and BOTOX® (Allergan). N: no injection; S: saline injection; B2: BTX-A-1, two units; D2: BTX-A-2, two units; B4: BTX-A-1, four units; D4: BTX-A-2, four units; B8: BTX-A-1, eight units; and D8: BTX-A-2, eight units.

[25] FIG. 5 shows the results of measuring conduction velocities caused by treatment with a botulinum toxin produced by the method of the present invention and BOTOX® (Allergan). N: no injection; S: saline injection; B2: BTX-A-1, two units; D2: BTX-

A-2, two units; B4: BTX-A-1, four units; D4: BTX-A-2, four units; B8: BTX-A-1, eight units; and D8: BTX-A-2, eight units.

[26]

[27] Detailed Description of Invention and Preferred Embodiments

[28] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Generally, the nomenclature used herein and the experiment methods are those well known and commonly employed in the art.

[29] In one aspect, the present invention is directed to a method for production of botulinum toxin, the method comprising the steps of: (a) treating a culture of a botulinum toxin-producing strain with acid to precipitate a botulinum toxin; (b) adding buffer to the precipitated botulinum toxin, followed by treatment with a protease inhibitor and nuclease, thereby extracting the botulinum toxin; (c) treating the extracted botulinum toxin with acid to precipitate the botulinum toxin and dissolving the precipitate in buffer; and (d) purifying the botulinum toxin by anion exchange chromatography (FIG. 1).

[30] The resulting botulinum toxin produced by the method of the present invention can be stored by various methods, including frozen storage and lyophilized storage.

[31] The method of the present invention may further comprise, after step (d), the steps of: (e) treating an anion exchange chromatography fraction containing the botulinum toxin with ammonium sulfate to form a precipitate, and dissolving the precipitate in buffer; and (f) purifying the botulinum toxin by anion exchange chromatography.

[32] In the present invention, the botulinum toxin-producing strain is preferably *Clostridium botulinum*, but is not limited thereto, and it will be apparent to those skilled in the art that any strain capable of producing a botulinum toxin may be used in the present invention.

[33] As used herein, the term "botulinum toxin" is means to include not only a neurotoxin produced by the *Clostridium botulinum* strain, but also modified, recombinant, hybrid and chimeric botulinum toxins. A recombinant botulinum toxin may have a light chain and/or heavy chain produced by non-Clostridium species in a recombinant manner. In addition, the term "botulinum toxin" as used herein is meant to include botulinum toxin serotypes A, B, C, D, E, F and G, botulinum toxin complexes (i.e., 300, 600 and 900 kDa complexes), and a pure botulinum toxin (i.e., 150 kDa neurotoxic molecule), which are all useful in the practice of the present invention.

[34] As used herein, the term "produced botulinum toxin" means a pure botulinum toxin or a botulinum toxin complex, which is separated or substantially separated from other proteins or impurities that can be accompanied by the botulinum toxin when the botulinum toxin is collected from a culture or fermentation process. Thus, the produced

botulinum toxin has a purity of at least 90%, preferably at least 95%, and most preferably at least 98%. Particularly, the produced botulinum toxin in the present invention may be a botulinum type A toxin protein having a purity of at least 98%.

[35] Culture of the *Clostridium botulinum* strain for producing the botulinum toxin can be performed using a conventional method known in the art and a conventional medium that may be used for culture.

[36] By way of non-limiting example, a medium for culture of *Clostridium botulinum* strain may include a casein hydrolysate, a yeast extract, glucose and the like, and the culture is performed at a temperature of 25 to 40℃ for 90-180 hours, and preferably 100-150 hours.

[37] The acid precipitation of step (a) may be performed by adding acid, preferably sulfuric acid or hydrochloric acid, to a culture of the strain, so that the culture reaches a pH of 3.0-4.5, preferably 3.3-4.0, and most preferably 3.4-3.6, as measured by a pH sensor.

[38] The acid precipitation of step (a) is based on the principle in which the addition of acid to a culture containing many kinds of proteins reduces the pH of the culture while killing botulinum bacteria remaining after culture so that the proteins reach the iso-electric point to precipitate. It includes crystallization in a broad sense, and the precipitation method is a method of roughly separating a desired material in a mixed state, unlike crystallization focused on purifying the desired material with high purity. In the precipitation method, impurities having a structure similar to the desired material are also precipitated. Herein, the pH is adjusted to about 3.0-4.5. The recovery rate of the botulinum toxin increases as the pH decreases. If the pH is 3.0 or lower, it will affect the botulinum toxin itself, and if the pH is 4.5 or higher, the recovery rate of the botulinum toxin will decrease. For these reasons, the pH is preferably within the above-specific range.

[39] Particularly, the pH is most preferably 3.4-3.6, because the recovery rate of the botulinum toxin is the highest in this pH range. When the pH of the botulinum strain culture reaches a suitable range after addition of acid, the acid is added to the culture until the change in the pH no longer appears, and then the culture is allowed to stand at room temperature for 15-30 hours, followed by removal of the supernatant.

[40] Step (b) of extracting the botulinum toxin comprises a step of dissolving the toxin resulting from step (a) in phosphate buffer, preferably sodium phosphate buffer, and removing the precipitate. Herein, the pH of the phosphate buffer is preferably about 4.0 to about 7.0. The resulting pH can be adjusted to 4.5-6.5, preferably 5.0-6.0, by addition of a base, preferably sodium hydroxide, and extraction of the toxin can be performed in the above-specified pH range.

[41] In addition, nuclease that is used in step (b) may be DNase and RNase, and the

protease inhibitor that is used in step (b) is preferably benzamidine HCl, but is not limited thereto, and any material capable of inhibiting protease activity, known in the art, may be used in the present invention. By treatment with nuclease in step (b) of extracting the botulinum toxin, impurities such as DNA and RNA contained in the precipitate formed by acid in step (a) can be degraded. If the step of treatment with the enzyme is carried out for 1.5 hours or less, degradation of DNA and RNA can be insufficient. For this reason, the step of treatment with the enzyme is carried out for 1.5-7 hours, preferably 3-6 hours, and DNase and RNase are preferably added at a concentration of 0.05-1.0 g/ℓ, preferably 0.1-0.5 g/ℓ.

[42] Because the extract obtained by the enzymatic treatment contains the botulinum toxin and a protein having polarity similar to that of the botulinum toxin, a step of precipitating the protein with hydrochloric acid should be performed. Specifically, step (c) of precipitation with hydrochloric acid is preferably performed by centrifuging the enzymatically treated extract, adjusting the pH of the supernatant to a pH of 2.5 to 4.5, preferably 3.0 to 4.0, by addition of hydrochloric acid (HCl), and then precipitating the protein in the supernatant with hydrochloric acid in a refrigerator at 4℃. Particularly, the pH in the step of precipitation with hydrochloric acid is most preferably adjusted to 3.3-3.8 in order to maintain the activity and recovery rate of the toxin at high levels. If the step of precipitation with hydrochloric acid is carried out in the above-described pH range, the titer of the botulinum toxin will be as high as 90% or more, and the coagulation of protein will significantly decrease. Next, the precipitate with hydrochloric acid may be dissolved in buffer, and the subsequent step may be performed.

[43] Step (d) that is the most important step is performed by chromatography using anion exchange resin after completion of the step of precipitation with hydrochloric acid in order to remove most major impurities other than the botulinum type A toxin. The anion exchange resin that is used in step (d) may be resin substituted with a diethylaminoethyl (DEAE) or quaternary ammonium (Q) group, but is not limited thereto. For example, the anion exchange resin may be DEAE-Sephadex as described in US Patent No. 5,696,077, International Patent Publication No. WO96/05222 and US Patent No. 5,846,929. Preferably, it is any one of anion exchange resins having a strongly basic quaternary ammonium group or weakly basic diethylaminoethyl (DEAE) group.

[44] Examples of the strongly basic anion exchange group that can be used in the present invention may include Q Sepharose Fast Flow, Q Sepharose High Performance, Resource Q, Source 15Q, Source 30Q, Mono Q, Mini Q, Capto Q, Capto Q ImpRes, Q HyperCel, Q Cermic HyperD F, Nuvia Q, UNOsphere Q, Macro-Prep High Q, Macro-Prep 25 Q, Fractogel EMD TMAE(S), Fractogel EMD TMAE Hicap (M), Fractogel EMD TMAE (M), Eshmono Q, Toyopearl QAE-550C, Toyopearl SuperQ-650C,

Toyopearl GigaCap Q-650M, Toyopearl Q-600C AR, Toyopearl SuperQ-650M, Toyopearl SuperQ-650S, TSKgel SuperQ-5PW (30), TSKgel SuperQ-5PW (20), or TSKgel SuperQ-5PW, but are not limited thereto and anion exchange resins known in the art may be used.

[45] The column buffer that is used in step (d) may be sodium phosphate buffer or citrate buffer. Preferably, sodium phosphate buffer is used. The concentration of the column buffer is controlled to 30-70 mM, preferably about 40-60 mM. The pH of the column is controlled to about 3.5-7.5, and the flow rate of the mobile phase is controlled to 0.5-5.0 mℓ/min, preferably 1.0-3.0 mℓ/min. Further, the conductivity of the buffer is adjusted to 3-30 mS/cm, and the sample is injected after completion of the equilibration of the column. The toxin is eluted as flow-through, and most major impurities are adsorbed. Specifically, in step (d) of purification by anion exchange chromatography, the botulinum type A toxin is not adsorbed onto the anion exchange resin, and most major impurities are removed by adsorption.

[46] In the present invention, the anion exchange chromatography in step (d) is preferably performed at a pH of 3.5-7.5, preferably 4.5-7.0, and a conductivity of 3-30 mS/cm, preferably 5-20 mS/cm.

[47] In order to completely remove impurities remaining after anion exchange chromatography of step (d), the method for producing the botulinum toxin according to the present invention may, if necessary, further comprise the steps of: (e) treating the anion exchange chromatography fraction containing the botulinum toxin with ammonium sulfate ($(NH_4)_2SO_4$) to form a precipitate, and dissolving the precipitate in buffer; and (f) purifying the botulinum toxin by secondary anion exchange chromatography.

[48] In step (e), the anion exchange chromatography fraction containing the botulinum toxin is treated with ammonium sulfate to form a precipitate, and the formed precipitate is dissolved in buffer. The step of precipitation with ammonium sulfate corresponds to a salting out process in which a salt (ammonium sulfate, etc.) that easily dissolves in water is added to a protein mixture to increase the ionic strength to thereby form a protein precipitate. If a desired protein precipitates mainly upon saturation with 30%(w/v) ammonium sulfate, the desired protein can be precipitated by precipitating out proteins other than the desired protein at a ammonium sulfate saturation concentration of 30% (w/v) or lower, and then adding ammonium sulfate to a saturation concentration of 30% (w/v) and can be collected by centrifugation. The salting out operation is frequently used as initial means. The ammonium sulfate solution that is used in step (e) may have an ammonium sulfate concentration of 10-50% (w/v), preferably 20-40% (w/v).

[49] Next, in step (f), the botulinum toxin can be purified by anion exchange chromatography. Purification of the high-purity botulinum toxin according to the present

invention is mostly performed by anion exchange chromatography (primary anion exchange chromatography) of step (d), and anion exchange chromatography (secondary anion exchange chromatography) in step (f) is performed in order to remove the remaining impurities and may be performed in the same manner as the anion exchange chromatography of step (d).

[50] The resulting fraction containing the botulinum type A protein, obtained by the above-described purification method, may be sterilized and filtered to prepare a crude liquid. The prepared crude liquid may be frozen and stored until use.

[51] In another aspect, the present invention is directed to a botulinum toxin produced by the method of the present invention. The purity of the botulinum toxin purified by HPLC was analyzed, and as a result, it could be seen that the purity of the botulinum toxin after primary anion exchange chromatography was 98.38% (FIG. 2), which is higher than the purity (about 95%) of BOTOX® commercially available from Allergan Inc., suggesting that most impurities were removed in the primary anion exchange chromatography step. Also, it was shown that the purity of the botulinum toxin after secondary anion exchange chromatography was 98.99% (FIG. 3).

[52] Moreover, the method for production of the botulinum toxin according to the present invention has an advantage in that the steps of filtration, dialysis and ethanol precipitation can be omitted, and thus the process for production of the botulinum toxin is easy and simple. Particularly, the method of the present invention has an advantage in that the filtration and ethanol precipitation steps in the method for production of BOTOX® of Allergan Inc. (US Patent Publication No. 2012-0156756; Non-APF method) can be omitted, and thus the number of the purification steps can be reduced (from five steps to three steps) to reduce the period for production of a crude liquid of the botulinum toxin (from 4 weeks to 2 weeks).

[53] In another example of the present invention, an experiment for comparison with the safety of BOTOX® (Allergan, Inc.) was performed in order to confirm the safety of the botulinum type A toxin protein (DWP450) produced by the method of the present invention. As a result, it was shown that the botulinum toxin (DWP450) produced by the method of the present invention showed a NOAEL value of 60 U/kg for females, suggesting that it is two times safer than BOTOX® (Allergan, Inc.) showing a NOAEL value of 30 U/kg. The two-times higher safety of the botulinum toxin produced by the method of the present invention is believed to be because the botulinum toxin of the present invention has high purity, and thus has an increased ability to act in a local area so that the systemic circulation of the botulinum toxin, which can result in side effects, is reduced.

[54] The botulinum toxin produced by the method of the present invention is used as an active ingredient in a pharmaceutical composition. The botulinum toxin can be used

for treatment of neuromuscular disorders characterized by hyperactive skeletal muscles. In addition, it can be used for various conditions, including a headache, a migraine headache, tension headache, a sinus headache, a cervicogenic headache, a sweating disorder, axillary hyperhidrosis, palmar hyperhidrosis, plantar hyperhidrosis, Frey's syndrome, a hyperkinetic skin line, a facial wrinkle, glabellar lines, crow's feet, marionette lines, a nasolabial fold, a skin disorder, achalasia, strabismus, chronic anal fissure, blepharospasm, musculoskeletal pain, fibromyalgia, pancreatitis, tachycardia, prostatic enlargement, prostatitis, urinary retention, urinary incontinence, overactive bladder, hemifacial spasm, tremors, myoclonus, gastrointestinal disorders, diabetes, sialorrhea, detrusor-sphincter dyssynergia, post stroke spasticity, wound healing, juvenile cerebral palsy, smooth muscle spasm, restenosis, a focal dystonia, epilepsy, cervical dystonia, thyroid disorder, hypercalcemia, an obsessive compulsive disorder, arthritic pain, Raynaud's syndrome, striae distensae, peritoneal adhesion, vasospasms, rhinorrhea, muscle contracture, an injured muscle, laryngeal dystonia, writer's cramp and carpel tunnel syndrome.

[55] As used herein, the term "pharmaceutical composition" means a formulation comprising the botulinum toxin as an active ingredient, and the formulation may comprise at least one additional ingredient (excipient) in the pharmaceutical composition in addition to the botulinum neurotoxin active ingredient. The additional ingredient may be selected from the group consisting of albumin, human serum albumin, recombinant human serum albumin, gelatin, sucrose, trehalose, hydroxyethyl starch, collagen, lactose, sucrose sodium chloride, polysaccharide, caprylate, polyvinylpyrrolidone and sodium, but is not limited thereto. Also, a method of preparing a pharmaceutical composition comprising the botulinum toxin as an active ingredient comprises a step of combining the botulinum toxin with an additional ingredient (excipient), and the combining step may be selected from the group of processes consisting of freeze drying, lyophilization and vacuum drying.

[56] Therefore, a pharmaceutical composition is a formulation which is suitable for diagnostic, therapeutic or cosmetic administration (e.g. by intramuscular or subcutaneous injection or by insertion of a depot or implant) to a subject, such as a human patient. The pharmaceutical composition can be: in a lyophilized or vacuum dried condition, a solution formed after reconstitution of the lyophilized or vacuum dried pharmaceutical composition with saline or water, or; as a solution that does not require reconstitution. The active ingredient can be one of the botulinum toxin serotypes A, B, C_1, D, E, F or G or a botulinum toxin, all of which can be made natively by *Clostridial* bacteria. As stated, a pharmaceutical composition can be liquid or solid, for example vacuum-dried. Exemplary methods for formulating a botulinum toxin active ingredient pharmaceutical composition are disclosed in US Patent Publication No. 2003-0118598

published on November 5, 2002.

[57] In another example of the present invention, in order to confirm the effect of the botulinum type A toxin protein (DWP450) produced by the method of the present invention, the compound muscle action potential (CMAP) amplitude and the conduction velocities (tC) were measured. In the experiment, two different botulinum type A toxin proteins, BTX-A-1 (BOTOX®, Allergan Inc., California, USA) and BTX-A-2 (DWP450) produced by Example 2 of the present invention, were used in four divided groups. In group 1, 0.08 ㎖ of sodium chloride (NaCl) was administered to one TA muscle, and another muscle was not treated. In group 2, 0.02 ㎖ of BTX-A-1 (two units) was administered to one TA muscle, and 0.02 ㎖ of BTX-A-2 (two units) was administered to another TA muscle. In group 3, 0.04 ㎖ of BTX-A-1 (four units) was administered to one TA muscle, and 0.04 ㎖ of BTX-A-2 (four units) was administered to another TA muscle. In group 4, 0.08 ㎖ of BTX-A-1 (eight units) was administered to one TA muscle, and 0.08 ㎖ of BTX-A-2 (eight units) was administered to another muscle.

[58] One unit of botulinum toxin is defined as the LD50 upon intraperitoneal injection into female Swiss Webster mice weighing about 18-20 grams each. One unit of botulinum toxin is the amount of botulinum toxin that kills 50% of a group of female Swiss Webster mice.

[59] The compound muscle action potential (CMAP) amplitudes caused by administration of BTX-A-1 and BTX-A-2 were measured, and as a result, at a slow stimulus rate of 2 Hz, the groups administered with BTX-A-1 and BTX-A-2 showed a paralytic effect on the TA muscle (dY) at 3 days, 1 week, 2 weeks, 3 weeks and 4 weeks after administration (FIG. 4A), and at a fast stimulus rate of 20 Hz, the groups administered with BTX-A-1 and BTX-A-2 showed a paralytic effect on the TA muscle (dY) at 3 days, 1 week, 2 weeks, 3 weeks and 4 weeks after administration (FIG. 4B). There was no significant difference ($p < 0.05$) between BTX-A-1 and BTX-A-2 at a slow stimulus rate of 2Hz and a fast stimulus rater of 20 Hz, and the paralytic effect on the TA muscle (dY) in the groups administered with BTX-A-1 and BTX-A-2 was related to the dosage of botulinum toxin administered.

[60] The conduction velocities (tC) caused by administration of BTX-A-1 and BTX-A-2 were measured, and as a result, it was shown that the groups administered with BTX-A-1 and BTX-A-2 did not induce a delay in conduction velocity at a slow stimulus rate of 2 Hz and a fast stimulus rate of 20 Hz(FIG. 5).

[61] Specifically, the botulinum toxin produced by the method of the present invention exhibits an effect similar to that of commercially available BOTOX® (Allergan, Inc.) and is two times safer, because it has higher purity leading to a decrease in the systemic circulation property of the botulinum toxin. Thus, it can be used for various

purposes, including treatment of neuromuscular disorders, removal of wrinkles, and treatment of spastic hemiplegia and cerebral palsy.

[62]

[63] EXAMPLES

[64] Hereinafter, the present invention will be described in further detail with reference to examples. It will be obvious to a person having ordinary skill in the art that these examples are illustrative purposes only and are not to be construed to limit the scope of the present invention.

[65]

[66] Example 1: Culture of *Clostridium botulinum* strain

[67] 1-1: Composition of medium used in culture

[68] a medium having a composition comprising 2% casein hydrolysate, 1% yeast extract, 1% glucose and 0.5% thioglycollate was used for the seed culture and main culture of the Clostridium botulinum strain in order to produce a botulinum toxin.

[69]

[70] 1-2 : Seed culture of *Clostridium botulinum* strain

[71] 20 ㎕ of Clostridium botulinum (the Korean Centers for Disease Control and Prevention Accession No.: 4-029-CBB-IS-001) was inoculated into a culture tube containing 10 ml of a sterile medium having the composition described in Example 1-1 and was subjected to primary seed culture (stationary culture) at 35℃ for 22-30 hours under anaerobic conditions. When the growth of the strain in the primary seed culture was confirmed, 8 ml of the primary seed culture was inoculated into a 1ℓ culture bottle containing 800 ml of a sterile medium having the same medium composition and was subjected to secondary seed culture (stationary culture) at 35℃ for 8-15 hours under anaerobic conditions.

[72]

[73] 1-3: Main culture of *Clostridium botulinum* strain

[74] In order to produce a botulinum toxin by culturing the *Clostridium botulinum* strain, the main culture of the strain was performed. Specifically, 9.3 ℓ of a medium having the composition described in Example 1-1 was prepared and placed in a 10ℓ incubator, followed by sterilization of the medium. Nitrogen was supplied to make anaerobic conditions, and the growth of the strain was performed at a temperature of 35℃ and an agitation speed of 50 rpm.

[75] The strain in the 1ℓ culture bottle subjected to secondary seed culture in Example 1-2 was inoculated into a 10ℓ incubator through an inoculation line connected to the inoculation port of the 10ℓ incubator. The *Clostridium botulinum* strain in the 10ℓ incubator was cultured under the conditions of 35℃ and 50 rpm and the set culture conditions were maintained, checked and recorded. When the strain was cultured for

100 hours or more, the main culture was completed.

[76]

[77] Example 2: Production of botulinum toxin

[78] 2-1: Step of precipitation with sulfuric acid

[79] The step of precipitation with sulfuric acid is a protein separation process in which sulfuric acid is added to a culture containing many kinds of proteins to reduce the pH of the culture while killing botulinum bacteria remaining after culture so that the proteins reach the isoelectric point to precipitate. The main culture was performed as described in Example 1-3 and after completion of the main culture, 5N sulfuric acid was added to the culture by an automatic pump so as to reach a pH of 3.4-3.6 as measured by the pH sensor of the 10ℓ incubator, and then the culture was transferred to a 20ℓ container (AS ONE, Cat. No AS5.372.06) through the harvest line of the incubator, and the 20ℓ container containing the sulfuric acid precipitate was transferred to a biological safety cabinet (BSC). Then, precipitation with sulfuric acid was performed in the BSC.

[80]

[81] 2-2: Enzymatic treatment and toxin extraction

[82] After removal of the supernatant from the sulfuric acid precipitate, 700 ml of 1M sodium phosphate buffer (pH 5.3) was added to the precipitate. Then, the solution was adjusted to a pH of 5.0-6.0 by addition of 5N sodium hydroxide (NaOH). To remove DNA and RNA from the precipitate, 60 ml of 0.4M benzamidine HCl, 1 g of DNase and 1 g of RNase were added to and reacted with the solution for about 5 hours to extract the botulinum toxin.

[83]

[84] 2-3: Hydrochloric acid precipitation and toxin dissolution

[85] The culture including toxin extract was centrifuged at 4℃ at 12000 x g for 15 minutes to separate it into pellets and a supernatant, and the separated supernatant was transferred to a fresh 10ℓ bottle (AS ONE, Cat. No AS5.372.04), and then adjusted to a pH of 3.4-3.6 by addition of 1N hydrochloric acid (HCl) and subjected to hydrochloric acid precipitation in a refrigerator at 4℃. The hydrochloric acid precipitate was centrifuged at 4 ℃ at 12000 x g for 15 minutes, and the supernatant was removed, after which 50 ml of sodium phosphate buffer (pH 6.5) was added to the toxin pellets to dissolve the toxin.

[86]

[87] 2-4: Primary anion exchange chromatography

[88] After completion of the precipitation process, in order to remove most major impurities other than the botulinum type A toxin, chromatography was performed using ion exchange resin. Specifically, anion exchange resin (Toyopearl SuperQ-650M,

Tosoh Bioscience, P/N 17228) was packed into a column, after which the sample that precipitated in Example 2-3 was injected into the column, and the toxin was eluted with 50 mM of sodium phosphate elution buffer. In the primary purification step, the botulinum type A toxin protein was not adsorbed onto the anion exchange resin, and most major impurities were removed by adsorption. For purification of a high-purity botulinum toxin, the sample was maintained at a pH of 4.5-7.0 and a conductivity of 5-20 mS/cm.

[89]

[90] 2-5: Ammonium sulfate precipitation

[91] Fractions containing the botulinum type A toxin protein purified by anion exchange chromatography were collected, and ammonium sulfate was added thereto at a concentration of 20-40% (w/v) to precipitate the botulinum type A toxin protein again. The precipitated botulinum type A toxin protein was dissolved again in 50 mM sodium phosphate (pH 6.5).

[92]

[93] 2-6: Secondary anion exchange chromatography

[94] After completion of the ammonium sulfate precipitation process, in order to remove minor impurities other than the botulinum type A toxin, chromatography using ion exchange resin was performed once more. Specifically, anion exchange resin (Toyopearl SuperQ-650M, Tosoh Bioscience, P/N 17228) was packed into a column, after which the ammonium sulfate precipitate dissolved in Example 2-5 was injected into the column, and the toxin was eluted with 50 mM sodium phosphate elution buffer. At this time, the botulinum type A toxin protein was not adsorbed, and minor impurities were removed by adsorption.

[95] The purification of a high-purity botulinum toxin is mostly achieved in the primary anion exchange chromatography step, and the purification step by secondary anion exchange chromatography is performed in order to remove the remaining impurities. For purification of a high-purity botulinum toxin, the ammonium sulfate precipitate was maintained at a pH of 4.5-7.0 and a conductivity of 5-20 mS/cm.

[96]

[97] 2-7: Preparation of crude liquid

[98] Fractions containing the botulinum type A toxin protein purified by anion exchange chromatography in Example 2-6 were collected, sterilized and filtered through a 0.2㎛ filter to prepare a crude liquid. The prepared crude liquid was stored below -70℃. The produced botulinum type A toxin protein was named "DWP450".

[99]

[100] Example 3: Analysis of purity of purified botulinum toxin

[101] HPLC (e2695, Waters) was performed by SEC (size exclusion chromatography). The

mobile phase used was 100 mM sodium phosphate buffer (pH 6.5), and a TSKgel G4000SWXL (Tosoh Bioscience, P/N 08542) column was connected to a guard column (Tosoh Bioscience, P/N 08543) for P/N 08542, and 20 μg of the botulinum type A toxin protein was loaded into the column and allowed to flow at a rate of 1 mL/min for 30 minutes. As a result, it was shown that the purity of the botulinum toxin after primary anion exchange chromatography was 98.38% (FIG. 2) and the purity of the botulinum toxin after secondary anion exchange chromatography was 98.99% (FIG. 3).

[102]

[103] Example 4: Evaluation of safety of purified botulinum toxin

[104] In order to confirm the safety of the botulinum type A toxin protein (DWP450) purified in Example 2, an experiment for comparison with the safety of BOTOX® (Allergan, Inc.) was performed.

[105] SD (Sprague-Dawley) white male rats were divided into the following five groups, each consisting of 10 males and 10 females: a group administered with 30 U/kg of a test material (DWP450); a group administered with 60 U/kg of the test material; a group administered with 30 U/kg of a comparative material (BOTOX® available from Allergan, Inc.); a group administered with 60 U/kg of the comparative material; and a control group (saline). Each of the test material, the comparative material and saline was administered intramuscularly a total of five times a time a week for 5 weeks.

[106] In the groups administered with 60 U/kg of the test material and 60 U/kg of the comparative material (BOTOX®), five females and five males were additionally added, and a comparative toxicity test was performed for a recovery period of 12 weeks in order to evaluate the reversibility of toxicity (Table 1).

[107]

[108] Table 1

[Table 1]

Evaluation of safety of purified botulinum toxin

Test	Species	Dosage (Units/kg)	NOAEL/MLD (Units/kg)	
			DWP450	Allergan
Comparative toxicity	SD rat	0, 30, 60	Male: <30Female: 60	Male: <30Female: 30

[109]

[110] As a result, it was shown that the botulinum toxin (DWP450) produced by the method of the present invention showed a NOAEL value of 60 U/kg for females, suggesting that it is two times safer than BOTOX® (Allergan, Inc.) showing a NOAEL

value of 30 U/kg. The two-times higher safety of the botulinum toxin of the present invention is believed to be because the botulinum toxin of the present invention had high purity, and thus had an increased ability to act in a local site so that the systemic circulation of the botulinum toxin, which could result in side effects, was reduced.

[111]

[112] Example 5: Examination of effect of purified botulinum toxin

[113] In order to examine the effect of the botulinum type A toxin protein (DWP450) purified in Example 2, the compound muscle action potential (CMAP) amplitudes and the conduction velocities (tC) were measured.

[114] Specifically, 24 SD (Sprague-Dawley) white rats were randomly divided into four groups, each consisting of six rats. Two different botulinum type A toxin proteins, BTX-A-1 (BOTOX®, Allergan Inc., California, USA) and BTX-A-2 (DWP450) purified in Example 2, were used. The two formulations had substantially identical characteristics as shown in Table 2 below, and the botulinum toxin diluted with 0.9% saline was used in all procedures. Rats were anesthetized by intraperitoneal injection of 10 ng/kg of ketamine hydrochloride, and then BTX-A-1 and BTX-A-2 were administered to the tibialis anterior (TA) muscle of the rats.

[115]

[116] Table 2

[Table 2]

Comparison of characteristics between BTX-A-1 and BTX-A-2

	BTX-A-1(Allergan)	BTX-A-2
Clostridium botulinum strain	Wild-type hall	Wild-type hall
Serotype	Botulinum toxin type A	Botulinum toxin type A
Complex molecular weight (kDa)	900	900
Package(units/vial)	100	100
Excipients - Stabilizer	Human serum albumin	Human serum albumin
Excipients - Isotonic agent	sodium chloride	sodium chloride
Form	Vacuum dried	Lyophilized
Storage condition (℃)	2 - 8	2 - 8
Shelf life (months)	36	36 (ongoing)
pH	6.0 ± 0.5	6.0 ± 0.5

[117]

[118] In group 1, 0.08 ㎖ of sodium chloride (NaCl) was administered to one TA muscle, and another TA muscle was not treated. In group 2, 0.02 ㎖ of BTX-A-1 (two units) was administered to one TA muscle, and 0.02 ㎖ of BTX-A-2 (two units) was administered to another TA muscle. In group 3, 0.04 ㎖ of BTX-A-1 (four units) was administered to one TA muscle, and 0.04 ㎖ of BTX-A-2 (four units) was administered to another TA muscle. In group 4, 0.08 ㎖ of BTX-A-1 (eight units) was administered to one TA muscle, and 0.08 ㎖ of BTX-A-2 (eight units) was administered to another muscle.

[119] Level of delay in CMAPs and conduction velocities were measured using CyberAmp380 and Digidata1320 (Axon Instruments. Inc, USA). The electrodes used were attached to the skin using an alligator clip. The negative electrode was attached to the popliteal muscle, and the positive electrode was attached to the retropubic space and the greater trochanter of the femur. The recording electrode was attached to the belly muscle of the tibia anterior muscle, and the reference recording electrode was attached to the left hind calcaneal tendon and the sole.

[120] An electric stimulation of 1-5 mA was applied at a slow stimulus rate of 2 Hz and a fast stimulus rate of 20 Hz. The paralytic effect on the TA muscle was determined by measuring the peak-to-peak amplitude of CMAPs (dY), and the delay in conduction velocity was determined by measuring the time gap between the stimulus point and negative peak point.

[121] Analysis by the electric stimulation was performed before administration of BTX-A-1 and BTX-A-2 and at 3 days, 1 week, 2 weeks, 3 weeks and 4 weeks after administration, and the CMAPs and conduction velocities caused by administration of BTX-A-1 and BTX-A-2 were analyzed by ANOVA using SAS (Version 9.2, SAS Institute Inc., USA).

[122] As a result, it was observed that, at a slow stimulus rate of 2 Hz, the groups administered with BTX-A-1 and BTX-A-2 showed a paralytic effect on the TA muscle (dY) at 3 days, 1 week, 2 weeks, 3 weeks and 4 weeks after administration (FIG. 4A), and at a fast stimulus rate of 20 Hz, the groups administered with BTX-A-1 and BTX-A-2 showed a paralytic effect on the TA muscle (dY) at 3 days, 1 week, 2 weeks, 3 weeks and 4 weeks after administration (FIG. 4B).

[123] There was no significant difference ($p < 0.05$) between BTX-A-1 and BTX-A-2 at a slow stimulus rate of 2Hz and a fast stimulus rater of 20 Hz, and the paralytic effect on the TA muscle (dY) in the groups administered with BTX-A-1 and BTX-A-2 was related to the dosage of botulinum toxin administered.

[124] The conduction velocities (tC) caused by administration of BTX-A-1 and BTX-A-2 were measured, and as a result, it was shown that the groups administered with BTX-A-1 and BTX-A-2 did not induce a delay in conduction velocity at a slow stimulus rate

of 2 Hz and a fast stimulus rate of 20 Hz.

[125] Specifically, it was found that the botulinum toxin produced by the method of the present invention exhibited an effect similar to that of commercially available BOTOX ® (Allergan, Inc.).

[126]

Industrial Applicability

[127] The use of the inventive method for production of a botulinum toxin makes it possible to produce a high-purity botulinum toxin by a simple process, suggesting that the inventive method is very economical and efficient. The botulinum toxin produced by the method of the present invention has high purity compared to botulinum toxins produced by conventional methods, and thus has an increased ability to act in a local area. Thus, the systemic circulation of the botulinum toxin, which can result in side effects, is reduced to increase the safety. Accordingly, the botulinum toxin of the present invention can be used for various purposes, including treatment of neuromuscular disorders, removal of wrinkles, and treatment of spastic hemiplegia and cerebral palsy.

[128] Although the present invention has been described in detail with reference to the specific features, it will be apparent to those skilled in the art that this description is only for a preferred embodiment and does not limit the scope of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

Claims

[Claim 1] A method for production of botulinum toxin, the method comprising the steps of:
(a) treating a culture of a botulinum toxin-producing strain with acid to precipitate a botulinum toxin;
(b) adding buffer to the precipitated botulinum toxin, followed by treatment with a protease inhibitor and nuclease, thereby extracting the botulinum toxin;
(c) treating the extracted botulinum toxin with acid to precipitate the botulinum toxin and dissolving the precipitate in buffer; and
(d) purifying the botulinum toxin by anion exchange chromatography.

[Claim 2] The method of claim 1, wherein the botulinum toxin-producing strain is *Clostridium botulinum*.

[Claim 3] The method of claim 1, wherein the purified botulinum toxin is a botulinum type A toxin protein having a purity of at least 98%.

[Claim 4] The method of claim 1, wherein the acid precipitation of step (a) is performed by adding sulfuric acid or hydrochloric acid, to the culture of the strain, so that the culture reaches a pH of 3.0-4.5.

[Claim 5] The method of claim 1, wherein the protease inhibitor in step (b) is benzamidine HCl.

[Claim 6] The method of claim 1, wherein the nuclease in step (b) is DNase and RNase.

[Claim 7] The method of claim 1, wherein the extraction of the botulinum toxin in step (b) is performed at a pH of 4.5-6.5.

[Claim 8] The method of claim 1, wherein the acid precipitation of step (c) is performed by adding sulfuric acid or hydrochloric acid, to the extracted botulinum toxin, so that the extracted botulinum toxin reaches a pH of 2.5-4.5.

[Claim 9] The method of claim 1, wherein the buffer in step (c) is sodium phosphate buffer.

[Claim 10] The method of claim 1, wherein the anion exchange chromatography in step (d) is performed at a pH of 3.5-7.5, and a conductivity of 3-30 mS/cm.

[Claim 11] The method of any one of claims 1 to 10, further comprising, after step (d), the steps of:
(e) treating the anion exchange chromatography fraction containing the botulinum toxin with ammonium sulfate to form a precipitate, and

dissolving the precipitate in buffer; and

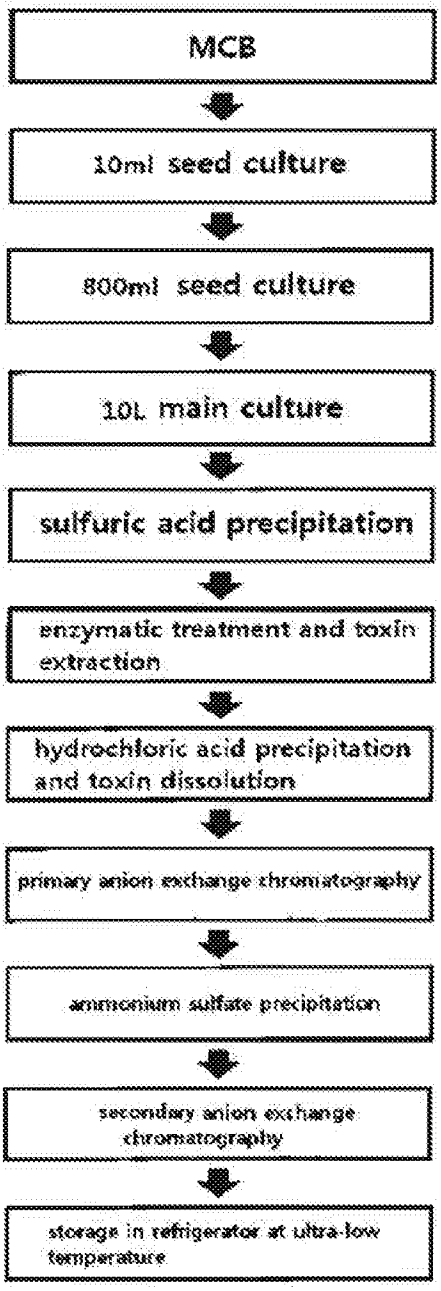
(f) purifying the botulinum toxin by anion exchange chromatography.

[Claim 12] The method of claim 11, wherein the ammonium sulfate in step (e) is added at a concentration of 10-50% (w/v).

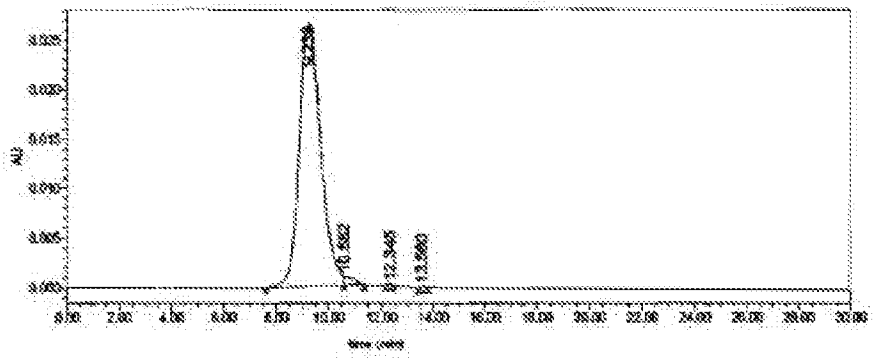
[Claim 13] The method of claim 11, wherein the buffer in step (e) is sodium phosphate buffer.

[Claim 14] The method of claim 11, wherein the anion exchange chromatography in step (e) is performed at a pH of 3.5-7.5, and a conductivity of 3-30 mS/cm.

[Fig. 1]

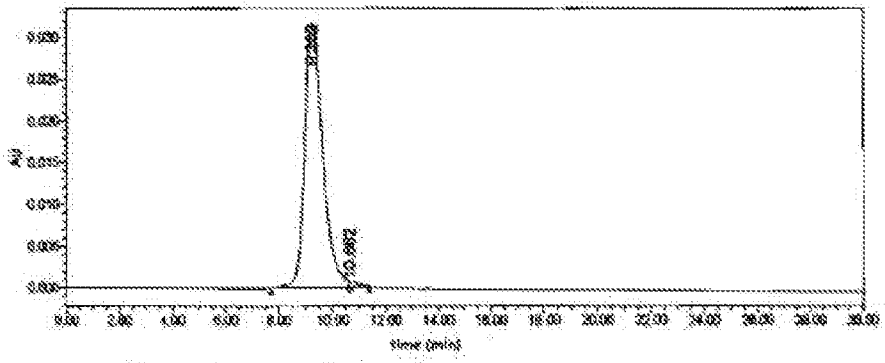

MCB
10ml seed culture
800ml seed culture
10L main culture
sulfuric acid precipitation
enzymatic treatment and toxin extraction
hydrochloric acid precipitation and toxin dissolution
primary anion exchange chromatography
ammonium sulfate precipitation
secondary anion exchange chromatography
storage in refrigerator at ultra-low temperature

[Fig. 2]



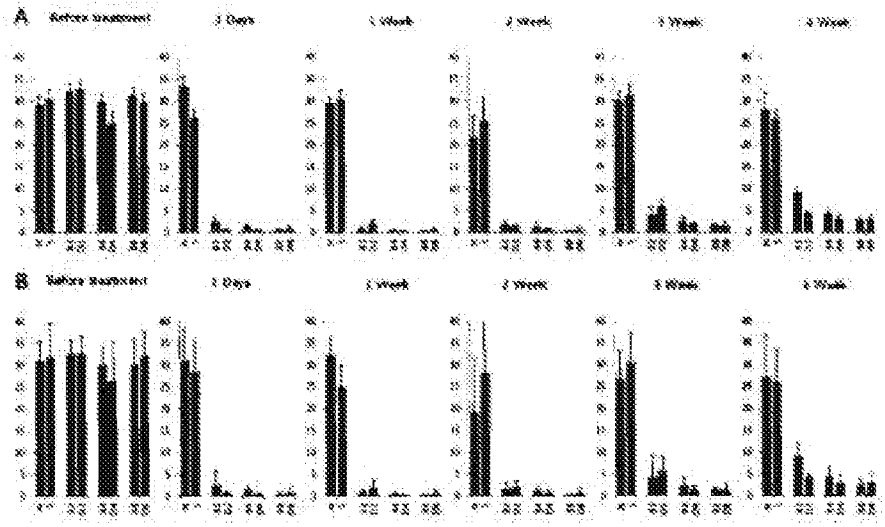
	retention time (min)	area (μV*sec)	% area
1	9.236	1506378	98.36
2	10.582	23129	1.51
3	12.345	744	0.05
4	13.580	936	0.06

[Fig. 3]

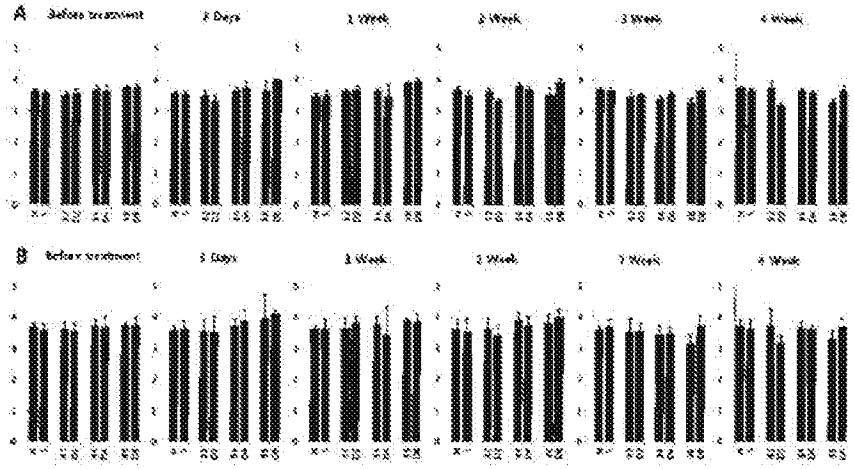


	retention time (min)	area (μV*sec)	% area
1	9.252	1488508	98.99
2	10.662	15187	1.01

[Fig. 4]



[Fig. 5]


A
Before treatment
3 Days
1 Week
2 Week
3 Week
4 Week
B
Before treatment
3 Days
1 Week
2 Week
3 Week
4 Week

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2014/004003

A. CLASSIFICATION OF SUBJECT MATTER

C07K 1/36(2006.01)i, C07K 1/30(2006.01)i, C07K 1/18(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K 1/36; C12P 21/00; C12P 21/04; C02F 1/28; C12N 1/00; C12P 21/02; C12N 1/20; B01D 15/08; C07K 1/30; C07K 1/18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: botulinum toxin, purification

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KR 10-2003-0060150 A (MEDYTOX, INC.) 16 July 2003 See claim 1.	1-14
Y	WO 2011-068072 A1 (REVANCE THERAPEUTICS, INC.) 09 June 2011 See paragraph [0041].	1-14
A	KR 10-2009-0091503 A (MEDYTOX, INC.) 28 August 2009 See claim 1.	1-14
A	KR 10-2011-0091492 A (MEDYTOX, INC.) 11 August 2011 See paragraphs [0028]-[0041].	1-14
A	US 7452697 B2 (LIU, SHIGUANG et al.) 18 November 2008 See abstract.	1-14

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
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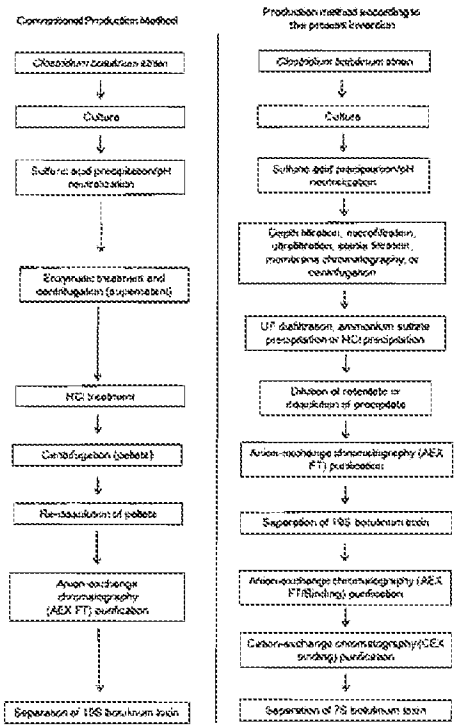
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[Continued on next page]

(54) Title: METHOD FOR PRODUCING BOTULINUM TOXIN



(57) Abstract: The present invention relates to a method for producing a botulinum toxin comprising: (a) treating a culture of a botulinum toxin-producing strain with acid to form a botulinum toxin-containing precipitate; (b) adding a buffer to the botulinum toxin-containing precipitate of step (a), followed by clarification by at least one method selected from the group consisting of depth filtration (DF), microfiltration (MF), ultrafiltration (UF), sterile filtration, membrane chromatography (MC) and centrifugation; (c) subjecting the botulinum toxin-containing solution of step (b) to UF diafiltration, ammonium sulfate precipitation or hydrochloric acid precipitation, and then diluting a retentate resulting from the UF diafiltration in a buffer or dissolving a precipitate resulting from the ammonium sulfate precipitation or hydrochloric acid precipitate in a buffer; and (d) subjecting the retentate dilution, ammonium sulfate precipitate solution or hydrochloric acid precipitate solution of step (c) to anion-exchange chromatography (AEX) to purify the botulinum toxin.

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Description

Title of Invention: METHOD FOR PRODUCING BOTULINUM TOXIN

Technical Field

[1] The present invention relates to a method for producing a botulinum toxin by an animal product-free (APF) process, and more particularly, to a method for producing a botulinum toxin, comprising the steps of:(a) treating a culture of a botulinum toxin-producing strain with acid to form a botulinum toxin-containing precipitate; (b) adding a buffer to the botulinum toxin-containing precipitate of step (a), and then clarifying by one or more method selected from the group consisting of depth filtration (DF), microfiltration (MF), ultrafiltration (UF), sterile filtration, membrane chromatography (MC) and centrifugation; (c) subjecting the botulinum toxin-containing solution of step (b) to UF diafiltration, ammonium sulfate precipitation or hydrochloric acid precipitation, and then diluting a retentate resulting from the UF diafiltration in a buffer or dissolving a precipitate resulting from the ammonium sulfate precipitation or hydrochloric acid precipitate in a buffer; and (d) subjecting the retentate dilution, ammonium sulfate precipitate solution or hydrochloric acid precipitate solution of step (c) to anion-exchange chromatography (AEX), thereby purifying the botulinum toxin.

[2]

Background Art

[3] A variety of *Clostridium* sp. strains that secrete neurotoxic toxins have been discovered since 1980s, and the characterization of toxins that are secreted from these strains has been made for the past 70 years.

[4] Neurotoxic toxins derived from the *Clostridium* sp. strains, that is, botulinum toxins, are classified into eight types (types A to H) depending on their serological properties. Each of the toxins has a toxin protein having a size of about 150 kDa and naturally contains a complex of several non-toxic proteins bound thereto. A medium complex (300 kDa) is composed of a toxin protein and a non-toxic non-hemagglutinin protein, and a large complex (450 kDa) and a very large complex (900 kDa) are composed of the medium-sized complex bound to hemagglutinin (Sugiyama, H., *Microbiol.* Rev., 44:419, 1980). Such non-toxic proteins are known to function to protect the toxin from low pH and various proteases in the intestines.

[5] The toxin is synthesized as a single polypeptide having a molecular weight of about 150 kDa in cells, and then cleaved at a position of 1/3 starting from the N-terminal end by the action of intracellular protease or treatment with an artificial enzyme such as trypsin into two units: a light chain (L; molecular weight: 50 kDa) and a heavy chain

(H; molecular weight: 100 kDa). The cleaved toxin has greatly increased toxicity compared to the single polypeptide. The two units are linked to each other by a disulfide bond and have different functions. The heavy chain binds to a receptor of a target cell (Park. M.K. *et al.*, FEMS Microbiol. Lett., 72:243, 1990) and functions to interact with a biomembrane at low pH (pH 4) to form a channel (Mantecucco, C. *et al.*, TIBS., 18:324, 1993), and the light chain has pharmacological activity, and thus imparts permeability to cells using a detergent or interferes with the secretion of a neurotransmitter when introduced into cells by, for example, electroporation (Poulain, B. *et al., Proc. Natl. Acad. Sci. USA*., 85:4090, 1988).

[6] The toxin inhibits the exocytosis of acetylcholine at the cholinergic presynapse of a neuromuscular junction to cause asthenia. It has been considered that even treatment with a very small amount of the toxin exhibits toxicity, suggesting that the toxin has any enzymatic activity (Simpson, L. L. *et al., Ann. Rev. Pharmacol. Toxicol.*, 26:427, 1986).

[7] According to a recent report, the toxin has metallopeptidase activity, and its substrates include composed of synaptobrevin, syntaxin, a synaptosomal associated protein of 25 KDa (SNAP25), etc., which are the unit proteins of an exocytosis machinery complex. Each type of toxin uses one of the above-described three proteins as its substrate, and it is known that type B, D, F and G toxins cleave synaptobrevin at a specific site, type A and E toxins cleave SNAP25 at a specific site, and type C cleaves syntaxin at a specific site (Binz, T. *et al., J. Biol. Chem.*, 265:9153, 1994).

[8] Particularly, botulinum toxin type A is known to be soluble in a dilute aqueous solution at a pH of 4.0-6.8. It is known that a stable non-toxic protein is separated from neurotoxin at a pH of about 7 or higher, and as a result, the toxicity is gradually lost. Particularly, it is known that the toxicity decreases as pH and temperature increase.

[9] The botulinum toxin is fatal to the human body even in small amounts and is easy to produce in large amounts. Thus, it constitutes four major bio-terror weapons together with *Bacillus* anthracis, *Yersinia* pestis and smallpox virus. However, it was found that, when botulinum toxin type A is injected at a dose that does not systematically affect the human body, it can paralyze local muscle in the injected site. Based on this characteristic, botulinum toxin type A can be used in a wide range of applications, including wrinkle removing agents, agents for treating spastic hemiplegia and cerebral palsy, etc. Thus, the demand for botulinum toxin type A has increased, and studies on methods of producing botulinum toxin so as to satisfy the demand have been actively conducted.

[10] A current typical commercial product is BOTOX® (a purified neurotoxin complex of botulinum toxin type A) that is commercially available from Allergan, Inc., USA. A 100-unit vial of BOTOX® is composed of about 5 ng of a purified neurotoxin complex of botulinum toxin type A, 0.5 mg of human serum albumin and 0.9 mg of sodium

chloride and is reconstituted using sterile saline without a preservative (injection of 0.9% sodium chloride). Other commercial products include Dysport® (a complex of *Clostridium* botulinum toxin type A and hemagglutinin, which has lactose and human serum albumin in a pharmaceutical composition containing botulinum toxin and is re-constituted using 0.9% sodium chloride before use) that is commercially available from Ipsen Ltd., UK and MyoBloc® (an injectable solution (a pH of about 5.6) comprising botulinum toxin type B, human serum albumin, sodium succinate and sodium chloride) that is commercially available from Solstice Neurosciences, Inc.

[11] Conventional methods used to produce botulinum toxins include an acid precipitation method, a method of precipitation with salt, and a chromatographic method.

[12] For example, Japanese Unexamined Patent Application Publication No. 1994-192296 discloses a method of producing a crystalline botulinum toxin type A by culturing *Clostridium* botulinum bacteria, followed by acid precipitation, extraction, addition of nuclease, and crystallization. Further, U.S. Patent No. 5696077 discloses a method of producing a crystalline botulinum toxin type B by culturing *Clostridium* botulinum bacteria, followed by acid precipitation, extraction, ion exchange chromatography, gel filtration chromatography and crystallization.

[13] Simpson et al. produced a botulinum toxin type A by purifying botulinum neurotoxin by gravity flow chromatography, followed by HPLC, capture using affinity resin, size exclusion chromatography, and ion (anion and cation) exchange chromatography including the use of two different ion exchange columns (Method in Enzymology, 165:76, 1988), and Wang et al. used precipitation and ion chromatography to purify a botulinum toxin type A (Dermatol Las Faci Cosm Surg., 2002:58, 2002).

[14] Moreover, U.S. Patent No. 6818409 discloses the use of ion exchange and lactose columns to purify a botulinum toxin, and U.S. Patent No. 7452697 discloses a method of preparing a botulinum toxin type A by ion exchange chromatography and hydrophobic chromatography. Korean Patent Unexamined Patent Application Publication No. 2009-0091501 discloses a method of purifying a botulinum toxin by acid precipitation and anion exchange chromatography, and U.S. Patent Publication No. 2013-0156756 discloses a method of purifying a botulinum toxin by anion exchange chromatography and cation exchange chromatography.

[15] However, the conventional methods have problems in that the use of anion exchange chromatography adversely affects the gel banding pattern of botulinum toxins (U.S. Patent No. 7452697) and in that these conventional methods are difficult to apply commercially, due to a long purification time. In addition, because *Clostridium* botulinum that is a botulinum toxin-producing strain is an anaerobic bacterium, there is a problem in that fermentation of the bacterium should be performed in an anaerobic system, and thus it is difficult to produce botulinum toxins in large amounts. In addition, there is a

problem in that the active ingredient botulinum toxin purified by the above-described purification method is not clearly separated and identified, and thus contains impurities. Additionally, the conventional methods for producing botulinum toxins have a problem in that a filtration or dialysis process is necessarily performed to purify a high-purity botulinum toxin, suggesting that the purification process is complex and difficult.

[16] In addition, in conventional processes for producing botulinum toxin, enzymes such as DNase or RNase were used to remove nucleic acids such as DNA or RNA (see, e.g., Korean Patent No. 10-1339349, and a conventional method for producing botulinum toxin as shown in FIG. 1). However, because enzymes such as DNase or RNase are of animal origin, these enzymes have the potential to contain various disease-causing substances, particularly abnormal prion proteins of animal origin known to cause transmissible spongiform encephalopathy, and thus have problems in terms of safety.

[17] Transmissible spongiform encephalopathy (TSE) is known as a neurodegenerative disorder causing serious degeneration of neurons, and examples thereof includes bovine spongiform encephalopathy (BSE), Scrapie, Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker syndrome, Kuru, transmissible mink encephalopathy, chronic wasting disease, feline spongiform encephalopathy, etc., which affect humans and animals. It was reported that BSE crosses the species barrier and affects even humans.

[18] The agent that causes transmissible spongiform encephalopathy (TSE) has characteristics in that it has no immunogenicity and the incubation period is long. From histopathological analysis of BSE-affected bovine brain tissue, it can be seen that special spongiform vacuoles were formed in the brain due to damage to neurons and deposition of abnormal protein fibers.

[19] The cause of TSE is an infectious protein known as the abnormal prion. Unlike general viruses that require nucleic acid, the abnormal prion is an infectious particle composed of protein alone without containing nucleic acid. Regarding TSE, it is known that, when an abnormal prion (PrP^{sc}) that is an infectious particle binds to a normal prion (PrP^{c}), it is converted to a pathogenic prion which is then accumulated in the brain (Prusiner SB, Alzheimer Dis Assoc Disord., 3:52-78, 1989).

[20] Creutzfeldt-Jakob disease is a rare neurodegenerative disorder of human transmissible spongiform encephalopathy (TSE) where the transmissible agent is apparently an abnormal isoform of a prion protein. An individual with Creutzfeldt-Jacob disease can deteriorate from apparent perfect health to akinetic mutism within six months. Thus, a potential risk may exist of acquiring a prion mediated disease, such as Creutzfeldt-Jacob disease, from the administration of a pharmaceutical composition which contains a biologic, such as a *botulinum* toxin, obtained using animal-derived

products. Thus, if a pharmaceutical composition is prepared using drug substance produced using animal-derived components, it can subject the patient to a potential risk of receiving various qpathogens or infectious agents.

[21] Thus, a method of producing botulinum toxin by an animal product-free (APF) process is urgently needed to solve safety problems such as transmissible spongiform encephalopathy infection caused by such animal-derived components worldwide.

[22] Under such a technical background, the present inventors have made extensive efforts to develop a method capable of preventing the risk of exposure to prion-mediated disease (transmissible spongiform encephalopathy (TSE)) and increasing the purity of botulinum toxin, and as a result, have found that when a culture of a botulinum toxin-producing strain is treated with acid to form a botulinum toxin precipitate and the formed botulinum toxin precipitate is clarified using at least one technique selected from the group consisting of depth filtration (DF), microfiltration (MF), ultrafiltration (UF), sterile filtration, membrane chromatography (MC) and centrifugation, which are pretreatment processes, followed by performing a process selected from among UF diafiltration, ammonium sulfate precipitation and hydrochloric acid precipitation, followed by purification using anion/cation-exchange chromatography, an enzymatic treatment step that uses animal products can be omitted to eliminate the risk of causing prion-mediated disease, and the purity of the botulinum toxin can be increased, thereby completing the present invention.

[23]

[24]

[25] **Disclosure of Invention**

[26] **Technical Problem**

[27] It is an object of the present invention to provide a more efficient and safe method for producing botulinum toxin, which can be performed under animal product-free (APF) conditions.

[28] More specifically, an object of the present invention is to provide a method for producing botulinum toxin, in which an enzymatic treatment step using animal products (APs) is replaced by clarification of the botulinum toxin using at least one technique selected from the group consisting of depth filtration (DF), microfiltration (MF), ultrafiltration (UF), sterile filtration, membrane chromatography (MC) and centrifugation, which are pretreatment processes, followed by performing one process selected from among UF diafiltration, ammonium sulfate precipitation and hydrochloric acid precipitation, followed by further purification using anion/cation-exchange chromatography, thereby ensuring safety with very high yield.

[29]

[30] **Technical Solution**

[31] To achieve the above object, the present invention provides a method for producing a botulinum toxin, comprising the steps of:

[32] (a) treating a culture of a botulinum toxin-producing strain with acid to form a botulinum toxin-containing precipitate;

[33] (b) adding a buffer to the botulinum toxin-containing precipitate of step (a), and then clarifying by one or more method selected from the group consisting of depth filtration (DF), microfiltration (MF), ultrafiltration (UF), sterile filtration, membrane chromatography (MC) and centrifugation;

[34] (c) subjecting the botulinum toxin-containing solution of step (b) to UF diafiltration, ammonium sulfate precipitation or hydrochloric acid precipitation, and then diluting a retentate resulting from the UF diafiltration in a buffer, or dissolving a precipitate resulting from the ammonium sulfate precipitation or hydrochloric acid precipitate in a buffer; and

[35] (d) subjecting the retentate dilution, ammonium sulfate precipitate solution or hydrochloric acid precipitate solution of step (c) to anion-exchange chromatography (AEX), thereby purifying the botulinum toxin.

[36]

[37] **Brief Description of the Drawings**

[38] FIG. 1 is a schematic diagram comparing a process for producing a botulinum toxin according to the present invention with a conventional process.

[39] FIG. 2 shows a chromatogram of first anion-exchange chromatography purification (AEX) step and second anion-exchange chromatography purification (AEX) step for purifying a botulinum toxin.

[40] FIG. 3 shows the results of analyzing the purity of a botulinum toxin by SDS-PAGE after first anion-exchange chromatography purification (AEX) step and second anion-exchange chromatography purification (AEX) step.

[41] FIG. 4 shows a chromatogram of first anion-exchange chromatography purification (AEX), second anion-exchange chromatography purification (AEX, binding mode) and cation-exchange chromatography (CEX) steps for a botulinum toxin.

[42] FIG. 5 shows the results of analyzing the purity of a botulinum toxin by SDS-PAGE after first anion-exchange chromatography purification (AEX), second anion-exchange chromatography purification (AEX, binding mode) and cation-exchange chromatography (CEX) steps.

[43] FIG. 6 shows a chromatogram of first anion-exchange chromatography purification (AEX), second anion-exchange chromatography purification (AEX, flow through mode) and cation-exchange chromatography (CEX) steps for a botulinum toxin.

[44] FIG. 7 shows the results of analyzing the purity of a botulinum toxin by SDS-PAGE after first anion-exchange chromatography purification (AEX), second anion-exchange

chromatography purification (AEX, flow through mode) and cation-exchange chromatography (CEX) steps.

[45]

[46] **Best Mode for Carrying Out the Invention**

[47] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Generally, the nomenclature used herein and the experiment methods, which will be described below, are those well known and commonly employed in the art.

[48] In the present invention, in order to develop an APF (animal product-free) and low-molecular-weight botulinum toxin purification process, it was attempted to develop a process to replace the enzymatic treatment step of a conventional process, which uses animal products (APs). Thus, filtration using a depth filter, a conventional hydrochloric acid precipitation process, a process using ammonium sulfate and a process using a UF system were performed, and as a result, it was shown that the purity of the resulting botulinum toxin was higher than that of a botulinum toxin produced by the conventional method. This suggests that the use of the above-described method according to the present invention makes it possible to produce a high-purity botulinum toxin from a culture of a strain under TSE-free conditions in a safe manner.

[49] The terms "process" and "separation", as used interchangeably herein, refer to the use of at least one method or system to achieve a specific purpose (for example, botulinum toxin purification) in a purification process.

[50] In an example of the present invention, when clarification was performed by at least one method selected from the group consisting of depth filtration (DF), microfiltration (MF), ultrafiltration (UF), sterile filtration, membrane chromatography (MC) and centrifugation, followed by UF diafiltration, ammonium sulfate precipitation or acid precipitation, a botulinum toxin having satisfactory purity could be obtained even by an anion-exchange chromatography (AEX) process alone.

[51] Therefore, in one aspect, the present invention is directed to a method for producing a botulinum toxin, and more particularly, to a method for producing a botulinum toxin, comprising the steps of:

[52] (a) treating a culture of a botulinum toxin-producing strain with acid to form a botulinum toxin-containing precipitate;

[53] (b) adding a buffer to the botulinum toxin-containing precipitate of step (a), and then clarifying by one or more method selected from the group consisting of depth filtration (DF), microfiltration (MF), ultrafiltration (UF), sterile filtration, membrane chromatography (MC) and centrifugation;

[54] (c) subjecting the botulinum toxin-containing solution of step (b) to UF diafiltration,

ammonium sulfate precipitation or hydrochloric acid precipitation, and then diluting a retentate resulting from the UF diafiltration in a buffer, or dissolving a precipitate resulting from the ammonium sulfate precipitation or hydrochloric acid precipitate in a buffer; and

[55] (d) subjecting the retentate dilution, ammonium sulfate precipitate solution or hydrochloric acid precipitate solution of step (c) to anion-exchange chromatography (AEX), thereby purifying the botulinum toxin.

[56] The botulinum toxin purified according to the production method of the present invention may have a form of 7S or 19S (molecular weight: 150-900 kDa).

[57] In the present invention, the botulinum toxin-producing strain is preferably *Clostridium* botulinum or a mutant strain thereof, but is not limited thereto, and it will be obvious to those skilled in the art that any strain capable of producing a botulinum toxin may be used.

[58] As used herein, the term "botulinum toxin" is meant to include not only neurotoxins (NTXs) produced by the *Clostridium* botulinum strain or a mutant strain thereof, but also modified, recombinant, hybrid and chimeric botulinum toxins. A recombinant botulinum toxin may have a light chain and/or heavy chain produced by non-Clostridium species in a recombinant manner. In addition, the term "botulinum toxin" as used herein is meant to include botulinum toxin serotypes A, B, C, D, E, F and G, botulinum toxin complexes (i.e., 300, 600 and 900 kDa complexes), and a pure botulinum toxin (i.e., 150 kDa neurotoxic molecule), which are all useful in the practice of the present invention.

[59] NTXs (7S) that are the main components of botulinum toxins are associated with nontoxic components in cultures or in foods, and become large complexes (Oguma et al., "Structure and function of *Clostridium* botulinum progenitor toxin.", J. Toxical-Toxin Reviews, 18:17-34, 1999). Type A strain that produces botulinum toxin serotype A produces three forms of progenitor toxins designated as LL (19S, 900 kDa), L (16S, 500 kDa) and M (12S, 300 kDa) toxins, all of which are considered to be fully activated, while type B, C, and D strains produce two forms, L and M. In addition, type E, F and G produce only a single form of toxin; types E and F produce M toxin, and type G produces L toxin. M toxin consists of a NTX (7S, 150 kDa) and a nontoxic component showing no hemagglutinin (HA) activity, which is described as non-toxic non-HA (NTNH).

[60] As used herein, the term "produced botulinum toxin" means a pure botulinum toxin or a botulinum toxin complex, which is separated or substantially separated from other proteins or impurities that can be accompanied by the botulinum toxin when the botulinum toxin is collected from a culturing or fermentation process. Thus, the produced botulinum toxin has a purity of at least 90%, preferably at least 95%, and

most preferably at least 98%. Particularly, the produced botulinum toxin in the present invention may be a botulinum toxin type A protein having a purity of at least 98%.

[61] Culture of the *Clostridium* botulinum strain for producing the botulinum toxin can be performed using a conventional method known in the art and a conventional medium that may be used for culture.

[62] By way of non-limiting example, a medium for culture of *Clostridium* botulinum strain may include a casein hydrolysate, a yeast extract, glucose and the like, and the culture is performed at a temperature of 25 to 40℃ for 90-180 hours, preferably 100-150 hours.

[63] The acid precipitation of step (a) may be performed by adding acid, preferably sulfuric acid or hydrochloric acid, to a culture of the strain after culturing, so that the culture reaches a pH of 3.0-4.5, preferably 3.3-4.0, most preferably 3.4-3.6.

[64] The acid precipitation of step (a) is based on the principle in which the addition of acid to a culture containing many kinds of proteins reduces the pH of the culture while killing botulinum bacteria remaining after culture so that the proteins reach the iso-electric point to precipitate. It includes crystallization in a broad sense, and the precipitation method is a method of roughly separating a desired material in a mixed state, unlike crystallization focused on purifying the desired material with high purity. In the precipitation method, impurities having a structure similar to the desired material are also precipitated. Herein, the pH is adjusted to about 3.0-4.5. The recovery rate of the botulinum toxin increases as the pH decreases. If the pH is 3.0 or lower, it will affect the botulinum toxin itself, and if the pH is 4.5 or higher, the recovery rate of the botulinum toxin will decrease. For these reasons, the pH is preferably within the above-specific range. Particularly, the pH is most preferably 3.4-3.6, because the recovery rate of the botulinum toxin is the highest in this pH range. When the pH of the botulinum strain culture reaches a suitable range after addition of acid, the acid is added to the culture until the change in the pH no longer appears, and then the culture is allowed to stand at room temperature for 10-30 hours, followed by removal of the supernatant.

[65] In the present invention, the acid precipitation in step (a) may be performed once or more.

[66] As used herein, the term "clarification" means re-dissolving a precipitate or the like in a buffer, and then removing impurities from the solution resulting from the re-dissolution. The "clarification" step in the present invention may generally be performed using one or more steps of including any of the following alone or various combinations thereof, for example, filtration, precipitation, flocculation and settling. More specifically, the clarification step may be performed using at least one technique selected from the group consisting of depth filtration (DF), microfiltration (MF), ultra-

filtration (UF), sterile filtration, membrane chromatography (MC) and centrifugation. Through the clarification step in the present invention, impurities, particularly nucleic acid impurities, HCD, cell debris and endotoxin, contained in the re-dissolved toxin precipitate, may be removed.

[67] In some embodiments, the present invention provides an improvement over the conventional clarification step commonly used in various purification schemes. The clarification step generally involves the removal of one or more undesirable entities and is typically performed prior to a step involving capture of the desired target molecule. Another aspect of clarification is the removal of soluble and insoluble components in a sample which may later result in the fouling of a sterile filter in a purification process, thereby making the overall purification process more economical. Furthermore, methods for enhancing clarification efficiency, for example, precipitation can be used. Precipitation of impurities can be performed by various methods such as flocculation, pH adjustment (acid precipitation), temperature shifts, phase change due to stimulus-responsive polymers or small molecules, or any combinations of these methods.

[68] As used herein, the term "depth filtration (DF)" means removing particles (e.g., impurities) from a solution using a series of filters with decreasing pore size. The term "depth filter" as used herein achieves filtration within the depth of the filter material. Such filters are those that comprise a random matrix of fibers bonded to form a complex, tortuous maze of flow channels. Particle separation in these filters results from entrapment by or adsorption to, the fiber matrix. The most frequently used depth filter media for bioprocessing of cell culture broths and other feedstocks consist of cellulose fibers, a filter aid such as DE, and a positively charged resin binder. Depth filter media, unlike absolute filters, retain particles throughout the porous media allowing for retention of particles both larger and smaller than the pore size. Particle retention is thought to involve both size exclusion and adsorption through hydrophobic, ionic and other interactions. Commercially available depth filters include Millistak+ Pod depth filter system, XOHC media(Millipore Corporation), Zeta Plus™ Depth Filter(3M Purification Inc.), etc. In the present invention, depth filtration may be performed using two or more depth filters arranged in parallel. In this case, commercially available depth filters may include Millistak+ mini DOHC (Millipore Corporation) and XOHC filters(Millipore Corporation).

[69] In the present invention, the depth filter generally has a nominal pore size of 0.01-20 μm, preferably 0.1-8 μm, is used for removal of flocculated cell debris and colloidal particulates having a size larger than the above pore size and cellular biomass including particulates smaller than the above pore size, and may include a porous depth filter media having porous layers of varying pore ratings.

[70] Thus, when depth filtration is used in the present invention, it is easy to remove im-

purities (e.g., nucleic acids), cell debris, endotoxin, etc., from the botulinum toxin precipitate.

[71] As used herein, the term "microfiltration (MF)" or "ultrafiltration (UF)" is a process that fractionates a target solute (e.g., botulinum toxin) through the pores of a membrane under a certain pressure according to the size and structure of the solute of a mixture solution. For example, clarification of a solution can be performed at a pressure of 5-40 psig and a temperature of 4 to 60°C by use of a PS (polysulfone) membrane for 0.1 μm size or 750 kDa molecular weight cut-off (MWCO) separation.

[72] Generally, microfiltration is a process which is carried out prior to ultrafiltration, and is used to separate particles having a size of 0.1-10 μm from a solution. It is generally used to separate a polymer having a molecular weight of 1×10^6 g/mol. In addition, microfiltration may be used to remove sediments, protozoan animals, large bacteria, etc. In the present invention, microfiltration may be easily used to remove polymers or cell debris. Generally, the microfiltration process is performed using a pressure pump or a vacuum pump at a velocity of 0.1-5 m/s, preferably 1-3 m/s, and a pressure of 50-600 kPa, preferably 100-400 kPa.

[73] Ultrafiltration is used to separate particles having a size of 0.01-0.1 μm from a solution, and the particles generally correspond to polymers having a molecular weight of 1×10^3-1×10^6 Da. Ultrafiltration is used to remove proteins, endotoxin, virus, silica, etc. In the present invention, when an ultrafiltration membrane for 100-300 K MWCO is used, it can remove impurities from the botulinum toxin precipitate and concentrate the botulinum toxin.

[74] As used herein, "sterile filtration" is a process that uses a microfilter or a membrane filter, can replace heating, irradiation or chemical treatment, and can safely clarify solutions containing target substances (e.g., biologics, botulinum toxins, etc.). For removal of microorganisms that can be contained in solutions, a microfilter having a pore size of 0.1-0.3 μm, preferably 0.15-0.25 μm, most preferably 0.2-0.22 μm, is used, and for removal and inactivation of virus, a nanofilter having a pore size of 20-50 nm is used. In addition, for removal of microorganisms, virus, etc., a clarification process may also be performed using a membrane filter which has a specific pore size and which is made of cellulose ester or PES (polyethersulfone).

[75] In the present invention, "membrane chromatography (MC)" may be used as a process for separation of a target substance (e.g., botulinum toxin), which is contrasted with "resin chromatography". Resin chromatography generally uses a spherical porous resin and is based on diffusion greater than convection of solution, whereas membrane chromatography uses a planar macroporous membrane and is based on convection greater than diffusion of solution. Thus, in membrane chromatography, the separation efficiency of solution is relatively high, and thus the access of viruses, plasmids, large

protein complexes, etc., to the membrane, is easy, thereby making separation easy. Commercially available membrane chromatography may include Mustang Q membrane chromatography capsule(Pall Corporation), Sartobind Q(Sartorius Stedim Biotech GmbH), but is not limited thereto.

[76] In the present invention, centrifugation may preferably be performed at a centrifugal force of preferably at 12,000~15,000 g.

[77] In the present invention, the botulinum toxin clarification of step (b) comprises a step of dissolving the toxin precipitate resulting from step (a) by addition of phosphate buffer, preferably sodium phosphate buffer, and clarifying the precipitate. Herein, the pH of the phosphate buffer is about 3.0-8.0, preferably about 4.0-7.0, and the solution is adjusted to the final pH of 4.5-6.5, preferably 5.5-6.2, more preferably 4.8-5.8. In this pH range, clarification of the toxin may be performed.

[78] In the present invention, dilution or dissolution of the botulinum toxin in step (c) comprises a step of adding phosphate buffer, preferably sodium phosphate buffer, to the toxin resulting from step (b) to dilute the retentate or dissolve the precipitate. Herein, the pH of the phosphate buffer is preferably about 4.0-8.5, and a base may be added to adjust the final pH to 4.5-8, preferably 5.5-7.5, more preferably 6-7. In this pH range, dilution or dissolution of the toxin may be performed.

[79] In the present invention, depth filtration (DF) of the botulinum toxin in step (b) may be performed using a peristaltic pump, and UF diafiltration, ammonium sulfate precipitation or hydrochloric acid precipitation in step (c) may be performed once or more.

[80] As used herein, the term "UF diafiltration" means performing diafiltration using the above-described ultrafiltration (UF), but is not limited thereto. "Diafiltration" means a technique that removes or collects any component (e.g., particles) from a target substance (solution) using a permeable filter capable of achieving separation according to the molecular weight (molecular size) of the component, thereby increasing the purity of the target substance.

[81] The step of precipitation with ammonium sulfate corresponds to a salting out process in which a salt (ammonium sulfate, etc.) that easily dissolves in water is added to a protein mixture to increase the ionic strength to thereby to form a protein precipitate. If a desired protein precipitates mainly upon saturation with 30%(w/v) ammonium sulfate, the desired protein can be precipitated by precipitating out proteins other than the desired protein at a ammonium sulfate saturation concentration of 30% (w/v) or lower, and then adding ammonium sulfate to a saturation concentration of 30% (w/v), and can be collected by centrifugation. The salting out operation is frequently used as initial means for purification. The ammonium sulfate solution used may have an ammonium sulfate concentration of 10-50% (w/v), preferably 20-40% (w/v). In

addition, hydrochloric acid precipitation in step (c) may be performed by adding hydrochloric acid to reach a pH of 2-5, preferably 2.5-4.5.

[82] The anion-exchange chromatography in step (d) may be performed at a pH of 2-9, preferably 3-8, and a conductivity of 2-40 mS/cm, preferably 3-30 mS/cm. In step (d), botulinum toxin may be collected as a botulinum toxin-containing fraction (flow-through mode) of a flow-through (FT) fraction eluted from anion-exchange chromatography or as a fraction containing the botulinum toxin bound to anion exchange chromatography resin. The finally purified botulinum toxin may have a form of 7S or 19S.

[83] The term "FT (flow-through)", "flow-through process" or "flow-through purification", as used interchangeably herein, means a separation process in which at least one target molecule (e.g., botulinum toxin) contained in a biopharmaceutical formulation together with one or more impurities passes through a material and one or more impurities generally bind to the material and the target molecule generally does not bind to the material (that is, flows through the materials).

[84] The term "conductivity" refers to the ability of an aqueous solution to conduct an electric current between two electrodes. In solution, the current flows by ion transport. Therefore, with an increasing amount of ions present in the aqueous solution, the solution will have a higher conductivity. The basic unit of measure for conductivity is the Siemen (or mho), mho (mS/cm), and can be measured using a conductivity meter, such as various models of Orion conductivity meters. Because electrolytic conductivity is the capacity of ions in a solution to carry electrical current, the conductivity of a solution may be altered by changing the concentration of ions therein. For example, the concentration of a buffering agent and/or the concentration of a salt (e.g. sodium chloride, sodium acetate, or potassium chloride) in the solution may be altered in order to achieve the desired conductivity. Preferably, the salt concentration of the various buffers may be modified to achieve the desired conductivity.

[85] In the present invention, a column buffer that is used for the anion-exchange chromatography in step (d) may be sodium phosphate buffer, citrate buffer or Tris-HCl buffer, but is not limited thereto. The concentration of the column buffer is adjusted to 15-70 mM, preferably about 20-60 mM. The pH of the column buffer is adjusted to 2-9, preferably 3-8, and the flow rate of the mobile phase is controlled to 0.5-5.0 mL/min, preferably 1.0-3.0 mL/min. Herein, the conductivity of the buffer is adjusted to 2-40 mS/cm, preferably 3-30 mS/cm, and and the sample is loaded into a column after completion of equilibration of the column.

[86] In addition, in the present invention, it could be seen that, when the pH of the botulinum toxin-containing anion exchange chromatography fraction obtained through steps (a) to (d) was adjusted upward and when anion-exchange chromatography was

performed again, followed by cation-exchange chromatography (CEX), a botulinum toxin having a very high purity, particularly a pure botulinum neurotoxin could be obtained.

[87] Therefore, in another aspect, the present invention provides a method for producing a botulinum toxin, further comprising, after step (d), the steps of:

[88] (e) adjusting the pH of the botulinum toxin-containing anion exchange chromatography fraction upward;

[89] (f) purifying the pH-adjusted anion exchange chromatography fraction resulting from step (e) by anion exchange chromatography; and

[90] (h) purifying the diluted botulinum toxin resulting from step (f) using cation exchange chromatography (CEX).

[91] Preferably, the method may further comprise, between step (f) and step (h), step (g) of adding a buffer to the purified anion exchange chromatography fraction to dilute the botulinum toxin.

[92] In the present invention, when steps (e) to (h) are additionally performed, the anion-exchange chromatography (AEX) process in step (d) is referred to as "first anion-exchange chromatography process" for convenience of explanation, and the AEX process in step (f) is referred to as "second anion-exchange chromatography process". Unless specified otherwise, the AEX process in step (d) refers to first anion-exchange chromatography in step (d). In addition, unless specified otherwise, TQ in the examples and the like means the first anion-exchange chromatography process, HQ means the second anion-exchange chromatography process, and XS means the cation-exchange chromatography process.

[93] In the present invention, the "pH" of a solution means the acidity or alkalinity relative to the ionization of a water sample. The pH of water is neutral, i.e., 7. Most pH readings range from 0 to 14. Solutions with a higher [H+] than water (pH less than 7) are acidic; solutions with a lower [H+] than water (pH greater than 7) are basic or alkaline. pH can be measured using a pH meter. Buffer pH may be adjusted using an acid or base such as HCl or NaOH.

[94] In the present invention, the upward adjustment of the pH of the anion-exchange chromatography fraction in step (e) may be performed once or more, and the upward adjustment of the pH in step (e) may be performed using at least one technique selected from the group consisting of UF diafiltration, pH titration, dialysis, and buffer exchange column chromatography. Preferably, upward adjustment of pH of the anion-exchange chromatography fraction in step (e) is performed using Tris-HCl buffer. Herein, the Tris-HCl buffer has a pH of 7.0-8.5, preferably 7.3-8.3. Thus, the pH may be adjusted to 7.0~8.5, prefereable 7.3~8.3, more prefereable 7.7~7.9.

[95] The second anion-exchange chromatography in step (f) may be performed at a pH of

2-9, preferably 3-8, and a conductivity of 2-40 mS/cm, preferably 3-30 mS/cm. In step (f), a botulinum toxin maybe collected as a botulinum toxin-containing fraction (flow-through mode) of a flow-through (FT) fraction eluted from anion-exchange chromatography or may be collected as a fraction containing the botulinum toxin bound to anion exchange chromatography resin.

[96] In the present invention, the buffer in step (g) is preferably a sodium phosphate buffer and has a pH of 6-8, preferably 6.5-7.5.

[97] In the present invention, the cation-exchange chromatography in step (h) may be performed at a pH of 2-9, preferably 3-8. In the cation-exchange chromatography of step (h), the botulinum toxin may be collected as a fraction containing the botulinum toxin bound to the cation-exchange chromatography resin, and the finally purified botulinum toxin may have a form of 7S (molecular weight: 150 kDa).

[98] In the method for producing the botulinum toxin according to the present invention, the resin that is used in the anion-exchange chromatography process includes diethylaminoethyl (DEAE), quaternary aminoethyl (QAE) and quaternary amine (Q) groups, but is not limited thereto. Preferably, TQ650, HQ, XQ, etc., may be used.

[99] In the method for producing the botulinum toxin according to the present invention, the resin that is used in the cation-exchange chromatography process preferably includes carboxymethyl (CM), sulphoethyl (SE), sulfopropyl (SP), phosphate (P), and sulfonate (S), but is not limited thereto. More preferably, HS, XS, etc., may be used.

[100] Cellulose ion exchange resin, for example, DE23™, DE32™, DE52™, CM-23™, CM-32™, and CM-52™ are available from a manufacturer (GE Healthcare, Lindesnes, Norway), and SEPHADEX-based and cross-linked ion exchangers are also known. For example, DEAE-, QAE-, CM-, SP- SEPHADEX and DEAE-, Q-, CM- and S- SEPHAROSE and SEPHAROSE Fast Flow are all available from a manufacturer (GE Healthcare Bio-Sciences). In addition, both DEAE and CM derivatized ethylene glycol-methacrylate copolymers (for example, TOYOPEARL™ DEAE-650S or M and TOYOPEARL™ CM-650S or M) are available from a manufacturer (Tosoh Bioscience LLC, King of Prussia, PA).

[101]

[102] EXAMPLES

[103] Hereinafter, the present invention will be described in further detail with reference to examples. It will be obvious to a person having ordinary skill in the art that these examples are illustrative purposes only and are not to be construed to limit the scope of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

[104]

[105] Example 1: Culture of *Clostridium* botulinum Strain

[106] A medium for culture of the *Clostridium* botulinum strain for producing a botulinum toxin had a composition comprising 2% casein hydrolysate, 1% yeast extract, 1% glucose and 0.5% thioglycollate, and sterilized at 121°C for 30 minutes. Then, 20 μL of *Clostridium* botulinum (the Korean Centers for Disease Control and Prevention Accession No.: 4-029-CBB-IS-001) was inoculated into a culture tube containing 10 ml of the medium, and was subjected to primary seed culture (stationary culture) at 35°C for 22-30 hours under anaerobic conditions. When the growth of the strain in the primary seed culture process was confirmed, 8 mL of the primary seed culture was inoculated into a culture bottle containing 800 ml of a sterile medium having the same medium composition and was subjected to secondary seed culture (stationary culture) at 35°C for 8-15 hours under anaerobic conditions. When the growth of the strain in the secondary seed culture process was confirmed, 800 mL of the secondary seed culture was inoculated into a 10 L culture bottle containing 10L of a sterile medium having the same sterilized medium composition and subjected to culture at 35°C for 4-6 days under anaerobic conditions.

[107]

[108] Example 2: Production of Botulinum Toxin

[109] 2-1: Sulfuric Acid Precipitation and pH neutralization

[110] The step of precipitation with sulfuric acid is a protein separation process in which sulfuric acid is added to a culture containing many kinds of proteins to reduce the pH of the culture while killing botulinum bacteria remaining after culture so that the proteins reach the isoelectric point to precipitate. The main culture was performed as described in Example 1, and after completion of the main culture, the culture was collected in a 10 L culture container (10 L SUS pot). Then, 5N sulfuric acid was added to the culture so as to reach a pH of 3.4-3.6, and the culture was allowed to stand at room temperature for 12-20 hours so that it would be separated into a supernatant and a precipitate. The supernatant was removed, and 2.5-3.0 L of the sulfuric acid precipitate finally remained.

[111] pH neutralization of the sulfuric acid precipitate remaining after removal of the supernatant was performed in the following manner. 700 mL of 1 M sodium phosphate (pH 5.3) was added to the sulfuric acid precipitate, followed by stirring. 5N NaOH was added to adjust the pH of the sulfuric acid precipitate to 5.9-6.1. The pH-neutralized sulfuric acid precipitate was collected.

[112]

[113] 2-2: Pretreatment Process

[114] 1) Conventional Process

[115] The sulfuric acid precipitates resulting from Example 2-1 was treated according to a conventional process. Specifically, to remove DNA and RNA remaining in the pre-

cipitate, 60 mL of 0.4M benzamidine HCl, 100 mg of DNase and 300 mg of RNase were added, and to extract the botulinum toxin, the precipitate solution was incubated for about 3-7 hours. Then, the incubated precipitate solution was centrifuged at 4℃ and 12,000 x g for 15 minutes, followed by collection of the supernatant. The pH of the supernatant was lowered to 3.4-3.6 by addition of 1N hydrochloric acid, and then the supernatant was allowed to stand at a temperature of 3 to 5℃ for 12-20 hours, thereby performing the process of precipitation with hydrochloric acid. The hydrochloric acid precipitate formed by the above-described process was centrifuged at 4℃ and 12000 x g for 15 minutes to remove the supernatant, and the remaining toxin pellets were dissolved in 30 mL of sodium phosphate buffer (pH 6.5).

[116]

[117] 2) Depth Filtration Process

[118] The sulfuric acid precipitates resulting from Example 2-1 was filtered using Zeta Plus™ Encapsulated Capsule depth filter effective filtration (3M, BC0025S60SP05A) connected to a peristaltic pump.

[119]

[120] i) UF Following Depth Filtration

[121] The depth filtrate was subjected 10 times to diafiltration with 50 mM sodium phosphate (pH 6.5) using a UF system (PALL, TFF cassette 30 kDa), and then adjusted to a final volume of 30 mL.

[122] ii) Ammonium Sulfate Precipitation Following Depth Filtration

[123] Ammonium sulfate was added to the depth filtrate to a concentration of 30% (w/v), and then the filtrate was allowed to stand at a temperature of 3 to 5℃ for 12-20 hours. Then, the solution was centrifuged at 4℃ and 12,000 x g for 15 minutes to remove the supernatant, and then the centrifuged pellets were re-dissolved in 30 mL of 50 mM sodium phosphate (pH 6.5).

[124] iii) Hydrochloric Acid Precipitation Following Depth Filtration

[125] 1N HCl was added to the depth filtrate to lower the pH of the filtrate to 3.4-3.6, and then the solution was allowed to stand at a temperature of 3 to 5℃ for 12-20 hours. Next, the solution was centrifuged at 4℃ and 12,000 x g for 15 minutes to remove the supernatant, and the centrifuged pellets were re-dissolved in 30 mL of 50mM sodium phosphate (pH 6.5).

[126]

[127] 2-3: Purification Process

[128] Measurement of the purity of the botulinum toxin purified by the TQ process (first anion-exchange chromatography process) was performed by a SEC (size exclusion chromatography) method using HPLC (Waters e2695). Herein, the mobile phase used was 100 mM sodium phosphate (pH 6.5), and a TSKgel G4000SWxl (Tosoh

Bioscience, P/N 08542) column was connected to a guard column (Tosoh Bioscience, P/N 08543), and 20 μg of the botulinum toxin protein was loaded into the column and allowed to flow at a rate of 1 mL/min for 30 minutes.

[129] In order to visually examine the mixed state/purity/content of the botulinum toxin contained in the sample collected in each purification (process) step for the botulinum toxin purified by the TQ process (first anion-exchange chromatography process), the HQ process (second anion-exchange chromatography process) and the XS process (cation-exchange chromatography process), protein electrophoresis (SDS-PAGE) was performed. Specifically, the samples considered to contain the botulinum toxin were quantified by the Bradford method, and then a certain amount (e.g., 40 μg protein) of each sample was taken and suitably heated and/or dissolved, and 4-12% SDS-PAGE was performed using the denatured protein. The electrophoresed gel was stained with a suitable dye (silver nitrate or Coomassie Blue), and then the purity of the botulinum toxin in each purification step was visually examined.

[130]

[131] 2-3-1: TQ Purification (Anion Exchange Chromatography Flow-Through (AEX FT))

[132] After completion of the pretreatment process of Example 2-2, in order to remove most major impurities other than the botulinum toxin, chromatography was performed in the following manner using ion-exchange resin.

[133] (1) TQ resin was packed into an XK 26/40 column to a height of 30-34 cm, and then the column was mounted in AKTA Prime Plus.

[134] (2) The column was equilibrated with equilibrium/elution buffer (50 mM sodium phosphate (pH 6.4-6.6, and 10±5 mS/cm)).

[135] (3) 10 mL of the sample pretreated by the conventional process of Example 2-2 was loaded into the column and allowed to flow at a rate of 2 mL/min. After completion of the injection, 160-180 mL of equilibrium/elution buffer was loaded, and fractions were sequentially collected until the peak at a UV wavelength of 280 nm decreased to the baseline (the fractions were analyzed by SDS-PAGE).

[136] (4) For regeneration of the column after collection of the fractions, 200 mL or more of washing buffer (50 mM sodium phosphate, pH 6.4~6.6, 1M NaCl) was loaded at a flow rate of 5 mL/min to wash the column.

[137] (5) According to the procedures of (2) to (4) above, TQ purification was performed using the samples pretreated by other three methods (UF following depth filtration, ammonium sulfate precipitation following depth filtration, and hydrochloric acid precipitation following depth filtration) instead of the sample pretreated by the conventional process. Herein, for purification of a high-purity botulinum toxin, pH was maintained at 6.4-6.6, and conductivity was maintained at 10±5 mS/cm.

[138] As a result, it was shown that the botulinum toxin was not adsorbed onto the TQ

resin, and most major impurities were removed by adsorption, and thus a botulinum toxin could be produced with high purity. In addition, the resulting botulinum toxin had a form of 19S (molecular weight: about 900 kDa).

[139]

[140] 2-3-2: HQ Purification (Anion Exchange Chromatography: AEX, Biding Mode)

[141] After completion of TQ purification, in order to further purify the botulinum toxin, chromatography using HQ ion-exchange resin (hereinafter referred to as "HQ process") was performed. In the HQ process, the sample resulting from TQ purification was purified in binding or FT (flow-through) mode. As used herein, the term "HQ (binding)" refers to a process that separates (or elutes) a target substance (e.g., botulinum toxin) based on the difference in quantity of electric charge of HQ resin capable of binding to the target substance, and the term "HQ (FT)" refers to a process in which, based on the difference in quantity of electric charge of HQ resin capable of binding to impurities which can mixed with a target substance (e.g., botulinum toxin), the target substance that flows through (FT) the HQ resin without binding to the HQ resin is separated from a sample.

[142]

[143] HQ (Binding) Purification (AEX, Biding Mode)

[144] (1) The sample, purified by TQ after pretreatment according to the conventional process, was subjected 10 times to diafiltration with 25mM Tris-HCl buffer (pH 7.7-7.9) using a UF system (PALL, TFF cassette 30 kDa MWCO). The final pH of the sample was 7.7-7.9, and the volume was adjusted to 30 mL.

[145] (2) HQ resin was packed in AKTA Prime Plus.

[146] (3) The column was equilibrated with 25mM Tris-HCl buffer (pH 7.7-7.9).

[147] (4) The sample prepared in (1) was loaded into the equilibrated column at a flow rate of 2.5 mL/min, and washed with 40 mL of 25mM Tris-HCl buffer (pH 7.7-7.9), and then eluted at 20 CV and 10% gradient. Fractions corresponding to the first peak were sequentially collected (the fractions were analyzed by SDS-PAGE).

[148] (5) For regeneration of the column after collection of the fractions, 120 mL or more of washing buffer (25 mM Tris-HCl, pH 7.7-7.9, 1M NaCl) was loaded at a flow rate of 2.5 mL/min to wash the column.

[149] (6) The procedures of (1) to (5) above were performed using the samples purified by TQ after treatment according to other three methods (TQ purification after UF following depth filtration, ammonium sulfate precipitation following depth filtration, and hydrochloric acid precipitation following depth filtration), instead of the sample purified by TQ after pretreatment according to the conventional method.

[150] As a result, it was shown that the botulinum toxin could be produced with high purity and that the resulting botulinum toxin had a form of 7S (molecular weight: about 150

kDa) (FIGS. 2 and 3).

[151]

[152] 2-3-3: HQ Purification (AEX, Biding Mode) and XS Purification (Binding Mode)

[153] After completion of the HQ purification according to Example 2-3-2, in order to further purify the botulinum toxin, XS cation-exchange chromatography (CEX, binding mode) was further performed.

[154] Specifically, the XS process was performed in the following manner.

[155] (1) The sample, purified by HQ after pretreatment according to the conventional process of Example 2-2, was diluted to 1/4 using 50 mM sodium phosphate buffer (pH 6.9-7.1).

[156] (2) XS resin was packed in AKTA Prime Plus.

[157] (3) The column was equilibrated with equilibrium/elution buffer (50 mM sodium phosphate, pH 6.9-7.1).

[158] (4) The diluted and HQ-purified sample of (1) was loaded into the equilibrated column at a flow rate of 1.6 mL/min, and washed with 50 mL of 50 mM sodium phosphate buffer (pH 6.9-7.1). And then, the sample was eluted at 20 CV and 12% gradient. Fractions corresponding to the first peak were sequentially collected (the fractions were analyzed by SDS-PAGE).

[159] (5) For regeneration of the column after collection of the fractions, 20 mL or more of washing buffer (50 mM sodium phosphate, pH 6.9-7.1, 1M NaCl) was loaded at a flow rate of 1.6 mL/min to wash the column.

[160] (6) The procedures of (1) to (5) above were performed using the samples purified by TQ and HQ after treatment according to other three methods (TQ purification after UF following depth filtration, ammonium sulfate precipitation following depth filtration, and hydrochloric acid precipitation following depth filtration), instead of the sample purified by HQ after pretreatment according to the conventional method.

[161] As a result, it was shown that the botulinum toxin could be produced with high purity and that the final botulinum toxin had a form of 7S (molecular weight: about 150 kDa) (FIGS. 4 and 5).

[162]

[163] 2-3-4: HQ Purification (AEX, FT Mode) and XS Purification (Binding Mode)

[164] According to the botulinum toxin production method described in Example 2-3-3 above, a botulinum toxin was produced using the flow-through mode instead of the binding mode in the HQ (AEX) purification process.

[165]

[166] HQ (AEX, FT Mode) Process

[167] HQ purification (AEX, FT mode) was performed in the following manner.

[168] The sample, purified by TQ after pretreatment according to the conventional process,

was subjected 10 times to diafiltration with 25 mM Tris-HCl buffer (pH 7.7-7.9, conductivity 7.0-7.5 mS/cm) using a UF system (PALL, TFF cassette 30 kDa MWCO). The final pH of the sample was 7.7-7.9, and the volume of the sample was adjusted to 30 mL. Next, the sample was subjected to a HQ (FT mode) process.

[169] i. HQ resin was packed in AKTA Prime Plus.

[170] ii. The column was equilibrated with 25 mM Tris-HCl buffer (pH 7.7-7.9, conductivity: 7.0-7.5 mS/cm).

[171] iii. The TQ-purified sample was loaded at a flow rate of 1.5 mL/min, and eluted with 40 mL of 25 mM Tris-HCl buffer (pH 7.7-7.9, conductivity: 7.0-7.5 mS/cm). Fractions corresponding to the peak were collected (the fractions were analyzed by SDS-PAGE).

[172] iv. For regeneration of the column after collection of the fractions, 40 mL or more of washing buffer(25 mM Tris-HCl, pH 7.7-7.9, 1M NaCl) was loaded at a flow rate of 1.5 mL/min to wash the column.

[173]

[174] XS Process using HQ (FT) Sample

[175] In order to increase the purity of the botulinum toxin, the sample purified by the HQ (FT) process was further purified by an XS (binding) process.

[176] (1) The sample purified by the HQ (FT) process was diluted to 1/4 using 50 mM sodium phosphate buffer (pH 6.9-7.1).

[177] (2) XS resin was loaded in AKTA Prime Plus.

[178] (3) The column was equilibrated with 50 mM sodium phosphate buffer (pH 6.9-7.1).

[179] (4) The HQ-purified sample diluted in (1) above, was loaded into the column at a flow rate of 1.5 mL/min, and washed with 50 mL of 50 mM sodium phosphate buffer (pH 6.9-7.1). And then, the sample was eluted at 60 CV and 12% gradient. Fractions corresponding to the first peak were collected (the fractions were analyzed by SDS-PAGE).

[180] (5) For regeneration of the column after collection of the fractions, 20 mL or more of washing buffer (50 mM sodium phosphate, pH 6.9-7.1, 1M NaCl) was loaded at a flow rate of 1.5 mL/min to wash the column.

[181] As a result, as shown in FIGS. 6 and 7, it was found that the botulinum toxin in the form of 7S (molecular weight: about 150 kDa) could be produced with a purity of 98% or higher.

[182] Taken together, as shown in FIGS. 2 to 7, in the botulinum toxin-containing sample obtained by anion-exchange chromatography purification (TQ (binding or FT mode) purification) after performing either the conventional process of Example 2 or the hydrochloric acid precipitation process following depth filtration, impurities other than 138 kDa (NTNH), 98 kDa (heavy chain), 52 kDa (light chain), 50 kDa (HA), 33 kDa (HA), 20 kDa (HA) and 17 kDa (HA), which are naturally observed in toxin 19S, were

observed in the smallest amount.

[183] Furthermore, in the botulinum toxin-containing sample obtained by anion-exchange chromatography (TQ (binding or FT mode) purification) after UF diafiltration following depth filtration, an impurity protein band of about 13 kDa was observed.

[184] In addition, in the botulinum toxin-containing sample obtained by anion-exchange chromatography (TQ (binding or FT mode) purification) after ammonium sulfate precipitation following depth filtration, an impurity protein band of about 37 kDa was observed.

[185] However, as can be seen from the results of HQ and XS purification processes after dissociation of toxin complex for purification of the low-molecular-weight botulinum toxin, no impurity protein band was observed in all the botulinum toxin-containing samples regardless of the kind of four pretreatment processes (the conventional process, UF following depth filtration, ammonium sulfate precipitation following depth filtration, and hydrochloric acid precipitation following depth filtration), and only low-molecular-weight botulinum toxins with 98 kDa (heavy chain) and 52 kDa (light chain) were observed.

[186] Particularly, the most efficient process capable of replacing enzymatic treatment and extraction processes for botulinum toxin-containing samples in order to separate botulinum toxin 19S is anion-exchange chromatography purification (TQ (binding or FT mode) performed after each of sulfuric acid precipitation, depth filtration and hydrochloric acid precipitation.

[187] On the other hand, in the process for separation of botulinum toxin 7S (low-molecular-weight botulinum toxin), the highest purification efficiency appeared when anion-exchange chromatography (HQ (binding mode or FT mode)) and cation-exchange chromatography (XS (binding mode)) were sequentially performed after each of the four pretreatment processes (the conventional process, UF following depth filtration, ammonium sulfate precipitation following depth filtration, and hydrochloric acid precipitation following depth filtration).

[188]

Industrial Applicability

[189] The novel method for producing the botulinum toxin according to the present invention makes it possible to fundamentally prevent the entry of animal-derived components, thereby ensuring increased safety. In addition, the use of the method of the present invention makes it possible to produce a high-purity botulinum toxin by a simple process, suggesting that the method is very economical and efficient. The botulinum toxin produced by the method of the present invention has a relatively high purity compared to botulinum toxins produced by conventional methods, and thus has

an increased ability to act in a local area. Thus, the systemic circulation of the botulinum toxin, which can result in side effects, is reduced to increase the safety. Accordingly, the botulinum toxin of the present invention can be used for various purposes, including treatment of neuromuscular disorders, removal of wrinkles, and treatment of spastic hemiplegia and cerebral palsy.

[190]

[191] Although the present invention has been described in detail with reference to the specific features, it will be apparent to those skilled in the art that this description is only for a preferred embodiment and does not limit the scope of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

[192]

Claims

[Claim 1] A method for producing a botulinum toxin comprising:
(a) treating a culture of a botulinum toxin-producing strain with acid to form a botulinum toxin-containing precipitate;
(b) adding a buffer to the botulinum toxin-containing precipitate of step (a), and then clarifying by one or more method selected from the group consisting of depth filtration (DF), microfiltration (MF), ultrafiltration (UF), sterile filtration, membrane chromatography (MC) and centrifugation;
(c) subjecting the botulinum toxin-containing solution of step (b) to UF diafiltration, ammonium sulfate precipitation or hydrochloric acid precipitation, and then diluting a retentate resulting from the UF diafiltration in a buffer, or dissolving a precipitate resulting from the ammonium sulfate precipitation or hydrochloric acid precipitate in a buffer; and
(d) subjecting the retentate dilution, ammonium sulfate precipitate solution or hydrochloric acid precipitate solution of step (c) to anion-exchange chromatography (AEX), thereby purifying the botulinum toxin.

[Claim 2] The method of claim 1, wherein the botulinum toxin-producing strain is *Clostridium botulinum* or a mutant strain thereof.

[Claim 3] The method of claim 1, wherein the botulinum toxin is selected from the group consisting of botulinum toxin serotypes A, B, C, D, E, F and G.

[Claim 4] The method of claim 1, wherein the purified botulinum toxin is a botulinum toxin type A protein having a purity of at least 98%.

[Claim 5] The method of claim 1, wherein the acid treating in step (a) is carried out by adding sulfuric acid or hydrochloric acid to the culture of the strain, thereby the culture reaches a pH of 3.0-4.5.

[Claim 6] The method of claim 1, wherein the acid precipitation in step (a) is performed once or more.

[Claim 7] The method of claim 1, wherein the depth filtration (DF) in step (b) is carried out using a peristaltic pump, and a filter used for the depth filtration has a nominal pore size of 0.01-20 μm.

[Claim 8] The method of claim 1, wherein UF diafiltration, ammonium sulfate precipitation or hydrochloric acid precipitation in step (c) is performed once or more.

[Claim 9] The method of claim 1, wherein ammonium sulfate for the ammonium sulfate precipitation in step (c) is added to reach a concentration of 10-50% (w/v).

[Claim 10] The method of claim 1, wherein hydrochloric acid precipitation in step (c) is carried out by adding hydrochloric acid to reach a pH of 2-5.

[Claim 11] The method of claim 1, wherein the buffer in step (b) and step (c) is a sodium phosphate buffer.

[Claim 12] The method of claim 11, wherein the sodium phosphate buffer in step (b) has a pH of 4.5-6.5, and the sodium phosphate buffer in step (c) has a pH of 6-7.

[Claim 13] The method of claim 1, wherein the anion-exchange chromatography in step (d) is carried out at a pH of 2-9 and a conductivity of 2-40 mS/cm.

[Claim 14] The method of claim 1, wherein the botulinum toxin in step (d) is collected as a botulinum toxin-containing fraction of flow-through (FT) fraction eluted from anion-exchange chromatography.

[Claim 15] The method of claim 1, wherein the purified botulinum toxin has a form of 7S or 19S.

[Claim 16] The method of claim 1, further comprising, after step (d:
(e) adjusting the pH of the botulinum toxin-containing anion exchange chromatography fraction upward;
(f) purifying the pH-adjusted anion exchange chromatography fraction resulting from step (e) by anion exchange chromatography; and
(h) purifying the diluted botulinum toxin resulting from step (f) using cation exchange chromatography (CEX).

[Claim 17] The method of claim 16, wherein the upward adjustment of the pH of the anion-exchange chromatography fraction in step (e) is performed once or more.

[Claim 18] The method of claim 16, further comprising, between step (f) and step (h), step (g) of adding a buffer to the purified anion exchange chromatography fraction to dilute the botulinum toxin.

[Claim 19] The method of claim 16, wherein the upward adjustment of the pH in step (e) is carried out using one or more technique selected from the group consisting of UF diafiltration, pH titration, dialysis, and buffer exchange column chromatography.

[Claim 20] The method of claim 18, wherein the buffer in step (g) is a sodium phosphate buffer.

[Claim 21] The method of claim 20, wherein the sodium phosphate buffer in step (g) has a pH of 6.5-7.5.

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[Claim 22] The method of claim 19, wherein the upward adjustment of pH of the anion-exchange chromatography fraction in step (e) is carried out using Tris-HCl buffer.

[Claim 23] The method of claim 22, wherein the Tris-HCl buffer in step (e) has a pH of 7.3-8.3.

[Claim 24] The method of claim 16, wherein the anion-exchange chromatography in step (f) is carried out at a pH of 2-9 and a conductivity of 2-40 mS/cm.

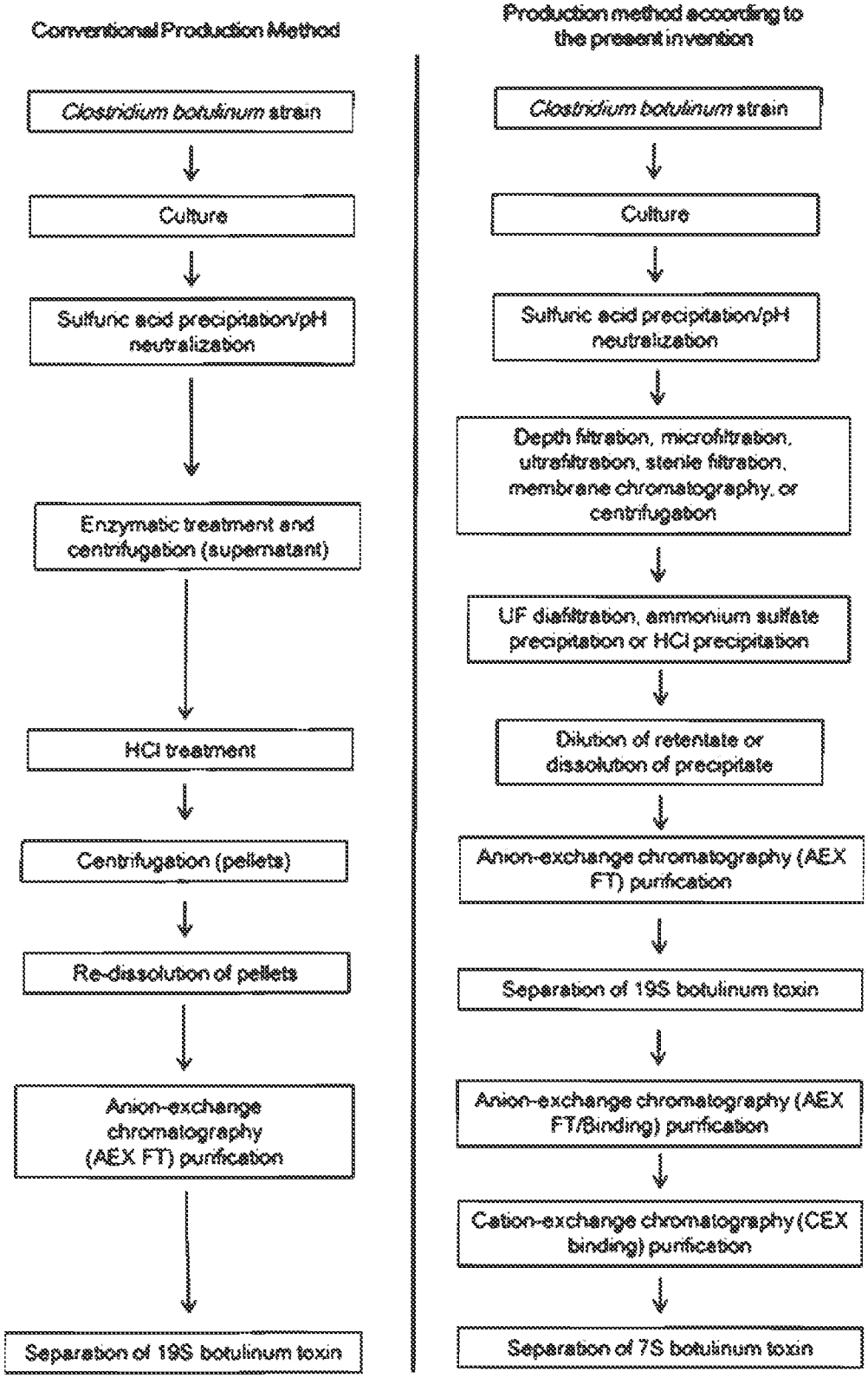
[Claim 25] The method of claim 16, wherein the botulinum toxin in step (f) is collected as a botulinum toxin-containing fraction of a flow-through (FT) fraction eluted from anion-exchange chromatography, or collected as a fraction containing the botulinum toxin bound to anion exchange chromatography resin.

[Claim 26] The method of claim 16, wherein the cation-exchange chromatography in step (h) is carried out at a pH of 2-9 and a conductivity of 2-40 mS/cm.

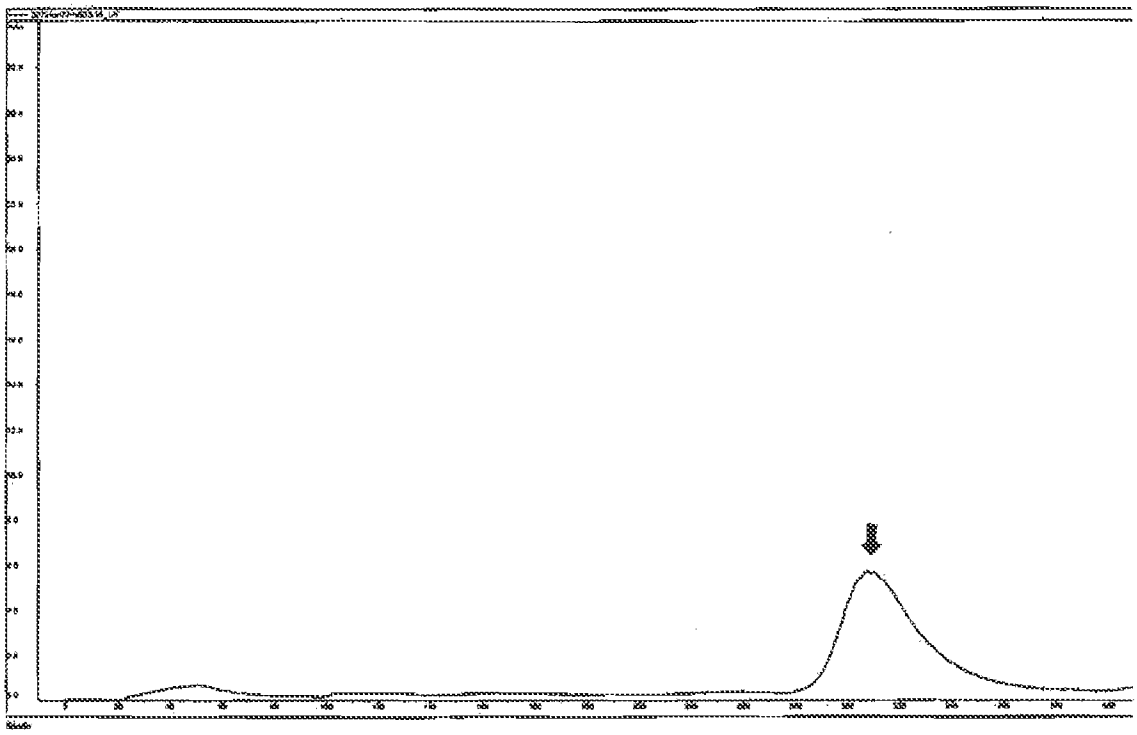
[Claim 27] The method of claim 16, wherein the botulinum toxin in step (h) is collected as a fraction containing the botulinum toxin bound to the cation-exchange chromatography resin.

[Claim 28] The method of claim 16, wherein the finally purified botulinum toxin has a form of 7S.

[Fig. 1]

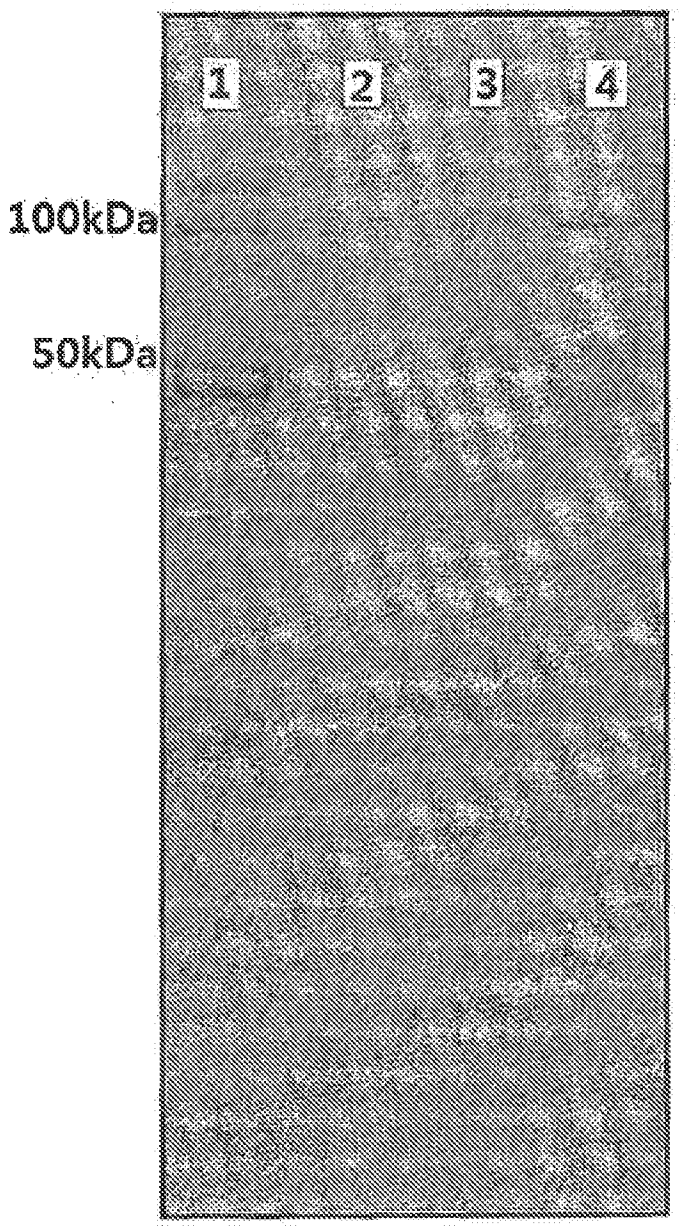

Conventional Production Method
Clostridium botulinum strain
Culture
Sulfuric acid precipitation/pH neutralization
Enzymatic treatment and centrifugation (supernatant)
HCl treatment
Centrifugation (pellets)
Re-dissolution of pellets
Anion-exchange chromatography (AEX FT) purification
Separation of 19S botulinum toxin
Production method according to the present invention
Clostridium botulinum strain
Culture
Sulfuric acid precipitation/pH neutralization
Depth filtration, microfiltration, ultrafiltration, sterile filtration, membrane chromatography, or centrifugation
UF diafiltration, ammonium sulfate precipitation or HCl precipitation
Dilution of retentate or dissolution of precipitate
Anion-exchange chromatography (AEX FT) purification
Separation of 19S botulinum toxin
Anion-exchange chromatography (AEX FT/Binding) purification
Cation-exchange chromatography (CEX binding) purification
Separation of 7S botulinum toxin

[Fig. 2]



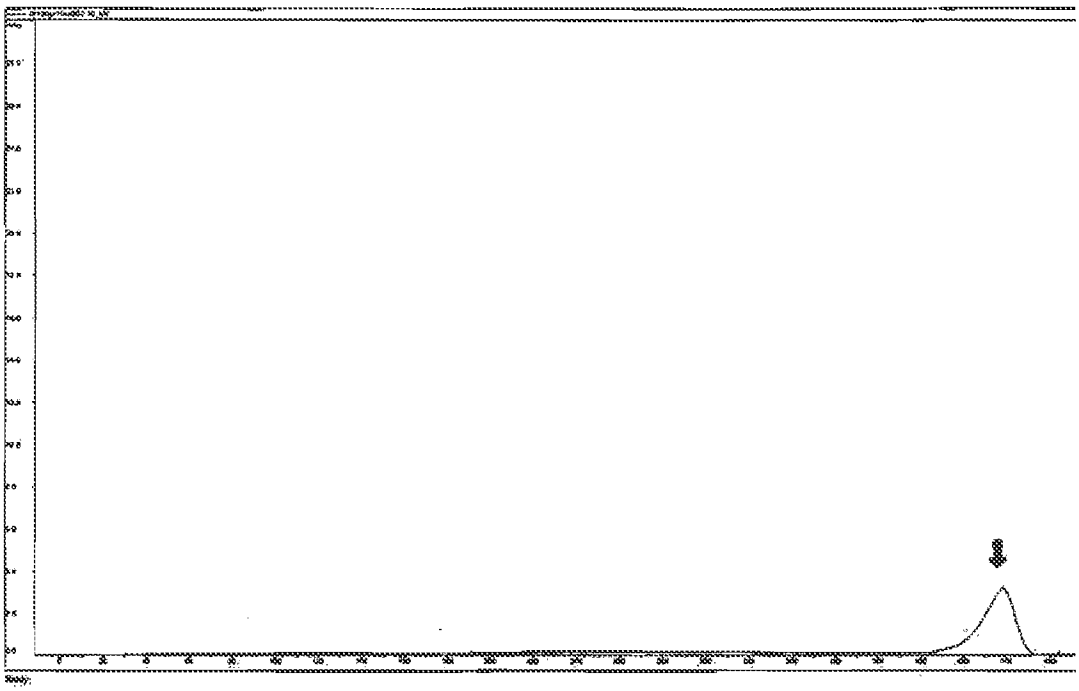
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[Fig. 3]



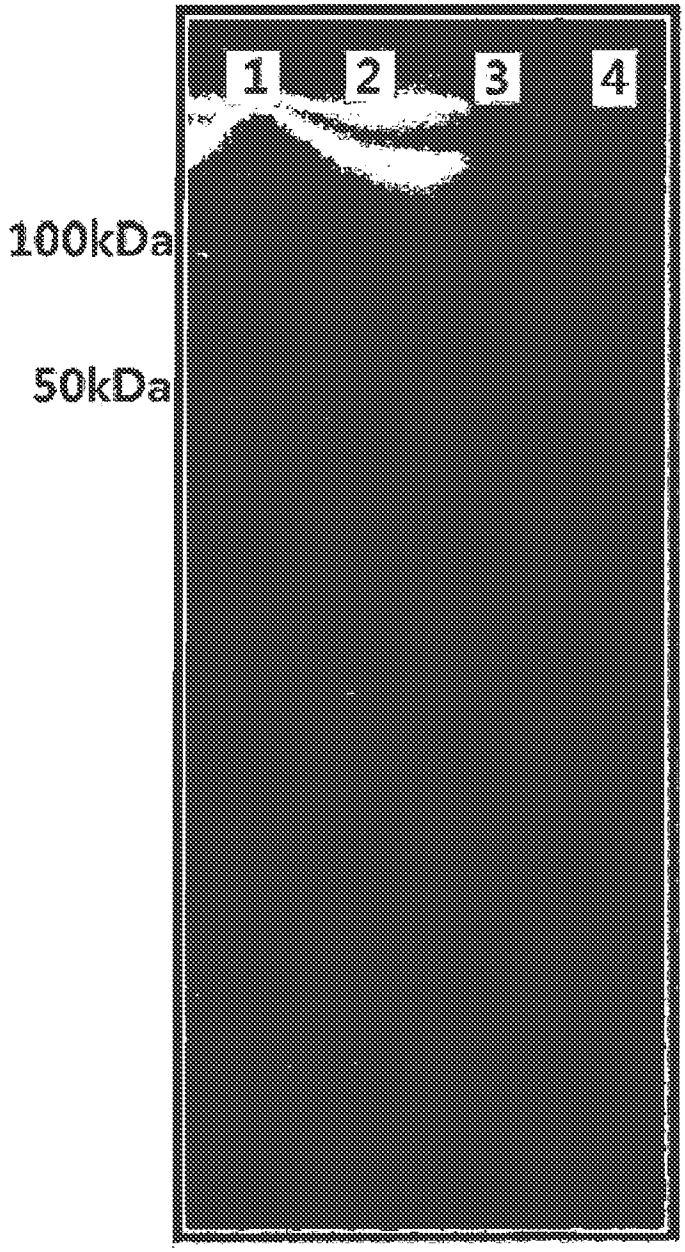
BNSDOCID: <WO 2017095062 A1_I_>
Hugel EX-1006 Page 464 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

[Fig. 4]



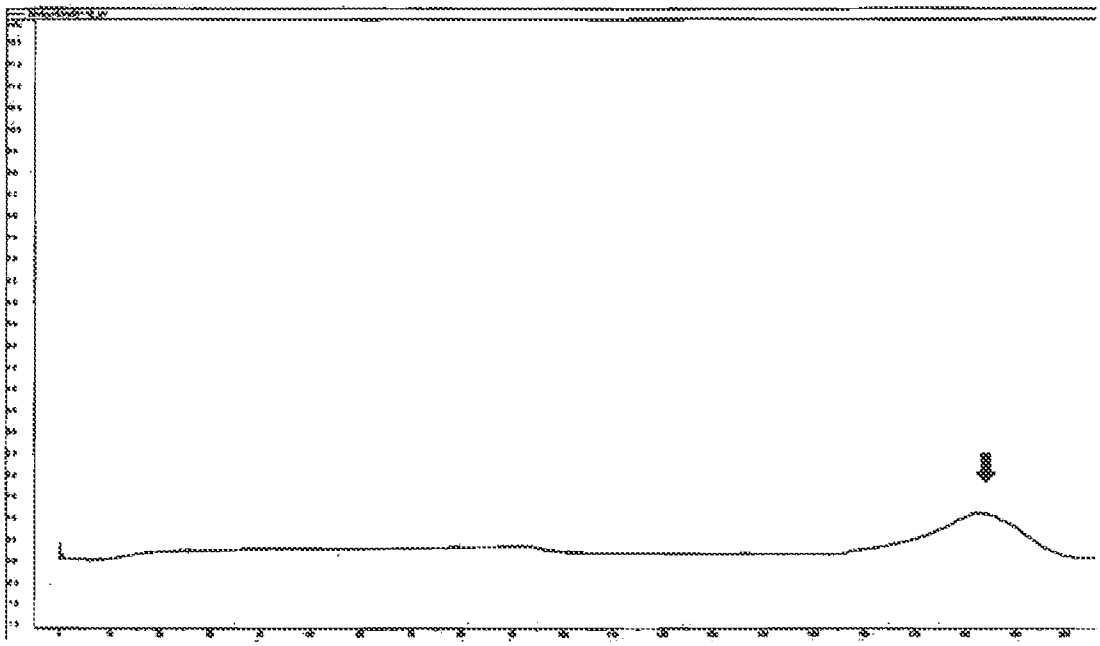
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[Fig. 5]



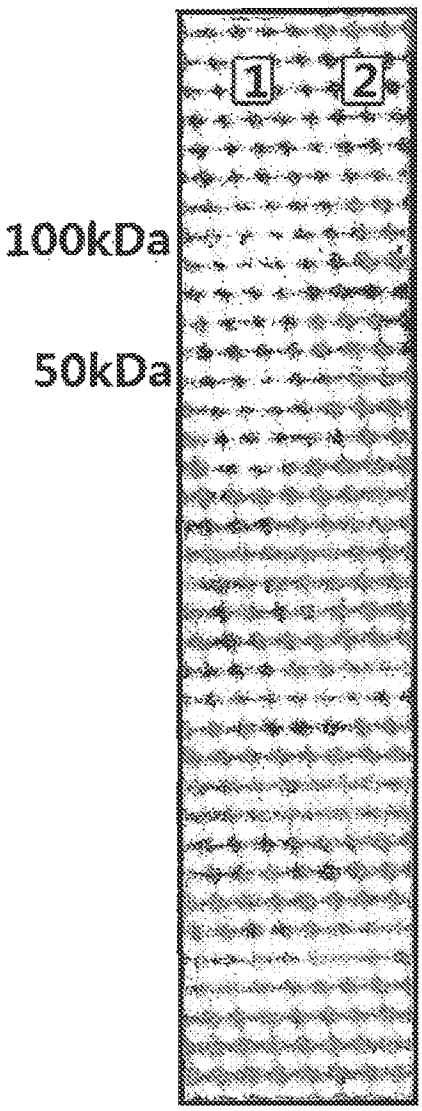
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[Fig. 6]



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[Fig. 7]



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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2016/013506

A. CLASSIFICATION OF SUBJECT MATTER

C07K 14/33(2006.01)i, C07K 1/16(2006.01)i, C07K 1/18(2006.01)i, C07K 1/30(2006.01)i, C07K 1/34(2006.01)i, C07K 1/36(2006.01)i, B01D 15/36(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K 14/33; C12N 9/52; C02F 1/28; A61K 39/08; C12P 21/06; B01D 15/00; C07K 1/16; C07K 1/18; C07K 1/30; C07K 1/34; C07K 1/36; B01D 15/36

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: botulinum toxin, acid precipitation, anion exchange chromatography, animal product free

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y		5,9-14,24-27
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A	US 2015-0184141 A1 (ALLERGAN, INC.) 02 July 2015 See the whole document.	1-28
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☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 February 2017 (21.02.2017)	**21 February 2017 (21.02.2017)**

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Electronic Acknowledgement Receipt

EFS ID:	40121261
Application Number:	16338561
International Application Number:	
Confirmation Number:	3639
Title of Invention:	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
First Named Inventor/Applicant Name:	Hyun Ho JUNG
Customer Number:	137713
Filer:	Judith Unjin Kim/Jessica LaBossiere
Filer Authorized By:	Judith Unjin Kim
Attorney Docket Number:	2479-0007US01
Receipt Date:	28-JUL-2020
Filing Date:	01-APR-2019
Time Stamp:	16:03:56
Application Type:	U.S. National Stage under 35 USC 371

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2	Foreign Reference	EP1185291A0.pdf	1377015 241267d3c3cc490069cec4145c920225a1bc5a59	no	24
Warnings:					
Information:					
3	Foreign Reference	WO2011050072A1.pdf	2314042 592de9a5ec294928ff20ee6c72f2d596ac106820	no	35
Warnings:					
Information:					
4	Foreign Reference	WO2015016462A1.pdf	3909995 5fefab3c8a8f036e84f0abdbc11a69a6e93806d2	no	27
Warnings:					
Information:					
5	Foreign Reference	WO2017095062A1.pdf	2434121 0e07bc60ca135b7894fa56d8693c7d4db691136f	no	40
Warnings:					
Information:					
6	Non Patent Literature	NPL_DASGUPTA_1968.pdf	923614 bf9df4449ba5b2261cd88ef12faa9598cfe673e7	no	10
Warnings:					
Information:					
7	Non Patent Literature	NPL_DASGUPTA_1970.pdf	534789 eeb21defec7500cc6a6de3939b0e32099d4c7dfb	no	7
Warnings:					
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8	Non Patent Literature	NPL_EESR_2020-05-04.pdf	671352 e9f83fd689f47d9b712a8ecad3ca6c93b82b8bf4	no	14
Warnings:					
Information:					
9	Non Patent Literature	NPL_SIMPSON_1988.pdf	601639 7882f31555874412b4b03fcb8ccbe92eface70e2	no	10
Warnings:					
Information:					
		Total Files Size (in bytes):	13820563		

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PTO/SB/06 (09-11)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 16/338,561	Filing Date 04/01/2019	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $460 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	09/18/2020	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 9	Minus	** 20	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0		x $460 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/SUSAN A HAY/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
MEDYTOX INC.

Confirmation No.: 3639

Application No.: 16/338,561
Int'l Filing Date: November 29, 2017

Art Unit: 1777

For: **METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION**

Examiner: Ryan Huang

Amendment and Reply under 37 C.F.R. § 1.111

Mail Stop Amendment

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Commissioner:

In reply to the Office Action dated May 18, 2020, Applicant submits the following amendments and remarks.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks and Arguments begin on page 6 of this paper.

A one-month Extension of Time under 37 C.F.R. § 1.136(a) is being submitted herewith. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any additional fees required to continue prosecution or appeal of this application (including issue fee, fees for net addition of claims or forwarding to appeal) are hereby authorized to be charged to our Deposit Account No. 50-5875.

Amendments to the Claims

This listing of claims will replace all prior versions, and listings, of claims in the application.

1. (Currently amended) A method of isolating a botulinum toxin type A macro complex from a botulinum toxin-containing solution, the method comprising:

(a) contacting the botulinum toxin-containing solution with an anion exchange chromatography medium at a pH lower than an isoelectric point (PI) of botulinum toxin;

contacting [[the]] a flow-through portion of the botulinum toxin-containing solution, which is not bound to the anion exchange chromatography medium [[,]] with a cation exchange chromatography medium at a pH lower than the PI of botulinum toxin; and

separating the botulinum toxin type A macro complex from the cation exchange chromatography medium; or

(b) contacting the botulinum toxin-containing solution with [[the]] a cation exchange chromatography medium at a pH lower than [[the]] a PI of botulinum toxin;

separating botulinum toxin from the cation exchange chromatography medium;

contacting [[the]] a solution containing the botulinum toxin separated from the cation exchange chromatography medium [[,]] with [[the]] an anion exchange chromatography medium at a pH lower than the PI of botulinum toxin; and

separating the botulinum toxin type A macro complex from a flow-through portion of the solution containing the botulinum toxin, which is not bound to the anion exchange chromatography medium.

2. (Original) The method of claim 1, wherein the pH lower than the PI of botulinum toxin is pH 3.5 to 6.0.

3. (Currently amended) The method of claim 1, further comprising

adjusting [[the]] a pH of ~~the botulinum toxin or~~ the botulinum toxin-containing solution to a pH lower than the PI of botulinum toxin, before the contacting of the botulinum toxin-containing solution with the anion exchange chromatography medium at the pH lower than the PI of botulinum toxin in (a);

adjusting a pH of the flow-through portion of the botulinum toxin-containing solution that is not bound to the anion exchange chromatography medium, before the contacting of the flow-through portion of the botulinum toxin-containing solution with the cation exchange chromatography medium at the pH lower than the PI of botulinum toxin in (a);

adjusting a pH of the botulinum toxin-containing solution, before the contacting of the botulinum toxin-containing solution with the cation exchange chromatography medium at the pH lower than the PI of botulinum toxin in (b); or

adjusting a pH of the solution containing the botulinum toxin separated from the cation exchange chromatography medium, before the contacting of the solution containing the botulinum toxin separated from the cation exchange chromatography medium with the anion exchange chromatography medium at the pH lower than the PI of botulinum toxin in (b)~~, or before each of these two processes~~.

4. (Currently amended) The method of claim 3, wherein the adjusting comprises mixing ~~the botulinum toxin or~~ the botulinum toxin-containing solution, the flow-through portion

of the botulinum toxin-containing solution, or the solution containing the botulinum toxin with a buffer having a pH lower than the PI of botulinum toxin.

5. (Original) The method of claim 4, wherein the buffer is a phosphate buffer, a citrate buffer, or an acetate buffer.

6. (Currently amended) The method of claim 4, wherein the buffer has a pH of 3.5 to 6.0.

7. (Original) The method of claim 4, wherein the buffer comprises a 10 mM to 100 mM phosphate buffer, a 10 mM to 100 mM citrate buffer, or a 10 mM to 100 mM acetate buffer.

8. (Currently amended) The method of claim 1, wherein the anion exchange chromatography in the contacting of the botulinum toxin-containing solution with the anion exchange chromatography medium at the pH lower than the PI of botulinum toxin in (a) or in the contacting of the solution containing the botulinum toxin separated from the cation exchange chromatography medium with the anion exchange chromatography medium at the pH lower than the PI of botulinum toxin in (b) is DEAE or Q anion exchange chromatography.

9. (Currently amended) The method of claim 1, wherein the cation exchange chromatography in the contacting of the flow-through portion of the botulinum toxin-containing solution that is not bound to the anion exchange chromatography medium with the cation exchange chromatography medium at the pH lower than the PI of botulinum toxin in (a) or in the contacting

of the botulinum toxin-containing solution with the cation exchange chromatography medium at the pH lower than the PI of botulinum toxin in (b) is CM, S, or SP cation exchange chromatography.

Remarks

Upon entry of the foregoing amendments, claims 1-9 are pending in the application, with claim 1 being the independent claim. Claims 1, 3, 4, 6, 8, and 9 are sought to be amended. Support for "flow-through portion of the botulinum toxin-containing solution" can be found, *inter alia*, at paras. [0023] and [0059] of the published captioned application (US2020/0009473). Amendments were also made to better show antecedent basis and correct typographical errors.

These changes are believed to introduce no new matter, and their entry is respectfully requested.

Claim Objections

In view of the objections to claim 1 for informalities, claim 1 has been amended to remove the informalities. The objections should be withdrawn.

Rejection under 35 U.S.C. §112(b)

Claims 1-9 were rejected under 35 U.S.C. §112(b) as allegedly being indefinite. Claims 1, 3, 4, 6, 8, and 9 have been amended to clearly provide antecedent basis for the indicated terms. The rejection should be withdrawn.

Rejection under 35 U.S.C. §103

The rejection of claims 1-7 under 35 U.S.C. §103 as allegedly being unpatentable over TON (US9206409) with evidentiary support from THERMOSCI-AEX and THERMOSCI-CEX is respectfully traversed.

Claim 1 recites:

A method of isolating a botulinum toxin type A macro complex from a botulinum toxin-containing solution, the method comprising:

(a) contacting the botulinum toxin-containing solution with an anion exchange chromatography medium at a pH lower than an isoelectric point (PI) of botulinum toxin;

contacting a flow-through portion of the botulinum toxin-containing solution[[, which]] is not bound to the anion exchange chromatography medium, with a cation exchange chromatography medium at a pH lower than the PI of botulinum toxin; and

separating the botulinum toxin type A macro complex from the cation exchange chromatography medium; or

(b) contacting the botulinum toxin-containing solution with a cation exchange chromatography medium at a pH lower than a PI of botulinum toxin;

separating botulinum toxin from the cation exchange chromatography medium;

contacting a solution containing the botulinum toxin separated from the cation exchange chromatography medium with an anion exchange chromatography medium at a pH lower than the PI of botulinum toxin; and

separating the botulinum toxin type A macro complex from a flow-through portion of the solution containing the botulinum toxin, which is not bound to the anion exchange chromatography medium.

TON discloses:

An animal product free (APF) process for obtaining a botulinum neurotoxin, the process comprising the following steps: (a) providing an APF fermentation medium; (b) fermenting clostridium botulinum bacteria in the fermentation medium, wherein the clostridium botulinum bacteria produce a botulinum neurotoxin, and; (c) performing a first contacting step wherein the fermentation medium containing the botulinum neurotoxin is contacted with an anion exchange chromatography medium; (d) eluting the captured botulinum neurotoxin from the anion exchange medium to obtain a first eluate; (e) performing a second contacting step in which the first eluate is contacted with a cation exchange chromatography medium to obtain a second eluate; (f) contacting the second eluate with a hydrophobic interaction chromatography medium; and (g) eluting a third eluate from the hydrophobic interaction chromatography medium; thereby obtaining the botulinum neurotoxin.

See claim 1 of TON.

TON only discloses "(d) eluting the captured botulinum neurotoxin from the anion exchange medium to obtain a first eluate" and subsequent steps of processing the first eluate. Thus, TON does not suggest or provide a reason for "contacting a flow-through portion of the botulinum toxin-containing solution, which is not bound to the anion exchange chromatography medium, with a cation exchange chromatography medium at a pH lower than the PI of botulinum toxin" in claim 1(a) or "separating the botulinum toxin type A macro complex from a flow-through portion of the solution containing the botulinum toxin, which is not bound to the anion exchange chromatography medium" in claim 1(b) of the captioned application. Further, TON does not disclose "a pH lower than the PI of botulinum toxin,"

A "flow-through portion of the solution containing the botulinum toxin" differs from the "eluate" portion of the solution containing the botulinum toxin. The eluate portion refers to a solution comprising a material that was bound to the chromatography medium, for example, after flowing the solution containing the botulinum toxin to the chromatography medium and optionally washing the medium, and then separated from the chromatography medium by an elution process. In this regard, the material is still bound to the chromatography medium after flowing the solution containing the botulinum toxin to the chromatography medium and optionally washing. In contrast, a "flow-through portion" is a liquid flowing out of the chromatography without binding to the chromatography medium (see para. [0023] of the specification). This is indeed supported by the disclosure of TON itself:

> Particulars of the anion exchange step were: use of the POROS® 50HQ column using 0.1 N sodium hydroxide for a minimum contact time of 30 minutes (at least about 3 column volumes, at 230 cm/hour). The column was then equilibrated with a 50 mM sodium phosphate, pH 6.5 buffer (at least 5 column volumes). Next the clarified ultrafiltered and diluted material (i.e. processed lysate APF fermentation material) was loaded at 230 cm/hour onto the POROS® 50HQ anion exchange column, followed by washing with at least about 20 column volumes of 50 mM sodium phosphate, pH 6.5 at 230 cm/hour until absorbance at

> 280 nm of column effluent decreases to 0.10 AU, followed by eluting with 50 mM sodium acetate, pH 4.8 at 230 cm/hour. The product pool was collected, when the absorbance at 280 nm (A_{280}) increases to at least about 0.15 AU and through the peak maximum to equal or less than about 0.2 AU on the trailing edge, into a vessel containing 1 column volume of 50 mM sodium acetate, pH 4.8. This elution pool was stored at about 2° C. to about 8° C. for up to 48 hours.

Col. 31, line 51, to col. 32, line 3, of TON.

According to this disclosure in TON, APF fermentation material was loaded to an anion exchange column by flowing APF fermentation material through the anion exchange column, followed by washing with 50 mM sodium phosphate, and followed by eluting with 50 mM sodium acetate to obtain a product. In this process, the botulinum toxin should be bound to the anion exchange medium even after washing with 50 mM sodium phosphate.

Regarding the feature "a pH lower than the PI of botulinum toxin," the Office asserted that "TON teaches that botulinum neurotoxin type A is known to be soluble at pH 4-6.8 . . ., i.e., one of ordinary skill in the art would find it advantageous to maintain the harvested fermentation medium containing botulinum neurotoxin type A at a pH that would maintain its solubility in buffer (i.e., at pH 4-6.8)." OA, p. 4.

However, the fact that botulinum neurotoxin type A is known to be soluble at pH 4-6.8 would not have provided a reason for one of ordinary skill in the art to select "a pH lower than the PI of botulinum toxin" in the context of the process of TON. On the contrary, the pH in the context of the process of TON should be "a pH higher than the PI of botulinum toxin" because the botulinum toxin should be bound to the anion exchange medium after loading the fermentation material and optionally, even after washing with 50 mM sodium phosphate. The anion exchange medium comprise a positive charge in order to exchange an anion (see "Quaternary amine," THERMOSCI-AEX), and thus, the pH of the fermentation material should be higher than the PI of botulinum toxin to maintain its negative charge. Thus, TON does not disclose or provide a

reason to use "a pH lower than the PI of botulinum toxin," On the contrary, TON teaches away from "a pH lower than the PI of botulinum toxin."

In sum, in view of TON and the cited evidentiary support, one of ordinary skill in the art would not have a reason to modify "(d) eluting the captured botulinum neurotoxin from the anion exchange medium to obtain a first eluate" of TON into "contacting a flow-through portion of the botulinum toxin-containing solution, which is not bound to the anion exchange chromatography medium, with a cation exchange chromatography medium at a pH lower than the PI of botulinum toxin" of claim 1(a) or "separating the botulinum toxin type A macro complex from a flow-through portion of the solution containing the botulinum toxin, which is not bound to the anion exchange chromatography medium, in claim 1(b) of the captioned application. Further, TON does not disclose "a pH lower than the PI of botulinum toxin" as claimed. On the contrary, TON teaches away from the claimed invention since processing the captured botulinum neurotoxin cannot be compatible with processing a flow-through portion of the botulinum neurotoxin.

At least in view of the above, the rejection under 35 U.S.C. §103 should be withdrawn.

Conclusion

Prompt and favorable consideration of this Amendment and Reply is respectfully requested. Applicant believes the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Date: September 18, 2020

Respectfully submitted,

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By /Judith U. Kim/
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Atty. Dkt. No. 2479-0007US01

PTO/AIA/22 (03-13)
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PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)

Docket Number (Optional): 2479-0007US01

Application Number: 16/338,561

Filed: November 29, 2017

For: Method of Isolating Botulinum Toxin from Botulinum Toxin-Containing Solution

Art Unit: 1777

Examiner: Ryan HUANG

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	Micro Entity Fee	
☑	One month (37 CFR 1.17(a)(1))	$200	$100	$50	$ 200
☐	Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$
☐	Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$
☐	Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$
☐	Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 50-5875.

☑ Payment made via EFS-Web.

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I am the

☐ applicant.

☒ attorney or agent of record. Registration number 40679.

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____.

/Judith U. Kim/ — Signature

September 18, 2020 — Date

Judith U. Kim — Typed or printed name

(443) 926-6811 — Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

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2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal

Application Number:	16338561
Filing Date:	01-Apr-2019
Title of Invention:	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
First Named Inventor/Applicant Name:	Hyun Ho JUNG
Filer:	Judith Unjin Kim/Jessica LaBossiere
Attorney Docket Number:	2479-0007US01

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 1 month with $0 paid	1251	1	200	200
Miscellaneous:				
		Total in USD ($)		**200**

Electronic Acknowledgement Receipt

EFS ID:	40604306
Application Number:	16338561
International Application Number:	
Confirmation Number:	3639
Title of Invention:	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
First Named Inventor/Applicant Name:	Hyun Ho JUNG
Customer Number:	137713
Filer:	Judith Unjin Kim/Jessica LaBossiere
Filer Authorized By:	Judith Unjin Kim
Attorney Docket Number:	2479-0007US01
Receipt Date:	18-SEP-2020
Filing Date:	01-APR-2019
Time Stamp:	16:56:42
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$200
RAM confirmation Number	E20209HG56530171
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		2479-0007US01_Reply_to_OA_of_5-18-20.pdf	63729 3fece2b3d2cca598540a0108e141a6a96253461a	yes	10
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Amendment/Req. Reconsideration-After Non-Final Reject		1	1	
	Claims		2	5	
	Applicant Arguments/Remarks Made in an Amendment		6	10	
Warnings:					
Information:					
2	Extension of Time	2479-0007US01_EOT.pdf	141125 2bbaa08773ae0c301a1aa66721ff0e3565ca391a	no	2
Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	31113 9fd394619ee39bb2f22b04bbefdf0f53bb72a37f	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	235967		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/338,561	04/01/2019	Hyun Ho JUNG	2479-0007US01	3639

137713 7590 11/06/2020

Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

EXAMINER	
HUANG, RYAN	

ART UNIT	PAPER NUMBER
1777	

NOTIFICATION DATE	DELIVERY MODE
11/06/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

eofficeaction@appcoll.com
patents@potomaclaw.com

Office Action Summary	Application No. 16/338,561	Applicant(s) JUNG et al.	
	Examiner RYAN B HUANG	Art Unit 1777	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 18 September 2020.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-9 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-9 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date 28 July 2020.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Summary

This is a final Office action in reply to the amendments filed 18 September 2020 for the application filed 01 April 2019. Claims 1-9 are pending:

- Claims 1, 3, 4, 6, 8, and 9 have been amended.

Notice of Pre-AIA or AIA Status

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Priority

Applicant's claim for the benefit of a prior-filed application (371 of PCT/IB2017/057476, filed 29 November 2017) under 35 U.S.C. 119(e) or under 35 U.S.C. 120, 121, 365(c), or 386(c) is acknowledged.

Acknowledgment is made of applicant's claim for foreign priority (KR10-2016-0127540, filed 04 October 2016) under 35 U.S.C. 119 (a)-(d).

Response to Arguments

Applicant's amendments, filed 18 September 2020, with respect to the rejection of Claim 1 under 35 USC 103 as unpatentable over TON have been fully considered and are persuasive. This rejection has been withdrawn. However, upon further consideration and search, new grounds of rejection have been made under 35 USC 103 as unpatentable over KIM '741.

Applicant's amendments to Claim 1 have obviated the Claim Objections over Claim 1; however, Applicant has introduced amendments that warrant Claim Objections over Claim 1.

Applicant's amendments to Claims 1 and 3 have overcome the 35 USC 112(b) rejections of Claims 1 and 3; however, Applicant's amendments to Claims 8 and 9 have not addressed the lack of antecedent basis issues. Please refer to the relevant section below for proposed amendments.

All other arguments have been indirectly addressed.

Claim Interpretation

As noted in paragraph 21 of the Specification, the isoelectric point (pI) of botulinum toxin type A is about 6.06.

Claim Objections

Claim 1 is objected to because of the following informalities:

- Please add the removed comma in the phrase in lines 5-7: "contacting a flow-through portion of the botulinum toxin-containing solution, which is not bound to the anion exchange chromatography medium, with a cation exchange chromatography medium".

Appropriate correction is required.

Claim Rejections - 35 USC § 112

The following is a quotation of 35 U.S.C. 112(b):

(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

1. Claims 8 and 9 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

Regarding Claim 8, there is insufficient antecedent basis for "the anion exchange chromatography"; amend to remove this phrase, e.g., "The method of claim 1, wherein ~~the anion exchange chromatography~~ in the contacting of the botulinum toxin-containing solution…".

Regarding Claim 9, there is insufficient antecedent basis for "the cation exchange chromatography"; amend to remove this phrase, e.g., "The method of claim 1, wherein ~~the cation exchange chromatography~~ in the contacting of the flow-through portion of the botulinum toxin-containing solution…".

Claim Rejections - 35 USC § 103

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not

be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

The factual inquiries for establishing a background for determining obviousness under 35 U.S.C. 103 are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims the examiner presumes that the subject matter of the various claims was commonly owned as of the effective filing date of the claimed invention(s) absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and effective filing dates of each claim that was not commonly owned as of the effective filing date of the later invention in order for the examiner to consider the applicability of 35 U.S.C. 102(b)(2)(C) for any potential 35 U.S.C. 102(a)(2) prior art against the later invention.

2. Claims 1-9 is/are rejected under 35 U.S.C. 103 as being unpatentable over KIM et al. (US PGPub 2018/0251741 A1, effectively filed 30 November 2015).

Regarding Claim 1, KIM '741 discloses a method for producing and purifying botulinum toxin (p0023) including botulinum toxin type A complex (i.e., a method of isolating a botulinum toxin type A macro complex; p0048) whereby after an initial series of clarification and purification steps (steps a-e) of botulinum toxin produced from culture (i.e., a botulinum toxin-containing solution; p0041-0045), subsequent anion exchange (AEX, step f) and cation exchange (CEX, step h) purification steps are practiced (p0076, p0079-0081). The AEX chromatography step f is performed at a pH of 3-8, and botulinum toxin is collected in a flow-through fraction from the AEX column (i.e., contacting the botulinum toxin-containing solution with an anion exchange chromatography medium at a pH lower

than an isoelectric point of botulinum toxin; p0085). After diluting in a buffer in step g, the CEX chromatography step h is performed at a pH of 3-8 whereby botulinum toxin is collected as a fraction containing the botulinum toxin bound to the CEX resin (i.e., contacting a flow-through portion of the botulinum toxin-containing solution, which is not bound to the anion exchange chromatography medium with a cation exchange chromatography medium at a pH lower than the pI of botulinum toxin; separating the botulinum toxin type A macro complex from the cation exchange chromatography medium; p0087). Given the 6.06 isoelectric point (pI) of botulinum toxin type A, the taught pH ranges of 3-8 during AEX and CEX chromatography steps f and h overlap with the claimed ranges of "a pH lower than the pI of botulinum toxin" and therefore, establishes cases of *prima facie* obviousness (MPEP 2144.05).

Regarding Claim 2, as applied to the rejection of Claim 1, KIM '741 teaches the AEX and CEX chromatography steps f and h are performed at a pH of 3-8, which overlaps with the claimed range of 3.5 to 6.0 and therefore, establishes cases of *prima facie* obviousness (MPEP 2144.05).

Regarding Claim 3, as applied to the rejection of Claim 1, KIM '741 further discloses in step g the flow-through fraction of botulinum toxin collected from the AEX chromatography process of step f is diluted in a buffer of pH 6-8 (i.e., adjusting a pH of the flow-through portion of the botulinum toxin-containing solution that is not bound to the anion exchange chromatography medium before the contacting of the flow-through portion of the botulinum toxin-containing solution with the cation exchange chromatography medium at the pH lower than the pI of botulinum toxin; p0086).

Regarding Claim 4, as applied to the rejection of Claim 3, KIM '741 further discloses that step g utilizes a buffer of pH 6-8 (p0086), which overlaps with the claimed range of the buffer having a pH lower than the pI of botulinum toxin (i.e., less than 6.06) and therefore, establishes a case of *prima facie* obviousness (MPEP 2144.05).

Regarding Claim 5, as applied to the rejection of Claim 4, KIM '741 further discloses the buffer in step g is a sodium phosphate buffer (i.e., wherein the buffer is a phosphate buffer; p0086).

Regarding Claim 6, as applied to the rejection of Claim 4, KIM '741 further discloses that step g utilizes a buffer of pH 6-8 (p0086), which overlaps with the claimed range of the buffer having a pH of 3.5 to 6.0 and therefore, establishes a case of *prima facie* obviousness (MPEP 2144.05).

Regarding Claim 7, as applied to the rejection of Claim 4, KIM '741 further discloses 50 mM sodium phosphate buffer (p0134), which reads upon the claimed range of 10 mM to 100 mM.

Regarding Claim 8, as applied to the rejection of Claim 1, KIM '741 further discloses the anion exchange chromatography process utilizes diethylaminoethyl (DEAE) or quaternary amine (Q) resins (i.e., wherein the anion exchange chromatography… is DEAE or Q anion exchange chromatography; p0088).

Regarding Claim 9, as applied to the rejection of Claim 1, KIM '741 further discloses the cation exchange chromatography process utilizes carboxymethyl (CM), sulfonate (S), or phosphate (P) resins (i.e., wherein the cation exchange chromatography… is CM, S, or SP cation exchange chromatography; p0089).

Conclusion

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RYAN B HUANG whose telephone number is (571)270-0327. The examiner can normally be reached on 9 am-5 pm EST.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Vickie Kim can be reached on (571)272-0579. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or

Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Ryan B Huang/
Primary Examiner, Art Unit 1777

Notice of References Cited	Application/Control No. 16/338,561	Applicant(s)/Patent Under Reexamination JUNG et al.	
	Examiner RYAN B HUANG	Art Unit 1777	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-20180251741-A1	09-2018	Kim; Kyoung-Yun	B01D61/147	1/1
	B					
	C					
	D					
	E					
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/338,561	JUNG et al.
	Examiner	Art Unit
	RYAN B HUANG	1777

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	05/12/2020	11/03/2020							
	1	✓	✓							
	2	✓	✓							
	3	✓	✓							
	4	✓	✓							
	5	✓	✓							
	6	✓	✓							
	7	✓	✓							
	8	✓	✓							
	9	✓	✓							

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/338,561	JUNG et al.
	Examiner	Art Unit
	RYAN B HUANG	1777

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner
B01D15/36$ and A61K38/4893	05/12/2020	RBH
B01D15/36$	05/12/2020	RBH
B01D15/362 and B01D15/363	05/12/2020	RBH

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
Applicants search on EAST and PALM	05/12/2020	RBH
botulinum toxin generic search	05/12/2020	RBH
AEX flow-through, CEX bind-elute search	05/12/2020	RBH
Focused search on AEX flow through	11/03/2020	RBH
Considered PCT search report	11/03/2020	RBH

/Ryan B Huang/ Primary Examiner, Art Unit 1777	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/338,561	JUNG et al.
	Examiner	Art Unit
	RYAN B HUANG	1777

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/Ryan B Huang/ Primary Examiner, Art Unit 1777	

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	106	((("JUNG") near3 ("Hyun") near3 ("Ho")) OR (("YANG") near3 ("Gi") near3 ("Hyeok")) OR (("RHEE") near3 ("Chang") near3 ("Hoon")) OR (("KIM") near3 ("Hack") near3 ("Woo")) OR (("RYU") near3 ("Gi") near3 ("Hyuck")) OR (("HONG") near3 ("Hyung") near3 ("Pyo")) OR (("SONG") near3 ("Jung") near3 ("Hyun")) OR (("KONG") near3 ("Joon") near3 ("Chan"))).INV.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/05/12 11:49
S2	25	botulinum and S1	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/05/12 11:49
S3	0	B01D15/36$.cpc. and A61K38/4893.cpc.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/05/12 11:53
S4	3,952	B01D15/36$.cpc.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/05/12 11:53
S5	0	B01D15/36$.cpc. and A61K38/4893.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/05/12 11:53
S6	11,085	B01D15/36$.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/05/12 11:53
S7	2,024	B01D15/362.cpc. and B01D15/363.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/05/12 11:54

S8	83	botulinum and S7	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/05/12 11:56
S9	31	botulinum and S7	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/05/12 11:58
S10	46	botulinum same isoelectric adj point	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/05/12 15:30
S11	148	botulinum and (flow ajd through flow-through) with anion adj exchange and cation adj exchange	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/05/12 16:02
S12	113	botulinum and (flow ajd through flow-through) with anion adj exchange same cation adj exchange	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/05/12 16:03
S13	110	((("JUNG") near3 ("Hyun") near3 ("Ho")) OR (("YANG") near3 ("Gi") near3 ("Hyeok")) OR (("RHEE") near3 ("Chang") near3 ("Hoon")) OR (("KIM") near3 ("Hack") near3 ("Woo")) OR (("RYU") near3 ("Gi") near3 ("Hyuck")) OR (("HONG") near3 ("Hyung") near3 ("Pyo")) OR (("SONG") near3 ("Jung") near3 ("Hyun")) OR (("KONG") near3 ("Joon") near3 ("Chan"))).INV.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/11/03 15:35
S14	25	botulinum and S13	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/11/03 15:35
S15	0	B01D15/36$.cpc. and A61K38/4893.cpc.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/11/03 15:35
S16	4,136	B01D15/36$.cpc.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/11/03 15:35
S17	0	B01D15/36$.cpc. and A61K38/4893.cpc.	US-PGPUB; USPAT;	OR	ON	2020/11/03 15:35

			USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			
S18	11,578	B01D15/36$.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/11/03 15:35
S19	2,126	B01D15/362.cpc. and B01D15/363.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/11/03 15:35
S20	91	botulinum and S19	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/11/03 15:35
S21	31	botulinum and S19	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/11/03 15:35
S22	47	botulinum same isoelectric adj point	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/11/03 15:35
S23	156	botulinum and (flow ajd through flow-through) with anion adj exchange and cation adj exchange	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/11/03 15:35
S24	120	botulinum and (flow ajd through flow-through) with anion adj exchange same cation adj exchange	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/11/03 15:35
S25	61	botulinum and (flow adj through flow-through) with anion adj exchange and cation adj exchange	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/11/03 15:36
S26	33	botulinum and (flow adj through flow-through) with anion adj exchange same cation adj exchange	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/11/03 15:36
S27	2	"20150337281".pn.	US-PGPUB; USPAT;	OR	ON	2020/11/03 19:53

			USOCR; EPO; DERWENT			

EAST Search History (Interference)

<This search history is empty>

11/3/2020 8:47:29 PM
C:\Users\rhuang\Documents\EAST\Workspaces\16338561-Botox.wsp

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16338561
Filing Date	2017-11-29
First Named Inventor	Hyun Ho JUNG
Art Unit	1777
Examiner Name	Ryan HUANG
Attorney Docket Number	2479-0007US01

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	1185291	EP	A2	2002-03-13	BIOTECON GES FUER BIOTECHNOLOGISCHE ENTWICKLUNG &	English Abstract provided	
	2	2011050072	WO	A1	2011-04-28	REVANCE THERAPEUTICS, INC., et al.		
	3	2015016462	WO	A1	2015-02-05	DAEWOON CO., LTD.		

EFS Web 2.1.18

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /RBH/

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First Named Inventor	Hyun Ho JUNG
Art Unit	1777
Examiner Name	Ryan HUANG
Attorney Docket Number	2479-0007US01

	4	2017095062	WO	A1	2017-06-08	DAEWOONG CO., LTD.		

If you wish to add additional Foreign Patent Document citation information please click the Add button Add

NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1	DASGUPTA et al., "Cation-exchange chromatography of Clostridium botulinum type a toxin on amberlite IRC-50 resin at pH 5.55", Biochimica Et Biophysica ACTA - PROTEIN STRUCTURE, 1968, Vol. 168(3), pages 522-531, ELSEVIER SCIENCE BV, AMSTERDAM, NL	
	2	DASGUPTA et al., "Purification of Clostridium botulinum type a toxin", Biochimica Et Biophysica ACTA - PROTEIN STRUCTURE, 1970, Vol. 214(2), pages 343-349, ELSEVIER SCIENCE BV, AMSTERDAM, NL.	
	3	Extended European Search Report dated May 4, 2020, issued in European Application No. 17857967.8	
	4	SIMPSON et al., "Isolation and Characterization of the Botulinum Neurotoxins", METHODS IN ENZYMOLOGY, 1988, Vol. 165, pages 76-85, ELSEVIER, ACADEMIC PRESS, US.	

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature	/Ryan B Huang/	Date Considered	11/03/2020

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /RBH/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16338561
Filing Date	2017-11-29
First Named Inventor	Hyun Ho JUNG
Art Unit	1777
Examiner Name	Ryan HUANG
Attorney Docket Number	2479-0007US01

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☒ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Judith U. Kim/	Date (YYYY-MM-DD)	2020-07-28
Name/Print	Judith U. Kim	Registration Number	40679

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /RBH/

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 16/338,561	Filing Date 04/01/2019	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $460 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	02/08/2021	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 9	Minus	** 20	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0		x $480 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	SLIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/ALA H HUNTER/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/338,561	04/01/2019	Hyun Ho JUNG	2479-0007US01	3639

137713 7590 02/08/2021

Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

EXAMINER	
HUANG, RYAN	

ART UNIT	PAPER NUMBER
1777	

NOTIFICATION DATE	DELIVERY MODE
02/08/2021	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

eofficeaction@appcoll.com
patents@potomaclaw.com

Applicant-Initiated Interview Summary	**Application No.** 16/338,561	**Applicant(s)** JUNG et al.		
	Examiner RYAN B HUANG	**Art Unit** 1777	**AIA (First Inventor to File) Status** Yes	**Page** 1 of 1

All Participants (applicant, applicants representative, PTO personnel)	**Title**	**Type**
RYAN B HUANG	Primary Examiner	Telephonic
JUDITH KIM	Attorney of Record	

Date of Interview: 03 February 2021

Issues Discussed:

35 U.S.C. 112

Atty KIM asked about 112(b) rejections of Claims 8 and 9 regarding "anionic exchange chromatography" . Examiner HUANG explained that the method step of anionic exchange chromatography was never introduced in the claim or Claim 1; while the medium was introduced, such media can be used in any chromatography step, not necessarily only anionic exchange chromatography. Examiner HUANG suggested amending Claim 1 to introduce AEX chromatography steps.

No agreements were reached.

/Ryan B Huang/ Primary Examiner, Art Unit 1777	

Applicant is reminded that a complete written statement as to the substance of the interview must be made of record in the application file. It is the applicants responsibility to provide the written statement, unless the interview was initiated by the Examiner and the Examiner has indicated that a written summary will be provided. See MPEP 713.04
Please further see:
MPEP 713.04
Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews, paragraph (b)
37 CFR § 1.2 Business to be transacted in writing

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

Doc code: RCEX
Doc description: Request for Continued Examination (RCE)

PTO/SB/30EFS (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)

Application Number	16/338,561	Filing Date	2017-11-29	Docket Number (if applicable)	2479-0007US01	Art Unit	1777
First Named Inventor	Hyun Ho JUNG			Examiner Name	Ryan HUANG		

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV

SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

- ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
- ☐ Other _____

☒ Enclosed

- ☒ Amendment/Reply
- ☐ Information Disclosure Statement (IDS)
- ☐ Affidavit(s)/ Declaration(s)
- ☐ Other _____

MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____ (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

FEES

☒ **The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.**
The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to Deposit Account No 505875

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

☒ Patent Practitioner Signature

Applicant Signature

PTO/SB/30EFS (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Signature of Registered U.S. Patent Practitioner			
Signature	/Judith U. Kim/	Date (YYYY-MM-DD)	2021-02-08
Name	Judith U. Kim	Registration Number	40679

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

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6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

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9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal

Application Number:	16338561
Filing Date:	01-Apr-2019
Title of Invention:	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
First Named Inventor/Applicant Name:	Hyun Ho JUNG
Filer:	Judith Unjin Kim/Jessica LaBossiere
Attorney Docket Number:	2479-0007US01

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
RCE- 1ST REQUEST	1801	1	1360	1360
		Total in USD ($)		**1360**

Electronic Acknowledgement Receipt

EFS ID:	41864820
Application Number:	16338561
International Application Number:	
Confirmation Number:	3639
Title of Invention:	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
First Named Inventor/Applicant Name:	Hyun Ho JUNG
Customer Number:	137713
Filer:	Judith Unjin Kim/Jessica LaBossiere
Filer Authorized By:	Judith Unjin Kim
Attorney Docket Number:	2479-0007US01
Receipt Date:	08-FEB-2021
Filing Date:	01-APR-2019
Time Stamp:	15:59:42
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$1360
RAM confirmation Number	E202128F59522752
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		2479-007US01_Amdmt_Reply_to_FOA_filed_2-8-21.pdf	258917 2a0e101658831bb5a2f3458174b05b68ea9e9379	yes	10
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Response After Final Action		1	1	
	Claims		2	5	
	Applicant Arguments/Remarks Made in an Amendment		6	10	
Warnings:					
Information:					
2	Request for Continued Examination (RCE)	2479-0007US01_RCE.pdf	1364384 7aee2be766672ff052ec7a8136eaa30526f13680	no	3
Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	30869 68fefaffde23144fb12cbcfec7b1f3d96f3791cb	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	1654170		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
MEDYTOX INC.

Application No.: 16/338,561
Int'l Filing Date: November 29, 2017

For: **METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION**

Confirmation No.: 3639

Art Unit: 1777

Examiner: Ryan Huang

Atty Dkt. No. 2479-0007US01

Amendment and Reply under 37 C.F.R. § 1.114

Mail Stop RCE

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Commissioner:

In reply to the final Office Action dated November 6, 2020, Applicant submits the following amendments and remarks.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks and Arguments begin on page 6 of this paper.

It is not believed that extensions of time are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any additional fees required to continue prosecution or appeal of this application (including issue fee, fees for net addition of claims or forwarding to appeal) are hereby authorized to be charged to our Deposit Account No. 50-5875.

Hugel Ex. 1006 - Page 526 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

Amendments to the Claims

This listing of claims will replace all prior versions, and listings, of claims in the application.

1. (Currently amended) A method of isolating a botulinum toxin type A macro complex from a botulinum toxin-containing solution, the method comprising:

(a) contacting the botulinum toxin-containing solution with an anion exchange chromatography medium at a pH lower than an isoelectric point (PI) of botulinum toxin;

contacting a flow-through portion of the botulinum toxin-containing solution, which is not bound to the anion exchange chromatography medium, with a cation exchange chromatography medium at a pH lower than the PI of botulinum toxin; and

separating the botulinum toxin type A macro complex from the cation exchange chromatography medium; or

(b) contacting the botulinum toxin-containing solution with a cation exchange chromatography medium at a pH lower than a PI of botulinum toxin;

separating botulinum toxin from the cation exchange chromatography medium;

contacting a solution containing the botulinum toxin separated from the cation exchange chromatography medium with an anion exchange chromatography medium at a pH lower than the PI of botulinum toxin; and

separating the botulinum toxin type A macro complex from a flow-through portion of the solution containing the botulinum toxin, which is not bound to the anion exchange chromatography medium.

2. (Original) The method of claim 1, wherein the pH lower than the PI of botulinum toxin is pH 3.5 to 6.0.

3. (Previously presented) The method of claim 1, further comprising

adjusting a pH of the botulinum toxin-containing solution to a pH lower than the PI of botulinum toxin, before the contacting of the botulinum toxin-containing solution with the anion exchange chromatography medium at the pH lower than the PI of botulinum toxin in (a);

adjusting a pH of the flow-through portion of the botulinum toxin-containing solution that is not bound to the anion exchange chromatography medium, before the contacting of the flow-through portion of the botulinum toxin-containing solution with the cation exchange chromatography medium at the pH lower than the PI of botulinum toxin in (a);

adjusting a pH of the botulinum toxin-containing solution, before the contacting of the botulinum toxin-containing solution with the cation exchange chromatography medium at the pH lower than the PI of botulinum toxin in (b); or

adjusting a pH of the solution containing the botulinum toxin separated from the cation exchange chromatography medium, before the contacting of the solution containing the botulinum toxin separated from the cation exchange chromatography medium with the anion exchange chromatography medium at the pH lower than the PI of botulinum toxin in (b).

4. (Previously presented) The method of claim 3, wherein the adjusting comprises mixing the botulinum toxin-containing solution, the flow-through portion of the botulinum toxin-containing solution, or the solution containing the botulinum toxin with a buffer having a pH lower

than the PI of botulinum toxin.

5. (Original) The method of claim 4, wherein the buffer is a phosphate buffer, a citrate buffer, or an acetate buffer.

6. (Previously presented) The method of claim 4, wherein the buffer has a pH of 3.5 to 6.0.

7. (Original) The method of claim 4, wherein the buffer comprises a 10 mM to 100 mM phosphate buffer, a 10 mM to 100 mM citrate buffer, or a 10 mM to 100 mM acetate buffer.

8. (Currently amended) The method of claim 1, wherein the anion exchange chromatography medium ~~in the contacting of the botulinum toxin-containing solution with the anion exchange chromatography medium at the pH lower than the PI of botulinum toxin in (a) or in the contacting of the solution containing the botulinum toxin separated from the cation exchange chromatography medium with the anion exchange chromatography medium at the pH lower than the PI of botulinum toxin in (b) is~~ comprises DEAE or Q anion exchange resin ~~chromatography~~.

9. (Currently amended) The method of claim 1, wherein the cation exchange chromatography medium ~~in the contacting of the flow-through portion of the botulinum toxin-containing solution that is not bound to the anion exchange chromatography medium with the cation exchange chromatography medium at the pH lower than the PI of botulinum toxin in (a) or~~

~~in the contacting of the botulinum toxin-containing solution with the cation exchange chromatography medium at the pH lower than the PI of botulinum toxin in (b) is~~ comprises CM, S, or SP cation exchange resin ~~chromatography~~.

Remarks

Upon entry of the foregoing amendments, claims 1-9 are pending in the application, with claim 1 being the independent claim. Claims 1, 8, and 9 are sought to be amended. Amendments were made to better show antecedent basis and correct a typographical error.

These changes are believed to introduce no new matter, and their entry is respectfully requested.

Statement of Interview Summary

Applicant thanks Examiner Ryan Huang for the telephonic interview of February 3, 2021 with Applicant's undersigned representative. Clarification of the 112(b) rejection was requested and the Examiner explained that there was no antecedent basis for "chromatography" in claim 1. No agreement was reached.

Claim Objection

In view of the objection to claim 1, a comma has been inserted in claim 1. The objection should be withdrawn.

Rejection under 35 U.S.C. §112(b)

Claims 8 and 9 were rejected under 35 U.S.C. §112(b) as allegedly being indefinite. Claims 8 and 9 have been amended for sufficient antecedent basis to "chromatography medium" in claim 1. The rejection should be withdrawn.

Rejection under 35 U.S.C. §103

The rejection of claims 1-9 under 35 U.S.C. §103 as allegedly being unpatentable over KIM (US2018/0251741, effectively filed November 30, 2015 according to the Office), is respectfully traversed without Applicant's admission as to the effective date of November 30, 2015 for KIM.

As recited in amended claim 1, a flow-through portion is obtained from the anion exchange chromatography (AEX) at a pH lower than an isoelectric point (PI) of botulinum toxin and the bound portion is obtained from the cation exchange chromatography (CEX) at a pH lower than an isoelectric point (PI) of botulinum toxin to isolate a botulinum toxin type A macro complex.

The Office states that

> KIM '741 discloses a method for producing and purifying botulinum toxin (. . . p0023) including botulinum toxin type A complex (p0048) whereby after an initial series of clarification and purification steps (steps a-e) of botulinum toxin produced from culture (. . . p0041-p0045), subsequent anion exchange (AEX, step f) and cation exchange (CEX, step h) purification steps are practiced (. . . p0076, p0079-0081). The AEX chromatography step f is performed at a pH of 3-8, and botulinum toxin is collected in a flow-through fraction from the AEX column (. . . p0085). After diluting in a buffer in step g, the CEX chromatography step h is performed at a pH of 3-8 whereby botulinum toxin is collected as a fraction containing the botulinum toxin bound to the CEX resin (. . . p0087). Given the 6.06 isoelectric point (PI) of botulinum toxin type A, the taught pH ranges of 3-8 during AEX and CEX chromatography steps f and h overlap with the claimed ranges of "a pH lower than the PI of botulinum toxin" and therefore, establishes cases of *prima facie obviousness* (MPEP2144.05).

OA, para. bridging pgs. 4-5.

Applicant points out that an ion-exchange chromatography separates ions based on their affinity to the ion exchanger. The two types of ion chromatography are anion-exchange and cation-

exchange. Cation-exchange chromatography is used when the molecule of interest is positively charged. In this type of chromatography, the stationary phase is negatively charged and positively charged molecules are loaded to be attracted to it. Anion-exchange chromatography is used when the stationary phase is positively charged and negatively charged molecules are loaded to be attracted to it.

An isoelectric point (PI) of a protein is a pH at which the overall number of negative and positive charges is zero. Thus, a botulinum toxin molecule in a pH lower than an isoelectric point (PI) of botulinum toxin has an opposite charge compared to the same molecule in a pH higher than an isoelectric point (PI) of botulinum toxin. This means that when botulinum toxin is present in a flow-through portion at a pH lower than a PI of botulinum toxin in AEX, the botulinum toxin would not be present in a flow-through portion at a pH higher than a PI of botulinum toxin in AEX.

KIM discloses a broad pH range of 2-9, preferably 3-8, for anion exchange and cation exchange chromatographies. However, in the Examples, the actual pH during the anion and cation exchange chromatographies was higher than the PI of botulinum toxin:

- TQ purification (anion exchange chromatography flow-through (first AEX, FT)) at a pH higher than the PI, i.e., at pH 6.4-6.6 (see pg. 8, paras. 0107 and 0109),
- HQ purification (anion exchange chromatography binding mode (second AEX, binding mode)) using the samples purified by TQ at a pH higher than the PI, i.e., at pH 7.7-7.9 (see pg. 9, paras. 01113, and 0115), and
- XS purification (cation exchange chromatography binding mode (CEX, binding mode)) using the samples purified by HQ at a pH higher than the PI, i.e., at pH 6.9-7.1 (see pg. 9, paras. 0119 and 0122).

Further, KIM discloses that the final purified botulinum toxin is a form of 7S (molecular weight: about 150 kDa), not 19S (molecular weight: about 900 kDa) (see page 9, para. 0125).

The general disclosure in KIM of a pH of 2-9, preferably 3-8, in which the AEX and CEX can be performed does not provide a reason for one of ordinary skill in the art to select "a pH lower than the PI of botulinum toxin." Thus, it is clear that KIM does not disclose or provide a reason to perform AEX in flow through mode and CEX in binding mode, all at a pH lower than a PI of botulinum toxin. In view of KIM, one of skill also would not have had a reasonable expectation of success in the claimed method of the captioned application.

On the contrary, the pH used in the Examples of KIM for the AEX and CEX was a pH higher than the PI of botulinum toxin. Thus, KIM in fact teaches away from "a pH lower than the PI of botulinum toxin" as recited in the claims.

At least in view of the above, the rejection under 35 U.S.C. §103 should be withdrawn.

Conclusion

All of the stated grounds of objections and rejections have been properly traversed, accommodated, or rendered moot. Applicant therefore respectfully requests that the Examiner reconsider all objections and rejections and that they be withdrawn. Applicant believes that a full and complete reply has been made to the Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Date: February 8, 2021

Respectfully submitted,

Potomac Law Group, PLLC
8229 Boone Blvd.
Suite 430
Vienna, VA 22182
(443) 926-6811

By /Judith U. Kim/
Judith U. Kim
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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/338,561	04/01/2019	Hyun Ho JUNG	2479-0007US01	3639

137713 7590 02/10/2021

Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

EXAMINER	
HUANG, RYAN	

ART UNIT	PAPER NUMBER
1777	

NOTIFICATION DATE	DELIVERY MODE
02/10/2021	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

eofficeaction@appcoll.com
patents@potomaclaw.com

Applicant-Initiated Interview Summary	**Application No.** 16/338,561	**Applicant(s)** JUNG et al.		
	Examiner RYAN B HUANG	**Art Unit** 1777	**AIA (First Inventor to File) Status** Yes	**Page** 1 of 2

All Participants (applicant, applicants representative, PTO personnel)	**Title**	**Type**
RYAN B HUANG	Primary Examiner	Telephonic
JUDITH KIM	Attorney of Record	

Date of Interview: 05 February 2021

Issues Discussed:

35 U.S.C. 112

Proposed amendments for Claims 8 and 9 to state AEX/CEX "medium" would likely overcome 112b rejections.

35 U.S.C. 103

Atty KIM considered finality of previous Office action to be premature because the prior filed amendment did not change the scope/further limit the claim. Examiner HUANG disagreed - the amendment required that the "flow through" solution of botox in AEX be subsequently treated by CEX, whereas the original claim only required that the solution of botox in AEX be subsequently treated by CEX. Prior claim was broad - the botox solution could point toward either flow through or eluted solution from AEX; amended claim that was finally rejected further limited the scope to only the non-eluted flow through solution.

No agreements were reached.

Proposed Amendment(s)

See 112 above.

/Ryan B Huang/ Primary Examiner, Art Unit 1777	

Applicant is reminded that a complete written statement as to the substance of the interview must be made of record in the application file. It is the applicants responsibility to provide the written statement, unless the interview was initiated by the Examiner and the Examiner has indicated that a written summary will be provided. See MPEP 713.04
Please further see:
MPEP 713.04
Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews, paragraph (b)
37 CFR § 1.2 Business to be transacted in writing

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview.

Applicant-Initiated Interview Summary	**Application No.** 16/338,561	**Applicant(s)** JUNG et al.		
	Examiner RYAN B HUANG	**Art Unit** 1777	**AIA (First Inventor to File) Status** Yes	**Page** **2 of 2**

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
MEDYTOX INC.

Application No.: 16/338,561
Int'l Filing Date: November 29, 2017

For: **METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION**

Confirmation No.: 3639

Art Unit: 1777

Examiner: Ryan Huang

Atty Dkt. No. 2479-0007US01

Statement of Substance of Applicant-Initiated Interview Summary

Mail Stop RCE

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Commissioner:

Further to the Applicant-Initiated Interview Summary dated February 10, 2021, Applicant provides the following summary.

Applicant thanks Examiner Ryan Huang for the telephone interview with Applicant's undersigned representative on February 5, 2021.

Proposed amendments to claims 8 and 9 were discussed for antecedent basis to AEX/CEX "medium."

The Examiner also explained the reason for the finality of the Office Action: the amendment in the prior Amendment and Reply required the "flow through" solution whereas the original claim required the "solution."

It is not believed that extensions of time are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any additional fees required to continue prosecution or

appeal of this application (including issue fee, fees for net addition of claims or forwarding to appeal) are hereby authorized to be charged to our Deposit Account No. 50-5875.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Date: February 16, 2021

Respectfully submitted,

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(443) 926-6811

By /Judith U. Kim/
Judith U. Kim
Attorney for Applicant
Registration No.: 40,679

Electronic Acknowledgement Receipt

EFS ID:	41930740
Application Number:	16338561
International Application Number:	
Confirmation Number:	3639
Title of Invention:	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
First Named Inventor/Applicant Name:	Hyun Ho JUNG
Customer Number:	137713
Filer:	Judith Unjin Kim/Jessica LaBossiere
Filer Authorized By:	Judith Unjin Kim
Attorney Docket Number:	2479-0007US01
Receipt Date:	16-FEB-2021
Filing Date:	01-APR-2019
Time Stamp:	15:49:17
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant summary of interview with examiner	2479-007US01_Statement_of_Interview_Summary_filed_2-16-21.pdf	177464 b8b9dfa28f12524ba1e468c6dab4c422b41aef78	no	2

Warnings:

Information:	
Total Files Size (in bytes):	177464

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

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New International Application Filed with the USPTO as a Receiving Office

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/338,561	04/01/2019	Hyun Ho JUNG	2479-0007US01	3639

137713 7590 04/22/2021

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Suite 430
Vienna, VA 22182

EXAMINER	
HUANG, RYAN	

ART UNIT	PAPER NUMBER
1777	

NOTIFICATION DATE	DELIVERY MODE
04/22/2021	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

eofficeaction@appcoll.com
patents@potomaclaw.com

Office Action Summary	Application No. 16/338,561	Applicant(s) JUNG et al.	
	Examiner RYAN B HUANG	Art Unit 1777	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 08 February 2021.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-9 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-9 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Summary

This is a non-final Office action in reply to the Request for Continued Examination under 37 CFR 1.114 filed 08 February 2021 for the application filed 01 April 2019. Claims 1-9 are pending:

- Claims 1, 8, and 9 have been amended.

Notice of Pre-AIA or AIA Status

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Continued Examination Under 37 CFR 1.114

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 08 February 2021 has been entered.

Priority

Applicant's claim for the benefit of a prior-filed application (371 of PCT/IB2017/057476, filed 29 November 2017) under 35 U.S.C. 119(e) or under 35 U.S.C. 120, 121, 365(c), or 386(c) is acknowledged.

Acknowledgment is made of applicant's claim for foreign priority (KR10-2016-0127540, filed 04 October 2016) under 35 U.S.C. 119 (a)-(d).

Response to Arguments

Applicant's amendments and arguments, filed 08 February 2021, have been fully considered.

Regarding the Claim Objections over Claim 1, Applicant's amendments are persuasive; these objections have been withdrawn.

Regarding the 35 USC 112(b) rejections of Claims 8 and 9, Applicant's amendments are persuasive; these rejections have been withdrawn.

Regarding the 35 USC 103 rejections of Claims 1-9 as unpatentable over KIM '741, Applicant's arguments have been fully considered but are not persuasive.

Applicant argues that the examples provided in KIM teach pH values higher than the isoelectric point (PI) of botulinum toxin (pg. 8-9); and, apparently, Applicant seems to also argue that KIM fails to disclose isolation of botulinum toxin macro complex (pg. 7, middle; pg. 9, top). Applicant concludes that because KIM does not provide "reason for one of ordinary skill in the art to select 'a pH lower than the PI of botulinum toxin'… one of skill would not have had a reasonable expectation of success in the claimed method" and further "KIM… teaches away from 'a pH lower than the PI of botulinum toxin'" (pg. 9).

The Examiner respectfully disagrees.

Disclosed examples and preferred embodiments do not constitute a teaching away from a broader disclosure or nonpreferred embodiments (*In re* Susi, 440 F.2d 442, 169 USPQ 423 (CCPA 1971)). "A known or obvious composition does not become patentable simply because it has been described as somewhat inferior to some other product for the same use" (*In re* Gurley, 27 F.3d 551, 554, 31 USPQ2d 1130, 1132 (Fed. Cir. 1994); MPEP §2123 II).

While KIM may have disclosed higher pH solvents and buffers in the examples, KIM nonetheless does disclose pH ranges that encompass the claimed ranges. As noted in the 35 USC 103 rejection, The AEX chromatography step f is performed at a pH of 3-8, and botulinum toxin is collected in a flow-through fraction from the AEX column (p0085). In step h, CEX chromatography is performed at a pH of 3-8 whereby botulinum toxin is collected as a fraction containing the botulinum toxin bound to the CEX resin (p0087). Given the 6.06 isoelectric point (pI) of botulinum toxin type A, the taught pH ranges of 3-8 during AEX and CEX chromatography steps f and h overlap with the claimed ranges of "a pH lower than the pI of botulinum toxin" and therefore, establishes cases of *prima facie* obviousness (MPEP 2144.05). Absent showings of significance or criticality to the claimed "a pH lower than the pI of botulinum toxin", the disclosure of KIM would render the claimed invention obvious to one of ordinary skill in the art.

Please note: a properly filed declaration or 1.312 affidavit showing data comparing the relative efficiency (or similar measure) of botulinum toxin recovery between a process using pH lower than the pI of botulinum toxin and a process using pH higher than the pI of botulinum toxin would be considered by the Examiner. Data provided in the original Specification does not sufficiently show this comparison. Please note that statistical significance of the

comparison and nexus between such results and the claimed invention must be met to overcome this obviousness rejection.

Regarding Applicant's inference that KIM fails to disclose isolating botulinum toxin macro complex, the Examiner respectfully disagrees. p0050 of KIM explicitly discloses that the taught method isolates botulinum complex: "the term 'produced botulinum toxin' means a pure botulinum toxin or a botulinum toxin complex…". The previous paragraphs, p0048-0049 further indicate botulinum toxin complexes of 900 kDa (p0048) with non-toxic components (p0049). These citations align with Applicant's definition of "botulinum toxin macro complex" (see disclosure paragraph 6).

All other arguments have been indirectly addressed.

Claim Interpretation

As noted in paragraph 21 of the Specification, the isoelectric point (pI) of botulinum toxin type A is about 6.06.

As noted in paragraph 6 of the Specification, a botulinum toxin macro complex is 900 kDa and includes 150 kDa of botulinum toxin (A-G) and a complex of a variety of non-toxic proteins.

Claim Rejections - 35 USC § 103

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

The factual inquiries for establishing a background for determining obviousness under 35 U.S.C. 103 are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims the examiner presumes that the subject matter of the various claims was commonly owned as of the effective filing date of the claimed invention(s) absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and effective filing dates of each claim that was not commonly owned as of the effective filing date of the later invention in order for the examiner to consider the applicability of 35 U.S.C. 102(b)(2)(C) for any potential 35 U.S.C. 102(a)(2) prior art against the later invention.

1. Claims 1-9 is/are rejected under 35 U.S.C. 103 as being unpatentable over KIM et al. (US PGPub 2018/0251741 A1, effectively filed 30 November 2015).

Regarding Claim 1, KIM '741 discloses a method for producing and purifying botulinum toxin (p0023) including botulinum toxin type A macro complex (i.e., a method of isolating a botulinum toxin type A macro complex; p0048-0050) whereby after an initial series of clarification and purification steps (steps a-e) of botulinum toxin produced from culture (i.e., a botulinum toxin-containing solution; p0041-0045), subsequent anion exchange (AEX, step f) and cation exchange (CEX, step h) purification steps are practiced (p0076, p0079-0081).

The AEX chromatography step f is performed at a pH of 3-8, and botulinum toxin is collected in a flow-through fraction from the AEX column (i.e., contacting the botulinum toxin-containing solution with an anion exchange chromatography medium at a pH lower than an isoelectric point of botulinum toxin; p0085; "flow-through" defined in p0073). After diluting in a buffer in step g, the CEX chromatography step h is performed at a pH of 3-8 whereby botulinum toxin is collected as a fraction containing the botulinum toxin bound to the CEX resin (i.e., contacting a flow-through portion of the botulinum toxin-containing solution, which is not bound to the anion exchange chromatography medium with a cation exchange chromatography medium at a pH lower than the pI of botulinum toxin; separating the botulinum toxin type A macro complex from the cation exchange chromatography medium; p0087). Given the 6.06 isoelectric point (pI) of botulinum toxin type A, the taught pH ranges of 3-8 during AEX and CEX chromatography steps f and h overlap with the claimed ranges of "a pH lower than the pI of botulinum toxin" and therefore, establishes cases of *prima facie* obviousness (MPEP 2144.05).

(The Examiner further notes that KIM '741 seems to disclose in p0054 that botulinum toxin recovery rate is advantageously higher at pH 3.0-4.5, preferably 3.4-3.6. However, this is a recovery rate with respect to botulinum toxin recovery from bacterial culture, not chromatography.)

Regarding Claim 2, as applied to the rejection of Claim 1, KIM '741 teaches the AEX and CEX chromatography steps f and h are performed at a pH of 3-8, which overlaps with the claimed range of 3.5 to 6.0 and therefore, establishes cases of *prima facie* obviousness (MPEP 2144.05).

Regarding Claim 3, as applied to the rejection of Claim 1, KIM '741 further discloses in step g the flow-through fraction of botulinum toxin collected from the AEX chromatography process of step f is diluted in a buffer of pH 6-8 (i.e., adjusting a pH of the flow-through portion of the botulinum toxin-containing solution that is not bound to the anion exchange chromatography medium before the contacting of the flow-through portion of the botulinum toxin-containing solution with the cation exchange chromatography medium at the pH lower than the pI of botulinum toxin; p0086).

Regarding Claim 4, as applied to the rejection of Claim 3, KIM '741 further discloses that step g utilizes a buffer of pH 6-8 (p0086), which overlaps with the claimed range of the buffer having a pH lower than the pI of botulinum toxin (i.e., less than 6.06) and therefore, establishes a case of *prima facie* obviousness (MPEP 2144.05).

Regarding Claim 5, as applied to the rejection of Claim 4, KIM '741 further discloses the buffer in step g is a sodium phosphate buffer (i.e., wherein the buffer is a phosphate buffer; p0086).

Regarding Claim 6, as applied to the rejection of Claim 4, KIM '741 further discloses that step g utilizes a buffer of pH 6-8 (p0086), which overlaps with the claimed range of the buffer having a pH of 3.5 to 6.0 and therefore, establishes a case of *prima facie* obviousness (MPEP 2144.05).

Regarding Claim 7, as applied to the rejection of Claim 4, KIM '741 further discloses 50 mM sodium phosphate buffer (p0134), which reads upon the claimed range of 10 mM to 100 mM.

Regarding Claim 8, as applied to the rejection of Claim 1, KIM '741 further discloses the anion exchange chromatography process utilizes diethylaminoethyl (DEAE) or quaternary amine (Q) resins (i.e., wherein the anion exchange chromatography… is DEAE or Q anion exchange chromatography; p0088).

Regarding Claim 9, as applied to the rejection of Claim 1, KIM '741 further discloses the cation exchange chromatography process utilizes carboxymethyl (CM), sulfonate (S), or phosphate (P) resins (i.e., wherein the cation exchange chromatography… is CM, S, or SP cation exchange chromatography; p0089).

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RYAN B HUANG whose telephone number is (571)270-0327. The examiner can normally be reached on 9 am-5 pm EST.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Vickie Kim can be reached on (571)272-0579. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Ryan B Huang/
Primary Examiner, Art Unit 1777

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/338,561	JUNG et al.
	Examiner	Art Unit
	RYAN B HUANG	1777

CPC - Searched*		
Symbol	Date	Examiner
B01D15/36$	04/19/2021	RBH

CPC Combination Sets - Searched*		
Symbol	Date	Examiner
B01D15/36$ and A61K38/4893	05/12/2020	RBH
B01D15/36$	05/12/2020	RBH
B01D15/362 and B01D15/363	05/12/2020	RBH

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

/Ryan B Huang/ Primary Examiner, Art Unit 1777	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/338,561	JUNG et al.
	Examiner	Art Unit
	RYAN B HUANG	1777

Search Notes		
Search Notes	**Date**	**Examiner**
Applicants search on EAST and PALM	05/12/2020	RBH
botulinum toxin generic search	05/12/2020	RBH
AEX flow-through, CEX bind-elute search	05/12/2020	RBH
Focused search on AEX flow through	11/03/2020	RBH
Considered PCT search report	11/03/2020	RBH
Applicant is arguing KIM teaches away from claimed invention	04/19/2021	RBH
Reviewed KIM; examples may have disclosed higher pH, but general disclosure does not	04/19/2021	RBH
Affidavit/declaration with statistical significance may be sufficient to overcome rejection, allowance	04/19/2021	RBH
Additional CPC/text search	04/19/2021	RBH

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**

/Ryan B Huang/ Primary Examiner, Art Unit 1777	

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/338,561	JUNG et al.
	Examiner	Art Unit
	RYAN B HUANG	1777

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	05/12/2020	11/03/2020	04/19/2021						
	1	✓	✓	✓						
	2	✓	✓	✓						
	3	✓	✓	✓						
	4	✓	✓	✓						
	5	✓	✓	✓						
	6	✓	✓	✓						
	7	✓	✓	✓						
	8	✓	✓	✓						
	9	✓	✓	✓						

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	61	isoelectric same (botulinum botox)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/04/19 09:58
L2	11	(US-20200009473-$ or US-20150165003-$ or US-20140086900-$ or US-20110091937-$ or US-20100291136-$ or US-20180251741-$ or US-20150337281-$ or US-20110092682-$).did. or (US-9206409-$).did. or (US-20170327811-$ or US-20150322419-$).did.	US-PGPUB; USPAT; DERWENT	OR	ON	2021/04/19 09:58
L3	3	1 and 2	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/04/19 09:58
L5	2	isoelectric same (botulinum botox) same (CEX cation$3 adj exchange)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/04/19 10:03
L6	3	isoelectric same (botulinum botox) same (AEX anion$3 adj exchange)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/04/19 10:04
L7	4,328	B01D15/36$.cpc.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2021/04/19 10:07
L8	4	L7 and 1	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/04/19 10:07

L9	153	(botulinum botox) same (AEX anion$3 adj exchange)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/04/19 11:19
L10	114	(botulinum botox) same (CEX cation$3 adj exchange)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/04/19 11:19
L11	75	9 and 10	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/04/19 11:20
L12	55	pH and 11	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/04/19 11:20
L13	38	(low lower acid acidic) with pH and 11	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2021/04/19 11:20
S1	106	((("JUNG") near3 ("Hyun") near3 ("Ho")) OR (("YANG") near3 ("Gi") near3 ("Hyeok")) OR (("RHEE") near3 ("Chang") near3 ("Hoon")) OR (("KIM") near3 ("Hack") near3 ("Woo")) OR (("RYU") near3 ("Gi") near3 ("Hyuck")) OR (("HONG") near3 ("Hyung") near3 ("Pyo")) OR (("SONG") near3 ("Jung") near3 ("Hyun")) OR (("KONG") near3 ("Joon") near3 ("Chan"))).INV.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/05/12 11:49
S2	25	botulinum and S1	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/05/12 11:49
S3	0	B01D15/36$.cpc. and A61K38/4893.cpc.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/05/12 11:53

S4	3,952	B01D15/36$.cpc.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/05/12 11:53
S5	0	B01D15/36$.cpc. and A61K38/4893.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/05/12 11:53
S6	11,085	B01D15/36$.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/05/12 11:53
S7	2,024	B01D15/362.cpc. and B01D15/363.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/05/12 11:54
S8	83	botulinum and S7	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/05/12 11:56
S9	31	botulinum and S7	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/05/12 11:58
S10	46	botulinum same isoelectric adj point	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/05/12 15:30
S11	148	botulinum and (flow ajd through flow-through) with anion adj exchange and cation adj exchange	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/05/12 16:02
S12	113	botulinum and (flow ajd through flow-through) with anion adj exchange same cation adj exchange	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/05/12 16:03
S13	110	((("JUNG") near3 ("Hyun") near3 ("Ho")) OR (("YANG") near3 ("Gi") near3 ("Hyeok")) OR (("RHEE") near3 ("Chang") near3 ("Hoon")) OR (("KIM")	US-PGPUB; USPAT; USOCR; EPO;	OR	ON	2020/11/03 15:35

		near3 ("Hack") near3 ("Woo")) OR (("RYU") near3 ("Gi") near3 ("Hyuck")) OR (("HONG") near3 ("Hyung") near3 ("Pyo")) OR (("SONG") near3 ("Jung") near3 ("Hyun")) OR (("KONG") near3 ("Joon") near3 ("Chan"))).INV.	JPO; DERWENT			
S14	25	botulinum and S13	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/11/03 15:35
S15	0	B01D15/36$.cpc. and A61K38/4893.cpc.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/11/03 15:35
S16	4,136	B01D15/36$.cpc.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	ON	2020/11/03 15:35
S17	0	B01D15/36$.cpc. and A61K38/4893.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/11/03 15:35
S18	11,578	B01D15/36$.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/11/03 15:35
S19	2,126	B01D15/362.cpc. and B01D15/363.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/11/03 15:35
S20	91	botulinum and S19	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2020/11/03 15:35
S21	31	botulinum and S19	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/11/03 15:35

S22	47	botulinum same isoelectric adj point	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/11/03 15:35
S23	156	botulinum and (flow ajd through flow-through) with anion adj exchange and cation adj exchange	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/11/03 15:35
S24	120	botulinum and (flow ajd through flow-through) with anion adj exchange same cation adj exchange	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/11/03 15:35
S25	61	botulinum and (flow adj through flow-through) with anion adj exchange and cation adj exchange	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/11/03 15:36
S26	33	botulinum and (flow adj through flow-through) with anion adj exchange same cation adj exchange	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/11/03 15:36
S27	2	"20150337281".pn.	US-PGPUB; USPAT; USOCR; EPO; DERWENT	OR	ON	2020/11/03 19:53

EAST Search History (Interference)

<This search history is empty>

4/19/2021 11:23:36 AM
C:\Users\rhuang\Documents\EAST\Workspaces\16338561-Botox.wsp

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
MEDYTOX INC.

Application No.: 16/338,561
Int'l Filing Date: November 29, 2017

For: **METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION**

Confirmation No.: 3639

Art Unit: 1777

Examiner: Ryan Huang

Atty Dkt. No. 2479-0007US01

Amendment and Reply under 37 C.F.R. § 1.111

Mail Stop Amendment

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Commissioner:

In reply to the Office Action dated April 22, 2021, Applicant submits the following amendments and remarks.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks and Arguments begin on page 5 of this paper.

It is not believed that extensions of time are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any additional fees required to continue prosecution or appeal of this application (including issue fee, fees for net addition of claims or forwarding to appeal) are hereby authorized to be charged to our Deposit Account No. 50-5875.

Hugel Ex. 1006 - Page 559 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

Amendments to the Claims

This listing of claims will replace all prior versions, and listings, of claims in the application.

1. (Currently amended) A method of isolating a botulinum toxin type A macro complex from a botulinum toxin-containing solution, the method comprising:

(a) contacting the botulinum toxin-containing solution with an anion exchange chromatography medium at a pH lower than an isoelectric point (PI) of botulinum toxin;

contacting a flow-through portion of the botulinum toxin-containing solution, which is not bound to the anion exchange chromatography medium, with a cation exchange chromatography medium at a pH lower than the PI of botulinum toxin; and

separating the botulinum toxin type A macro complex from the cation exchange chromatography medium; or

(b) contacting the botulinum toxin-containing solution with a cation exchange chromatography medium at a pH lower than a PI of botulinum toxin;

separating botulinum toxin from the cation exchange chromatography medium;

contacting a solution containing the botulinum toxin separated from the cation exchange chromatography medium with an anion exchange chromatography medium at a pH lower than the PI of botulinum toxin; and

separating the botulinum toxin type A macro complex from a flow-through portion of the solution containing the botulinum toxin, which is not bound to the anion exchange chromatography medium,

wherein the pH lower than the PI of botulinum toxin is pH 3.5 to 5.5.

2. (Canceled).

3. (Previously presented) The method of claim 1, further comprising

adjusting a pH of the botulinum toxin-containing solution to a pH lower than the PI of botulinum toxin, before the contacting of the botulinum toxin-containing solution with the anion

exchange chromatography medium at the pH lower than the PI of botulinum toxin in (a);

adjusting a pH of the flow-through portion of the botulinum toxin-containing solution that is not bound to the anion exchange chromatography medium, before the contacting of the flow-through portion of the botulinum toxin-containing solution with the cation exchange chromatography medium at the pH lower than the PI of botulinum toxin in (a);

adjusting a pH of the botulinum toxin-containing solution, before the contacting of the botulinum toxin-containing solution with the cation exchange chromatography medium at the pH lower than the PI of botulinum toxin in (b); or

adjusting a pH of the solution containing the botulinum toxin separated from the cation exchange chromatography medium, before the contacting of the solution containing the botulinum toxin separated from the cation exchange chromatography medium with the anion exchange chromatography medium at the pH lower than the PI of botulinum toxin in (b).

4. (Previously presented) The method of claim 3, wherein the adjusting comprises mixing the botulinum toxin-containing solution, the flow-through portion of the botulinum toxin-containing solution, or the solution containing the botulinum toxin with a buffer having a pH lower than the PI of botulinum toxin.

5. (Original) The method of claim 4, wherein the buffer is a phosphate buffer, a citrate buffer, or an acetate buffer.

6. (Previously presented) The method of claim 4, wherein the buffer has a pH of 3.5 to 6.0.

7. (Original) The method of claim 4, wherein the buffer comprises a 10 mM to 100 mM phosphate buffer, a 10 mM to 100 mM citrate buffer, or a 10 mM to 100 mM acetate buffer.

8. (Previously presented) The method of claim 1, wherein the anion exchange chromatography medium comprises DEAE or Q anion exchange resin.

9. (Previously presented) The method of claim 1, wherein the cation exchange chromatography medium comprises CM, S, or SP cation exchange resin.

Remarks

Upon entry of the foregoing amendments, claims 1 and 3-9 are pending in the application, with claim 1 being the independent claim. Claim 2 has been canceled. Claim 1 is sought to be amended. Support can be found, for example, in para. [0020] of the published captioned application.

These changes are believed to introduce no new matter, and their entry is respectfully requested.

Rejection under 35 U.S.C. §103

The rejection of claims 1-9 under 35 U.S.C. §103 as allegedly being unpatentable over KIM (US2018/0251741, effectively filed November 30, 2015 according to the Office), is respectfully traversed without Applicant's admission as to the effective date of November 30, 2015 for KIM.

The Office alleged that KIM discloses a method for producing and purifying botulinum toxin (para. 0023) including botulinum toxin type A complex (para. 0048) whereby after an initial series of clarification and purification steps (steps a-e) of botulinum toxin produced from culture (para. 0041-0045), subsequent anion exchange (AEX, step f) and cation exchange (CEX, step h) purification steps are practiced (paras. 0076 and 0079-0081). The AEX chromatography step f is performed at a pH of 3-8, and botulinum toxin is collected in a flow-through fraction from the AEX column (para. 0085). After diluting in a buffer in step g, the CEX chromatography step h is performed at a pH of 3-8 whereby botulinum toxin is collected as a fraction containing the botulinum toxin bound to the CEX resin. Given the 6.06 isoelectric point (PI) of botulinum toxin

type A, the taught pH ranges of 3-8 during AEX and CEX chromatography steps f and h overlap with the claimed ranges of "a pH lower than the PI of botulinum toxin" and therefore, allegedly establishes a prima facie case of obviousness.

Ion-exchange chromatography separates ions based on their affinity to the ion exchanger. The two types of ion chromatography are anion-exchange and cation-exchange. Cation-exchange chromatography is used when the molecule of interest is positively charged. The molecule is positively charged because the pH for chromatography is less than the pI. In this type of chromatography, the stationary phase is negatively charged and positively charged molecules are loaded to be attracted to it. Anion-exchange chromatography is when the stationary phase is positively charged and negatively charged molecules (meaning that pH for chromatography is greater than the pI) are loaded to be attracted to it. In this regard, a molecule in a pH lower than an isoelectric point (PI) of botulinum toxin has an opposite charge properties compared to the same molecule in a pH higher than an isoelectric point (PI) of botulinum toxin. This means when botulinum toxin is present in a flow-through portion at a pH lower than an isoelectric point (PI) of botulinum toxin in AEX, then the botulinum toxin would not present in a flow-through portion at a pH higher than an isoelectric point (PI) of botulinum toxin in AEX. In this regard, even though KIM discloses that AEX chromatography step f is performed at a pH of 3-8 and the CEX chromatography step h is performed at a pH of 3-8, a person of ordinary skill in the art would not have had a reason to select a pH lower than an isoelectric point (PI) of botulinum toxin for the AEX chromatography step f from a pH of 3-8 and a pH lower than an isoelectric point (PI) of botulinum toxin for the CEX chromatography step h from a pH of 3-8.

Further, KIM simply discloses pH 3-8, which is simply the largest pH range that can be theoretically performed. Thus, KIM does not suggest or disclose that the second AEX and CEX can be performed a pH lower than an isoelectric point (PI) of botulinum toxin. Indeed, Example 2-3-3 of KIM discloses a process performing TQ purification (anion exchange chromatography flow-through (first AEX, FT)) at a pH higher than the PI, i.e., 6.4-6.6 (see p. 8, paras. 0107 and 0109), HQ purification (anion exchange chromatography binding mode (second AEX, binding mode) using the samples purified by TQ at a pH higher than the PI, i.e., 7.7-7.9 (see p. 9, paras. 01113 and 0115), and XS purification (cation exchange chromatography binding mode (CEX, binding mode) using the samples purified by HQ at a pH higher than the PI, i.e., 6.9-7.1 (see p. 9, paras. 0119 and 0122). Further, KIM discloses that the final purified botulinum toxin is a form of 7S (molecular weight: about 150 kDa), instead of 19S (molecular weight: about 900 kDa) (see p. 9, para. 0125).

In this regard, KIM only discloses a method for purifying the 7S form of botulinum toxin comprising first AEX in FT mode, second AEX in binding mode and CEX in binding mode, all performed at a pH higher than an isoelectric point (PI) of botulinum toxin. Thus, KIM does not provide a reason to use AEX in FT mode and CEX in binding mode using the samples purified from the AEX in FT mode, all performed in a pH lower than an isoelectric point (PI) of botulinum toxin.

The general disclosure of pH 3-8 in which the AEX and CEX can be performed does not provide a reason for one of ordinary skill in the art to select "a pH lower than the PI of botulinum toxin" in the context of the process of example 2-3-3 of KIM. On the contrary, the pH in the

context of the process of Example 2-3-3 of KIM should be "a pH higher than the PI of botulinum toxin." Thus, KIM teaches away from "a pH lower than the PI of botulinum toxin."

One of ordinary skill in the art would not have had a reason to modify the method for purifying the 7S form of botulinum toxin comprising first AEX in FT mode, second AEX in binding mode and CEX in binding mode, all performed at a pH higher than an isoelectric point (PI) of botulinum toxin to obtain a method for purifying the 19S form of botulinum toxin comprising first AEX in FT mode, and CEX in binding mode, all performed in a pH lower than an isoelectric point (PI) of botulinum toxin. Further, one of ordinary skill would not have had a reasonable expectation of success in a method for purifying 19S form botulinum toxin comprising first AEX in FT mode, and CEX in binding mode all performed in a pH lower than an isoelectric point (PI) of botulinum toxin because KIM only discloses a three-step method, i.e., comprising first AEX, second AEX, and CEX, and the final purified product is the 7S form of botulinum toxin. The claimed method comprise one AEX and CEX and the final purified product is the 19S form of botulinum toxin. In the claimed method, 7S form botulinum toxin was not detected (see, Table 1 of the specification).

Further, the Office states "[a]s noted in the 35 USC 103 rejection, the AEX chromatography step f is performed at a pH 3-8, and botulinum toxin in is collected in a flow-through fraction from the AEX column (p0085). In step h, CEX chromatography is performed at a pH 3-8 whereby botulinum toxin is collected as a fraction containing the botulinum toxin bound to the CEX resin in (para. 0087)" (see third para., p. 3 in the Office Action 04/22/2021). However, paragraph 0087 further reads: "and the finally purified botulinum toxin may have a form 7S (molecular weight: 150 kDa)." The disclosure of the reference documents should be considered as

a whole. However, the Office is mosaicking each claimed element from the disclosure after reading the claimed invention. Before step f and g, the method includes "(e) adjusting the pH of the botulinum toxin-containing anion exchange chromatography fraction upward." This upward adjustment of pH is for purifying a pure botulinum neurotoxin 7S, but not the 19S macro complex (see paras. 0076 and 0078). In this regard, the rejection is based improper hindsight.

Attached is a Declaration signed by a co-inventor, Jung Hyun SONG. In order to compare the relative efficiency of botulinum toxin recovery between an AEX using a pH lower than the PI of botulinum toxin and an AEX using pH higher than the PI of botulinum toxin, the inventor conducted experiments described in the Declaration. In the experiments, pH 5.5 and pH 6.5 were selected as a pH lower than the PI of botulinum toxin and a pH higher than the PI of botulinum toxin, respectively.

FIG. 1 of the Declaration shows a chromatogram for flow through solution and eluted solution from the AEX using pH 5.5, which is a pH lower than the PI of botulinum toxin. As shown in FIG. 1, the botulinum toxin containing sample was detected immediately after loading the sample. See the peak appearing near 0 ml. On the contrary, no significant peaks were appeared in the eluted solution with buffer B (pH 6.5). There was no significant fluctuation of the blue line at retention time between about 30 ml to 150 ml, i.e., there was no significant fluctuation of the absorbance at UVA278 when buffer B started to flow. This indicates that the botulinum toxin is contained in the flow through solution.

FIG. 3 of the Declaration shows a chromatogram for a flow through solution and eluted solution from the AEX using pH 6.5, which is a pH higher than the PI of botulinum toxin. As shown in FIG. 3, the botulinum toxin containing sample was detected at about 50 ml retention time

and after starting the elution with buffer B after loading the sample. This indicates that the botulinum toxin is not contained in the flow through solution, but contained in the eluted solution with buffer B. Thus, the claimed method would not work at a pH higher than the PI of botulinum toxin, as taught by KIM.

The Declaration states that "[a]s shown in Table I, the purity of botulinum toxin fraction no. 3, i.e., the flow through solution was 91.98%, which is significantly higher than the control sample purity, 74.09%." The Declaration also states that "[a]s shown in Table 2, the purity of botulinum toxin fraction no. 1, 3, 5, and 7, i.e., the eluted solution was about 30% to 88%. Even in the eluted solution, 19S is not purified but distributed to the all the fractions." These results from using pH 5.5 and pH 6.5 show that 19S can be purified from the flow through solution when pH 5.5 is used but at pH 6.5, 19S was not present in the flow through solution but present in the eluted solution, distributed in a wide retention time. In this regard, when pH 5.5 is used, 19S can be simply purified from the flow through solution with high purity and there is no need to add an additional eluting step. This is an unexpected benefit of the claimed invention.

The general disclosure in KIM of pH 2-9, preferably 3-8, in which the AEX and CEX can be performed does not provide a reason for one of ordinary skill in the art to select "a pH lower than the PI of botulinum toxin." Thus, it is clear that KIM does not disclose or provide a reason to perform AEX in flow through mode and CEX in binding mode, all at a pH lower than a PI of botulinum toxin. In view of KIM, one of skill also would not have had a reasonable expectation of success in the claimed method of the captioned application.

On the contrary, the pH used in the Examples of KIM for the AEX and CEX was a pH higher than the PI of botulinum toxin. Thus, KIM in fact teaches away from "a pH lower than the PI of botulinum toxin" as recited in the claims.

At least in view of the above, the rejection under 35 U.S.C. §103 should be withdrawn.

Conclusion

All of the stated grounds of objections and rejections have been properly traversed, accommodated, or rendered moot. Applicant therefore respectfully requests that the Examiner reconsider all objections and rejections and that they be withdrawn. Applicant believes that a full and complete reply has been made to the Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Date: July 22, 2021

Respectfully submitted,

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Judith U. Kim
Attorney for Applicant
Registration No.: 40,679

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Medytox Inc.	)	
	)	Group Art Unit: 1777
Serial No.: 16/338,561	)	
	)	Examiner: Huang, Ryan
Filed: November 29, 2017	)	
	)	
For: Method of isolating botulinum toxin from Botulinum toxin-containing solution	)	Confirmation No. 3639

DECLARATION UNDER 37 CFR 1.132

1. I, Jung Hyun SONG, hereby declare as follows.

2. I am a co-inventor of the captioned patent application.

3. The following experiments performed herein were performed at my direction.

4. In order to compare the relative efficiency of botulinum toxin recovery between a process using pH lower than the PI of botulinum toxin and a process using pH higher than the PI of botulinum toxin, I conducted following experiments.

1. Culture of *Clostridium botulinum*

In this experiment, a large amount of *Clostridium botulinum* type A toxin was expressed outside cells by culturing *Clostridium botulinum* in an animal product-free medium.

The used medium was a medium containing 4% yeast extract and 0.5% glucose, based on a total weight of the medium. The components of the medium were dissolved in water for injection (WFI), and pH was adjusted with NaOH to 7.2.

200 ml of the medium thus prepared was filtered through a sterile single use microfiltration system (0.2 μm Sartorius 2, manufactured by Sartorius), and injected into Cellbag™

(manufactured by GE healthcare, a single use fermentation bag). Then, the medium was inoculated with 1 ml of a seed culture of *Clostridium botulinum* at a density of 10^6 cells/ml~10^7 cells/ml through a sample injection port. Cellbag™ is a disposable bioreactor that uses rocking motion in order to cause mixing under low shear stress. Cellbag™ is a bag including an air inlet and outlet and a sample port, and is composed of a flexible material, and the bag is air impermeable. In addition, the bag is equipped with a pump combined thereto, which is used for bag inflation and cell supply. The used bag has a volume of 2 L.

Culturing was performed such that 2 L Cellbag™ containing 200 ml of the seed culture was seed-cultured at 37° C for 12 hours to 30 hours under rocking at 10 rpm by using a rocker unit in Cellbag™, and the resultant was inoculated in a bag containing 20 L of a main culture medium through a sterile single use connector (Kleenpack connector PALL) made of polycarbonate, and then cultured for 48 hours to 72 hours while maintaining anaerobic conditions. A sterile filter (0.2 μm) was installed at the sample injection port of the bag, and 200 ml of the seed culture medium was injected into the bag through the sterile filter. The injection of the medium was performed using a peristaltic pump.

The seed culture medium was injected to the bag without a headspace so as to maintain anaerobic conditions. The seed culture was also injected in the same manner. In addition, the main culture was performed by mounting the bag on a platform of the rocker unit of Cellbag™ and rocking the bag at 10 rpm at a temperature of 37° C. During the main culture, 1800 L of nitrogen was purged for 6 hours in order to maintain anaerobic conditions inside the bag.

After the culturing was terminated, a QDC connector (manufactured by Satorious) which is a sterile connector was connected to the sample port of the bag, and the culture was transferred to another disposable bag (flexible disposable bag) through the sterile connector. Then, a sterile disposable depthfilter (Sartoclear or SupraCap, PALL) and microfilter (0.2 μm, Sartopore 2, Sartorius) were connected to the disposable container containing the culture, and the culture was filtered therethrough to remove the cells. Then, the obtained supernatant was put in another disposable bag. From the supernatant, *Clostridium botulinum* toxin was purified.

2. Purification of Clostridium botulinum Toxin from Culture by anion exchange chromatography using pH lower than the PI of botulinum toxin and pH higher than the PI of botulinum toxin.

Hereinbelow, a method of producing type A toxin from the cell-removed culture will be described in detail.

(1) Acid Precipitation

1 N sulfuric acid was added to a bag containing 20 L of the culture supernatant obtained as above to adjust pH from 3.0 to 4.5, and as a result, toxin protein was precipitated. All procedures of this process were also performed in a disposable sample bag without being in contact with the outside.

Then, when the precipitation was completed, the resulting solution was filtered through a sterile disposable filtration system (disposable slice 200 0.2 μm filter, manufactured by Sartorius) without being in contact with the outside, thereby removing the supernatant, and the precipitate was collected in a disposable sample bag. 5 L of WFI was added to the collected precipitate to wash sulfuric acid, and a sterile disposable microfiltration system (disposable slice 200 0.2 μm filter) was connected to the bag and the supernatant was removed. As a result, the precipitate was collected into a disposable bag.

(2) Concentration and Filtration

10 L of citrate buffer (25 mM, pH 5.5) was added to the collected precipitate to redissolve toxin protein existing in the precipitate. This process was also performed in a disposable bag without being in contact with the outside. A sterile disposable filtration system (0.2 μm, ULTA Cap HC, GE, USA) was connected to the bag containing the re-dissolved sample, and filtration was performed without being in contact with the outside. As a result, the supernatant was collected in a sterile disposable bag and insoluble impurities were removed.

A sterile disposable ultrafiltration membrane (disposable slice 200 100 kDa, manufactured by Sartorius) having a molecular weight cut-off of 100 kDa was connected to the bag containing the sample from which the insoluble impurities were removed. Then, the sample was filtered without being in contact with the outside to be concentrated. Even in this process, toxin protein was in contact with an inner surface of the sterile container, and not in contact with the outside.

(3) Anion Exchange Chromatography using pH lower than the PI of botulinum toxin and pH higher than the PI of botulinum toxin.

In these experiments, pH 5.5 and pH 6.5 were selected as pH lower than the PI of botulinum toxin and pH higher than the PI of botulinum toxin, respectively.

(3.1) Anion Exchange Chromatography using pH lower than the PI of botulinum toxin

A disposable HiTrap® Q FF, which is prepacked with Q Sepharose® Fast Flow Sepharose anion exchange chromatography system (HiTrap® Q FF: Cytiva), was connected to the bag containing the sample after the concentration and buffer exchange were completed, and Q anion exchange chromatography was performed without being in contact with the outside.

In detail, the column packed with a Q anion exchange resin was pre-washed by flowing with 3 column volume (CV) of 0.5N sodium hydroxide, 10 CV of WFI, and then, 10 CV of 25 mM sodium citrate buffer containing 1M sodium chloride (pH 5.5)(hereinafter referred as "buffer B"). The column was equilibrated by flowing with 10 CV of 25 mM sodium citrate buffer (pH 5.5) (hereinafter referred as "buffer A"). Next, 5ml botulinum toxin containing sample prepared in above section (2) was loaded to the column by flowing the sample through the column. The column was washed by flowing 10 CV of buffer A. Then, the column was eluted by flowing 20 CV of buffer B with 0-100% gradient. The flow through solution was analyzed by cation exchange chromatography (CEX-HPLC).

For detail, the obtained sample containing fraction, i.e., fraction no. 3 was subjected to cation exchange chromatography (CEX-HPLC) and purity of the toxin was analyzed. As a mobile

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phase, mobile phase A - 2.4 mM Tris / 1.5mM imidazole / 11.6 mM piperazine pH3.7 and mobile phase B - 2.4 mM Tris / 1.5mM imidazole / 11.6 mM piperazine pH9.7 was used. ProPacTM SCX-10 LC column (manufactured by ThermoFisher Scientific, P/N 054995) was connected, and 20 μL of botulinum type A toxin protein containing fraction, i.e., fraction no. 3 was loaded and passed through the column for 30 minutes at 1.0 mL/min.

FIG. 1 is a chromatogram for flow through solution and eluted solution. In FIG.1, the blue line refers to absorbance at UVA278. The green line refers to buffer B concentration. The red line refers to a retention time for each fraction. The brown line refers to a conductivity. The purple line refers to pH. The pink line refers to injection mark. The pH where the peak near the 0 ml retention time was about 5.5. The seven red vertical lines refer to a fraction obtained from the botulinum toxin containing peak. Fraction no. 3 was further analyzed to determine the purity of the botulinum toxin.

As shown in FIG.1, the botulinum toxin containing sample was detected immediately after loading the sample. Please see a peak appearing near 0 ml. On the contrary, no significant peaks were appeared in the eluted solution with buffer B. there was no significant fluctuation of the blue line at retention time between about 30ml to 150ml. that is, there was no significant fluctuation of the absorbance at UVA278 when the buffer B was started to flow. This indicates that the botulinum toxin is contained in the flow through solution.

FIG. 2 is a chromatogram of CEX-HPLC fraction no. 3 obtained from the flow through sample after anion exchange chromatography of FIG.1 and sample obtained from section 2. Table 1 shows the purity determined by the CEX-HPLC.

Table 1.

Sample	HA complex	19S
Sample obtained from section 2	25.91	74.09
Fraction no. 3	8.02	91.98

As shown in Table 1, the purity of botulinum toxin fraction no. 3, i.e., the flow through solution was 91.98%, which is significantly higher than the control sample purity, 74.09%.

(3.2) Anion Exchange Chromatography using pH higher than the PI of botulinum toxin

A disposable HiTrap® Q FF, which is prepacked with Q Sepharose® Fast Flow Sepharose anion exchange chromatography system (HiTrap® Q FF: Cytiva), was connected to the bag containing the sample after the concentration and buffer exchange were completed, and Q anion exchange chromatography was performed without being in contact with the outside.

In detail, the column packed with a Q anion exchange resin was pre-washed by flowing with 3 column volume (CV) of 0.5N sodium hydroxide, 10 CV of WFI, and then, 10 CV of 20 mM MOPS buffer containing 1M sodium chloride (pH 6.5)(hereinafter referred as "buffer B"). The column was equilibrated by flowing with 10 CV of 20 mM sodium MOPS buffer (pH 6.5) (hereinafter referred as "buffer A"). Next, 5ml botulinum toxin containing sample prepared in above section (2) was loaded to the column by flowing the sample through the column. The column was washed by flowing 10 CV of buffer A. Then, the column was eluted by flowing 20 CV of buffer B with 0-100% gradient. The eluted solution was analyzed by cation exchange chromatography (CEX-HPLC).

For detail, the obtained sample containing fraction, i.e., fraction nos. 1, 3, 5, and 7 were subjected to CEX-HPLCE and purity of the toxin was analyzed. As a mobile phase, mobile phase A - 2.4 mM Tris / 1.5mM imidazole / 11.6mM piperazine pH3.7 and mobile phase B - 2.4 mM Tris / 1.5mM imidazole / 11.6mM piperazine pH9.7 was used. ProPac™ SCX-10 LC column (manufactured by ThermoFisher Scientific, P/N 054995) was connected, and 20 μL of botulinum type A toxin protein containing fraction, i.e., fraction no. 1, 3, 5, and 7 were loaded and passed through the column for 30 minutes at 1.0 mL/min.

FIG. 3 is a chromatogram for a flow through solution and eluted solution. In Fig. 3, the blue line refers to absorbance at UVA278. The green line refers to buffer B concentration. The red line

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refers to a retention time for each fraction. The brown line refers to a conductivity. The purple line refers to pH. The pink line refers to injection mark.

The pH where the peak containing botulinum toxin near 50 ml retention time was about 6.5. The red vertical lines refer to a fraction obtained from the botulinum toxin containing peak.

As shown in FIG. 3, the botulinum toxin containing sample was detected at about 50 ml retention time and after starting the elution with buffer B after loading the sample. This indicates that the botulinum toxin is not contained in the flow through solution, but contained in the eluted solution with buffer B.

FIG. 4 is a chromatogram of CEX-HPLC fraction no. 1, 3, 5, and 7 obtained from the eluted solution after anion exchange chromatography of FIG.3 and sample obtained from section 2. Table 2 shows the purity determined by the CEX-HPLC.

Table 2.

Sample	HA complex	19S
Sample obtained from section 2	25.91	74.09
Fraction no. 1	70.08	29.92
Fraction no. 3	31.12	68.88
Fraction no. 5	21.30	78.70
Fraction no. 7	11.62	88.38

As shown in Table 2, the purity of botulinum toxin fraction no. 1, 3, 5, and 7, i.e., the eluted solution was about 30% to 88%. However, 19 S is not purified but distributed to the all the fractions.

5. The undersigned declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Signed: _____ Dated: 2021 SEP. 24

Jung Hyun SONG

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FIG. 1

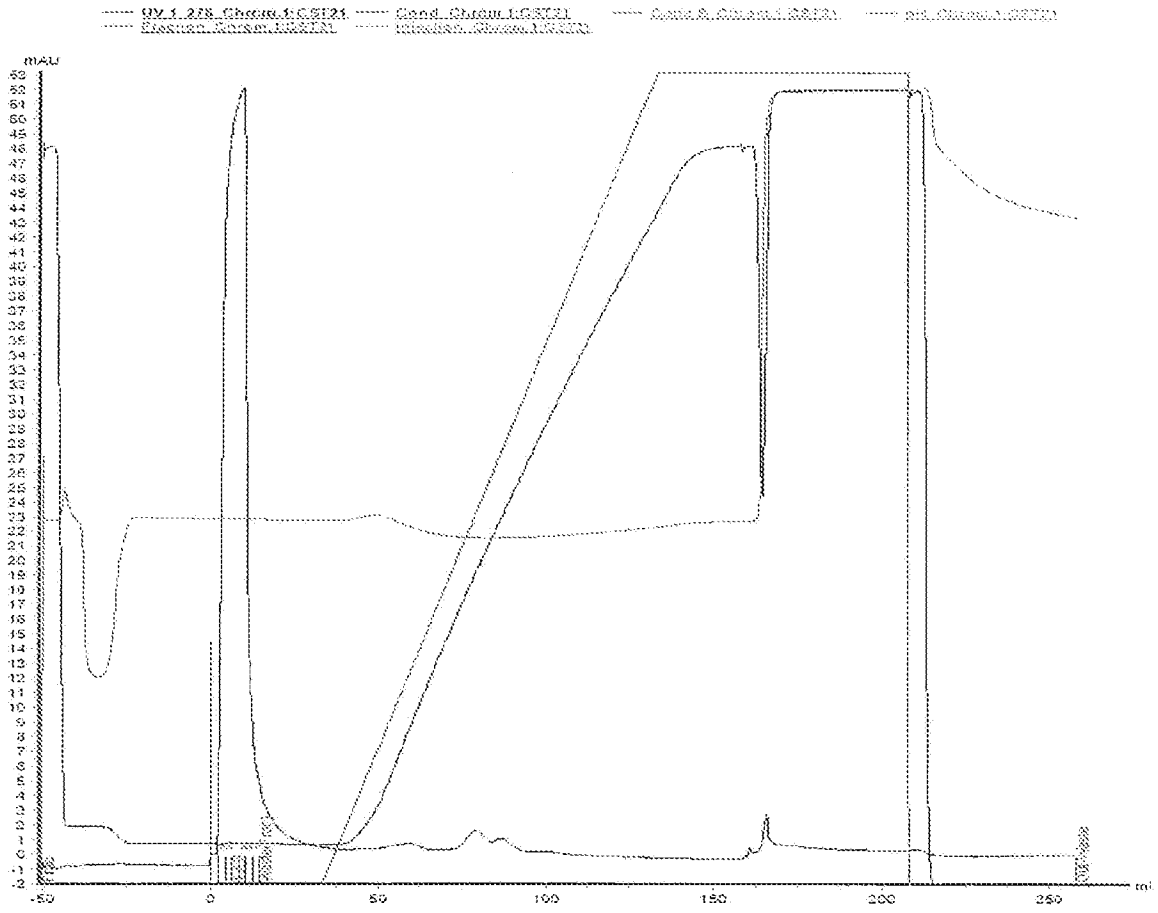


FIG. 2

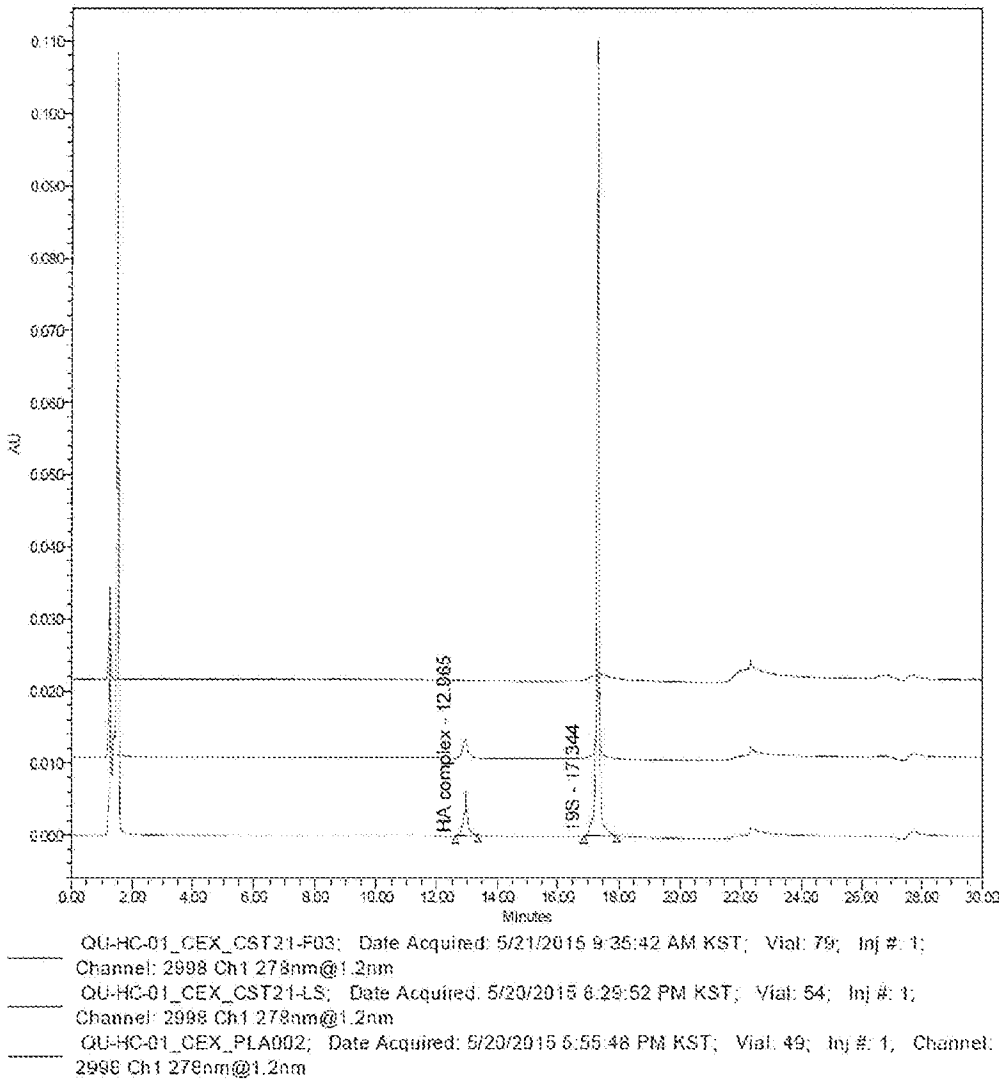

AU
Minutes
HA complex - 12.965
19S - 17.344
QU-HC-01_CEX_CST21-F03; Date Acquired: 5/21/2015 9:35:42 AM KST; Vial: 79; Inj #: 1; Channel: 2998 Ch1 278nm@1.2nm
QU-HC-01_CEX_CST21-LS; Date Acquired: 5/20/2015 6:29:52 PM KST; Vial: 54; Inj #: 1; Channel: 2998 Ch1 278nm@1.2nm
QU-HC-01_CEX_PLA002; Date Acquired: 5/20/2015 5:55:48 PM KST; Vial: 49; Inj #: 1; Channel: 2998 Ch1 278nm@1.2nm

FIG. 3

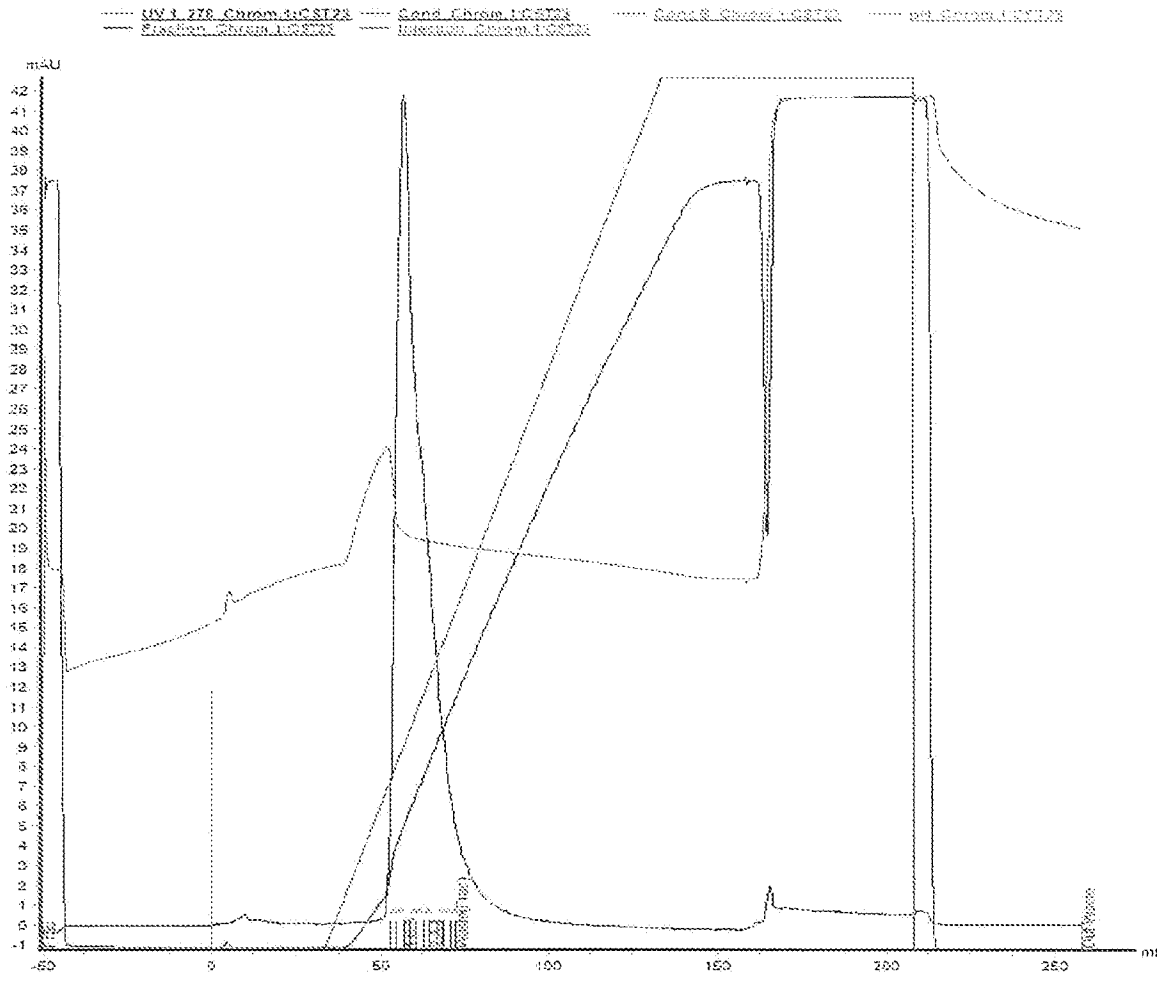
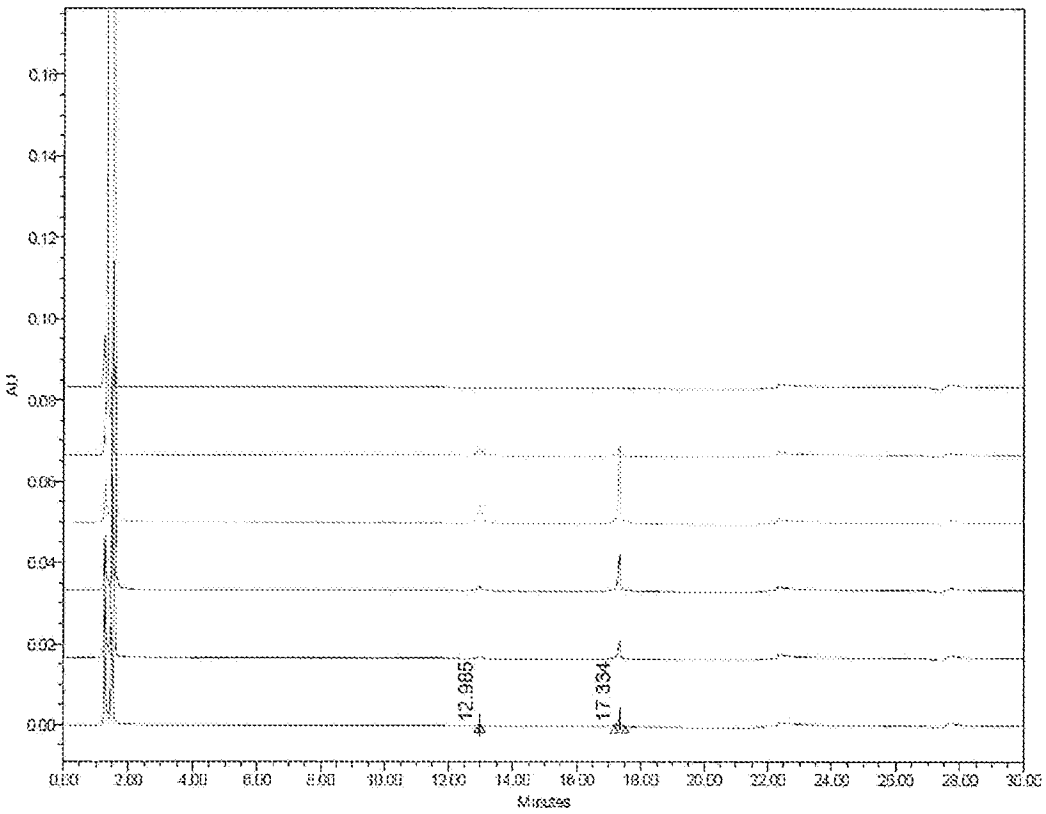


FIG. 4


AU
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
12.985
17.334
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
30.00
Minutes
QU-HC-01_CEX_CST23-F09; Date Acquired: 5/21/2015 9:34:33 PM KST; Vial: 100; Inj #: 1; Channel: 2998 Ch1 278nm@1.2nm
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QU-HC-01_CEX_CST23-F01; Date Acquired: 5/21/2015 7:28:25 PM KST; Vial: 96; Inj #: 1; Channel: 2998 Ch1 278nm@1.2nm
QU-HC-01_CEX_PLA003; Date Acquired: 5/21/2015 3:28:17 PM KST; Vial: 49; Inj #: 1; Channel: 2998 Ch1 278nm@1.2nm

Electronic Acknowledgement Receipt

EFS ID:	43320962
Application Number:	16338561
International Application Number:	
Confirmation Number:	3639
Title of Invention:	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
First Named Inventor/Applicant Name:	Hyun Ho JUNG
Customer Number:	137713
Filer:	Judith Unjin Kim/Jessica LaBossiere
Filer Authorized By:	Judith Unjin Kim
Attorney Docket Number:	2479-0007US01
Receipt Date:	22-JUL-2021
Filing Date:	01-APR-2019
Time Stamp:	15:48:47
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		2479-0007US01_Amdmt_Reply_to_OA_as_filed_7-22-21.pdf	227298 3e6b42fccd1b5e2e86d8727fc46330953c6994e7	yes	11

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Claims	2	4
	Applicant Arguments/Remarks Made in an Amendment	5	11

Warnings:					
Information:					
2	Affidavit-traversing rejectns or objectns rule 132	GN-55281-US_declaration_Signed_by_Kong_2021JUL22.pdf	17651485 ed4e0aba239de9cf38cac800e0facc57f25b613a	no	12
Warnings:					
Information:					
		Total Files Size (in bytes):	17878783		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/338,561	04/01/2019	Hyun Ho JUNG	2479-0007US01	3639

137713 7590 08/18/2021

Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

EXAMINER	
HUANG, RYAN	

ART UNIT	PAPER NUMBER
1777	

NOTIFICATION DATE	DELIVERY MODE
08/18/2021	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

eofficeaction@appcoll.com
patents@potomaclaw.com

Office Action Summary	Application No. 16/338,561	Applicant(s) JUNG et al.	
	Examiner RYAN B HUANG	Art Unit 1777	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 22 July 2021.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1 and 3-9 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1 and 3-9 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Summary

This is a final Office action in reply to the amendments filed 22 July 2021 filed 08 February 2021 for the application filed 01 April 2019. Claims 1 and 3-9 are pending:

- Claim 1 has been amended; and
- Claim 2 has been canceled.

Notice of Pre-AIA or AIA Status

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Priority

Applicant's claim for the benefit of a prior-filed application (371 of PCT/IB2017/057476, filed 29 November 2017) under 35 U.S.C. 119(e) or under 35 U.S.C. 120, 121, 365(c), or 386(c) is acknowledged.

Acknowledgment is made of applicant's claim for foreign priority (KR10-2016-0127540, filed 04 October 2016) under 35 U.S.C. 119 (a)-(d).

Response to Arguments

Applicant's amendments and arguments, filed 22 July 2021, have been fully considered.

Regarding the 35 USC 103 rejections of Claims 1-9 as unpatentable over KIM '741, Applicant's arguments have been fully considered but are not persuasive.

Applicant argues that (1) "[t]he general disclosure of pH 3-8 in which the AEX and CEX can be performed does not provide a reason for one of ordinary skill in the art" to practice the invention as claimed. Applicant further argues that (2) the examples of KIM, wherein the pH is higher than the pI of botulinum toxin, teaches away from the claimed invention (pg. 7-8).

The Examiner respectfully disagrees.

Regarding (1), based on the principles of ion exchange chromatography, a protein or target of interest will bind to either a cation or anion exchange resin based on its overall charge. In a buffered solution below the protein's

pI, the protein is positively charged and will bind to the negatively charged functional groups of a cation exchange resin and will not bind to the positively charged functional groups of an anion exchange resin. Because the claimed inventive method requires taking the flow-through portion of botulinum toxin-containing solution *not bound to* an anion exchange chromatography medium and separating botulinum toxin type A macro complex *bound to* a cation exchange chromatography medium, one of ordinary skill in the art of chromatography would find it obvious to perform such a method at a lower pH range to maximize the flow-through of botulinum toxin type A macro complex through the AEX resin and to maximize the binding of botulinum toxin type A macro complex to the CEX resin. Thus, it would be obvious to one of ordinary skill in the art at the time of the filing of the invention to use the lower end of the KIM '741 disclosed pH 3-8 range in AEX and CEX chromatography steps f and h, which overlaps with the claimed ranges.

Regarding (2), while KIM may have disclosed higher pH solvents and buffers in the examples, KIM nonetheless does disclose pH ranges that encompass the claimed ranges. Disclosed examples and preferred embodiments do not constitute a teaching away from a broader disclosure or nonpreferred embodiments (*In re* Susi, 440 F.2d 442, 169 USPQ 423 (CCPA 1971)). "A known or obvious composition does not become patentable simply because it has been described as somewhat inferior to some other product for the same use" (*In re* Gurley, 27 F.3d 551, 554, 31 USPQ2d 1130, 1132 (Fed. Cir. 1994); MPEP §2123 II).

Regarding the 1.312 affidavit filed by the Applicant 22 July 2021, the Examiner had requested Applicant show evidence of significance or criticality to the claimed "a pH lower than the pI of botulinum toxin" with respect to the claimed invention. However, Applicant has only provided comparisons of botulinum toxin recovery between pH lower than the pI and pH higher than the pI for only AEX chromatography, i.e., no nexus has been shown between the provided data and the claimed inventive method.

All other arguments have been indirectly addressed.

Claim Interpretation

As noted in paragraph 21 of the Specification, the isoelectric point (pI) of botulinum toxin type A is about 6.06.

As noted in paragraph 6 of the Specification, a botulinum toxin macro complex is 900 kDa and includes 150 kDa of botulinum toxin (A-G) and a complex of a variety of non-toxic proteins.

Claim Rejections - 35 USC § 103

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

The factual inquiries for establishing a background for determining obviousness under 35 U.S.C. 103 are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims the examiner presumes that the subject matter of the various claims was commonly owned as of the effective filing date of the claimed invention(s) absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and effective filing dates of each claim that was not commonly owned as of the effective filing date of the later invention in order for the examiner to consider the applicability of 35 U.S.C. 102(b)(2)(C) for any potential 35 U.S.C. 102(a)(2) prior art against the later invention.

1. Claims 1 and 3-9 is/are rejected under 35 U.S.C. 103 as being unpatentable over KIM et al. (US PGPub 2018/0251741 A1, effectively filed 30 November 2015).

Regarding Claim 1, KIM '741 discloses a method for producing and purifying botulinum toxin (p0023) including botulinum toxin type A macro complex (i.e., a method of isolating a botulinum toxin type A macro complex; p0048-0050) whereby after an initial series of clarification and purification steps (steps a-e) of botulinum toxin

produced from culture (i.e., a botulinum toxin-containing solution; p0041-0045), subsequent anion exchange (AEX, step f) and cation exchange (CEX, step h) purification steps are practiced (p0076, p0079-0081).

The AEX chromatography step f is performed at a pH of 3-8, and botulinum toxin is collected in a flow-through fraction from the AEX column (i.e., contacting the botulinum toxin-containing solution with an anion exchange chromatography medium at a pH lower than an isoelectric point of botulinum toxin; p0085; "flow-through" defined in p0073). After diluting in a buffer in step g, the CEX chromatography step h is performed at a pH of 3-8 whereby botulinum toxin is collected as a fraction containing the botulinum toxin bound to the CEX resin (i.e., contacting a flow-through portion of the botulinum toxin-containing solution, which is not bound to the anion exchange chromatography medium with a cation exchange chromatography medium at a pH lower than the pI of botulinum toxin; separating the botulinum toxin type A macro complex from the cation exchange chromatography medium; p0087). Given the 6.06 isoelectric point (pI) of botulinum toxin type A, the disclosed pH 3-8 ranges during AEX and CEX chromatography steps f and h overlap with the claimed ranges of "a pH lower than the pI of botulinum toxin" and "wherein the pH lower than the PI of botulinum toxin is pH 3.5 to 5.5".

Furthermore, based on the principles of ion exchange chromatography, a protein or target of interest will bind to either a cation or anion exchange resin based on its overall charge. In a buffered solution below the protein's pI, the protein is positively charged and will bind to the negatively charged functional groups of a cation exchange resin and will not bind to the positively charged functional groups of an anion exchange resin. Because the claimed inventive method requires taking the flow-through portion of botulinum toxin-containing solution *not bound to* an anion exchange chromatography medium and separating botulinum toxin type A macro complex *bound to* a cation exchange chromatography medium, one of ordinary skill in the art of chromatography would find it obvious to perform such a method at a lower pH range to maximize the flow-through of botulinum toxin type A macro complex through the AEX resin and to maximize the binding of botulinum toxin type A macro complex to the CEX resin. Thus, it would be obvious to one of ordinary skill in the art at the time of the filing of the invention to use the lower end of the KIM '741 disclosed pH 3-8 range in AEX and CEX chromatography steps f and h, which overlaps with the claimed ranges of "a pH lower than the pI of botulinum toxin" of 6.06 and "wherein the pH lower than the PI of botulinum toxin is pH 3.5 to 5.5" and therefore, establishes cases of *primafacie* obviousness (MPEP 2144.05).

Regarding Claim 3, as applied to the rejection of Claim 1, KIM '741 further discloses in step g the flow-through fraction of botulinum toxin collected from the AEX chromatography process of step f is diluted in a buffer of

pH 6-8 (i.e., adjusting a pH of the flow-through portion of the botulinum toxin-containing solution that is not bound to the anion exchange chromatography medium before the contacting of the flow-through portion of the botulinum toxin-containing solution with the cation exchange chromatography medium at the pH lower than the pI of botulinum toxin; p0086).

Regarding Claim 4, as applied to the rejection of Claim 3, KIM '741 further discloses that step g utilizes a buffer of pH 6-8 (p0086), which overlaps with the claimed range of the buffer having a pH lower than the pI of botulinum toxin (i.e., less than 6.06) and therefore, establishes a case of *prima facie* obviousness (MPEP 2144.05).

Regarding Claim 5, as applied to the rejection of Claim 4, KIM '741 further discloses the buffer in step g is a sodium phosphate buffer (i.e., wherein the buffer is a phosphate buffer; p0086).

Regarding Claim 6, as applied to the rejection of Claim 4, KIM '741 further discloses that step g utilizes a buffer of pH 6-8 (p0086), which overlaps with the claimed range of the buffer having a pH of 3.5 to 6.0 and therefore, establishes a case of *prima facie* obviousness (MPEP 2144.05).

Regarding Claim 7, as applied to the rejection of Claim 4, KIM '741 further discloses 50 mM sodium phosphate buffer (p0134), which reads upon the claimed range of 10 mM to 100 mM.

Regarding Claim 8, as applied to the rejection of Claim 1, KIM '741 further discloses the anion exchange chromatography process utilizes diethylaminoethyl (DEAE) or quaternary amine (Q) resins (i.e., wherein the anion exchange chromatography… is DEAE or Q anion exchange chromatography; p0088).

Regarding Claim 9, as applied to the rejection of Claim 1, KIM '741 further discloses the cation exchange chromatography process utilizes carboxymethyl (CM), sulfonate (S), or phosphate (P) resins (i.e., wherein the cation exchange chromatography… is CM, S, or SP cation exchange chromatography; p0089).

Conclusion

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the

shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RYAN B HUANG whose telephone number is (571)270-0327. The examiner can normally be reached on 9 am-5 pm EST.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Vickie Kim can be reached on (571)272-0579. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Ryan B Huang/
Primary Examiner, Art Unit 1777

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/338,561	JUNG et al.
	Examiner	**Art Unit**
	RYAN B HUANG	1777

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	05/12/2020	11/03/2020	04/19/2021	08/13/2021					
	1	✓	✓	✓	✓					
	2	✓	✓	✓	-					
	3	✓	✓	✓	✓					
	4	✓	✓	✓	✓					
	5	✓	✓	✓	✓					
	6	✓	✓	✓	✓					
	7	✓	✓	✓	✓					
	8	✓	✓	✓	✓					
	9	✓	✓	✓	✓					

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/338,561	JUNG et al.
	Examiner	Art Unit
	RYAN B HUANG	1777

CPC - Searched*		
Symbol	**Date**	**Examiner**
B01D15/36$	04/19/2021	RBH
B01D15/36$ and A61K38/4893	05/12/2020	RBH
B01D15/36$	05/12/2020	RBH
B01D15/362 and B01D15/363	05/12/2020	RBH

CPC Combination Sets - Searched*		
Symbol	**Date**	**Examiner**

US Classification - Searched*			
Class	**Subclass**	**Date**	**Examiner**

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

/Ryan B Huang/ Primary Examiner, Art Unit 1777	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/338,561	JUNG et al.
	Examiner	Art Unit
	RYAN B HUANG	1777

Search Notes		
Search Notes	**Date**	**Examiner**
Applicants search on EAST and PALM	05/12/2020	RBH
botulinum toxin generic search	05/12/2020	RBH
AEX flow-through, CEX bind-elute search	05/12/2020	RBH
Focused search on AEX flow through	11/03/2020	RBH
Considered PCT search report	11/03/2020	RBH
Applicant is argues KIM teaches away from claimed invention	04/19/2021	RBH
Affidavit/declaration with statistical significance may be sufficient to overcome rejection, allowance	04/19/2021	RBH
Additional CPC/text search	04/19/2021	RBH
Google search for ion exchange principle	08/13/2021	RBH

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**

/Ryan B Huang/ Primary Examiner, Art Unit 1777	

Web Search History

date, time	web site	search string
8/13/2021 2:45:51 PM	Google	pH and pI chromatography

Hugel Ex. 1006 - Page 595 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
MEDYTOX INC.

Application No.: 16/338,561
Int'l Filing Date: November 29, 2017

For: **METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION**

Confirmation No.: 3639

Art Unit: 1777

Examiner: Ryan Huang

Atty Dkt. No. 2479-0007US01

Amendment and Reply under 37 C.F.R. § 1.114

Mail Stop RCE

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Commissioner:

In reply to the final Office Action dated August 18, 2021, Applicant submits the following amendments and remarks.

Amendments to the Claims are reflected in the listing of claims, which begins on page 2 of this paper.

Remarks and Arguments begin on page 5 of this paper.

A one-month Extension of Time is submitted herewith on December 20, 2021 (December 18th was a Saturday). However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any additional fees required to continue prosecution or appeal of this application (including issue fee, fees for net addition of claims or forwarding to appeal) are hereby authorized to be charged to our Deposit Account No. 50-5875.

Hugel Ex. 1006 - Page 596 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 16/338,561	Filing Date 04/01/2019	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $460 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	12/20/2021	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 9	Minus	** 20	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0		x $480 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/BRUCE D HARRISON/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Doc code: RCEX
Doc description: Request for Continued Examination (RCE)

PTO/SB/30EFS (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)

Application Number	16/338,561	Filing Date	2017-11-29	Docket Number (if applicable)	2479-0007US01	Art Unit	1777
First Named Inventor	Hyun Ho JUNG			Examiner Name	Ryan HUANG		

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV

SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

- ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
- ☐ Other _____

☒ Enclosed

- ☒ Amendment/Reply
- ☐ Information Disclosure Statement (IDS)
- ☐ Affidavit(s)/ Declaration(s)
- ☐ Other _____

MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____ (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

FEES

☒ **The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.**
The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to Deposit Account No 505875

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

☒ Patent Practitioner Signature

Applicant Signature

PTO/SB/30EFS (02-18)
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Signature of Registered U.S. Patent Practitioner			
Signature	/Judith U. Kim/	Date (YYYY-MM-DD)	2021-12-20
Name	Judith U. Kim	Registration Number	40679

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/AIA/22 (10-20)
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PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)

Docket Number (Optional): 2479-0007US01

Application Number: 16/338,561

Filed: November 29, 2017

For: Method of Isolating Botulinum Toxin from Botulinum Toxin-Containing Solution

Art Unit: 1777

Examiner: Ryan HUANG

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	Micro Entity Fee	
☑	One month (37 CFR 1.17(a)(1))	$220	$110	$55	$ 220
☐	Two months (37 CFR 1.17(a)(2))	$640	$320	$160	$
☐	Three months (37 CFR 1.17(a)(3))	$1,480	$740	$370	$
☐	Four months (37 CFR 1.17(a)(4))	$2,320	$1,160	$580	$
☐	Five months (37 CFR 1.17(a)(5))	$3,160	$1,580	$790	$

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 505875.

☑ Payment made via EFS-Web.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant.

☒ attorney or agent of record. Registration number 40679.

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____.

/Judith U. Kim/ — Signature

December 20, 2021 — Date

Judith U. Kim — Typed or printed name

(443) 926-6811 — Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ * Total of _____ forms are submitted.

This collection of information is required by 37 CFR 1.136(a). The information is required to obtain or retain a benefit by the public, which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 6 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal

Application Number:	16338561
Filing Date:	01-Apr-2019
Title of Invention:	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
First Named Inventor/Applicant Name:	Hyun Ho JUNG
Filer:	Judith Unjin Kim/Jessica LaBossiere
Attorney Docket Number:	2479-0007US01

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 1 month with $0 paid	1251	1	220	220
Miscellaneous:				
RCE- 2ND AND SUBSEQUENT REQUEST	1820	1	2000	2000
		Total in USD ($)		**2220**

Electronic Acknowledgement Receipt

EFS ID:	44564421
Application Number:	16338561
International Application Number:	
Confirmation Number:	3639
Title of Invention:	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
First Named Inventor/Applicant Name:	Hyun Ho JUNG
Customer Number:	137713
Filer:	Judith Unjin Kim/Jessica LaBossiere
Filer Authorized By:	Judith Unjin Kim
Attorney Docket Number:	2479-0007US01
Receipt Date:	20-DEC-2021
Filing Date:	01-APR-2019
Time Stamp:	15:51:43
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$2220
RAM confirmation Number	E2021BJF51592028
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		2479-0007US01_Amdmt_Reply_to_FOA_as_filed_12-20-21.pdf	272722 56eaaf754f5ec4df1e23448064e3864dc5873933	yes	13
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Response After Final Action		1	1	
	Claims		2	4	
	Applicant Arguments/Remarks Made in an Amendment		5	13	
Warnings:					
Information:					
2	Request for Continued Examination (RCE)	2479-0007US01_RCE.pdf	1364375 45ddae4ab44aff409bdece1f53fb5d1636b49a9d	no	3
Warnings:					
Information:					
3	Extension of Time	2479-0007US01_EOT.pdf	182460 1b7a97836d3c73d6acf1224d37aaceac5de31b70	no	2
Warnings:					
Information:					
4	Fee Worksheet (SB06)	fee-info.pdf	40914 fa1182796fb412f85e35630b3a7be1e5b59cb50f	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	1860471		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
MEDYTOX INC.

Application No.: 16/338,561
Int'l Filing Date: November 29, 2017

For: **METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION**

Confirmation No.: 3639

Art Unit: 1777

Examiner: Ryan Huang

Atty Dkt. No. 2479-0007US01

Amendment and Reply under 37 C.F.R. § 1.114

Mail Stop RCE

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Commissioner:

In reply to the final Office Action dated August 18, 2021, Applicant submits the following amendments and remarks.

Amendments to the Claims are reflected in the listing of claims, which begins on page 2 of this paper.

Remarks and Arguments begin on page 5 of this paper.

A one-month Extension of Time is submitted herewith on December 20, 2021 (December 18th was a Saturday). However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any additional fees required to continue prosecution or appeal of this application (including issue fee, fees for net addition of claims or forwarding to appeal) are hereby authorized to be charged to our Deposit Account No. 50-5875.

Hugel Ex. 1006 - Page 608 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

Amendments to the Claims

This listing of claims will replace all prior versions, and listings, of claims in the application.

1. (Currently amended) A method of isolating a botulinum toxin type A macro complex from a botulinum toxin-containing solution, the method comprising:

culturing *Clostridium botulinum* in an animal product-free medium to obtain a culture;

removing *Clostridium botulinum* cells from the culture to obtain a botulinum toxin-containing solution;

adding an acid to the botulinum toxin-containing solution to precipitate the botulinum toxin and obtain a precipitate;

dissolving the precipitate in a medium to obtain a solution;

filtering the solution to obtain a botulinum toxin-containing solution; and

(a) contacting the botulinum toxin-containing solution with an anion exchange chromatography medium at a pH lower than an isoelectric point (PI) of botulinum toxin;

contacting a flow-through portion of the botulinum toxin-containing solution, which is not bound to the anion exchange chromatography medium, with a cation exchange chromatography medium at a pH lower than the PI of botulinum toxin; and

separating the botulinum toxin type A macro complex from the cation exchange chromatography medium; or

(b) contacting the botulinum toxin-containing solution with a cation exchange chromatography medium at a pH lower than a PI of botulinum toxin;

separating botulinum toxin from the cation exchange chromatography medium;

contacting a solution containing the botulinum toxin separated from the cation exchange chromatography medium with an anion exchange chromatography medium at a pH lower than the PI of botulinum toxin; and

separating the botulinum toxin type A macro complex from a flow-through portion of the solution containing the botulinum toxin, which is not bound to the anion exchange chromatography medium;

~~wherein the pH lower than the PI of botulinum toxin is pH 3.5 to 5.5~~.

2. (Canceled).

3. (Previously presented) The method of claim 1, further comprising

adjusting a pH of the botulinum toxin-containing solution to a pH lower than the PI of botulinum toxin, before the contacting of the botulinum toxin-containing solution with the anion exchange chromatography medium at the pH lower than the PI of botulinum toxin in (a);

adjusting a pH of the flow-through portion of the botulinum toxin-containing solution that is not bound to the anion exchange chromatography medium, before the contacting of the flow-through portion of the botulinum toxin-containing solution with the cation exchange chromatography medium at the pH lower than the PI of botulinum toxin in (a);

adjusting a pH of the botulinum toxin-containing solution, before the contacting of the botulinum toxin-containing solution with the cation exchange chromatography medium at the pH lower than the PI of botulinum toxin in (b); or

adjusting a pH of the solution containing the botulinum toxin separated from the cation exchange chromatography medium, before the contacting of the solution containing the botulinum toxin separated from the cation exchange chromatography medium with the anion exchange chromatography medium at the pH lower than the PI of botulinum toxin in (b).

4. (Previously presented) The method of claim 3, wherein the adjusting comprises mixing the botulinum toxin-containing solution, the flow-through portion of the botulinum toxin-containing solution, or the solution containing the botulinum toxin with a buffer having a pH lower than the PI of botulinum toxin.

5. (Original) The method of claim 4, wherein the buffer is a phosphate buffer, a citrate buffer, or an acetate buffer.

6. (Previously presented) The method of claim 4, wherein the buffer has a pH of 3.5

to 6.0.

7. (Original) The method of claim 4, wherein the buffer comprises a 10 mM to 100 mM phosphate buffer, a 10 mM to 100 mM citrate buffer, or a 10 mM to 100 mM acetate buffer.

8. (Previously presented) The method of claim 1, wherein the anion exchange chromatography medium comprises DEAE or Q anion exchange resin.

9. (Previously presented) The method of claim 1, wherein the cation exchange chromatography medium comprises CM, S, or SP cation exchange resin.

10. (New) The method of claim 1, wherein the pH lower than the PI of botulinum toxin is pH 3.5 to 6.0.

Remarks

Upon entry of the foregoing amendments, claims 1 and 3-10 are pending in the application, with claim 1 being the independent claim. Claim 2 was canceled. Claim 1 is sought to be amended, for which support can be found, e.g., in paras. [0031], [0031], and [0042] of the published captioned application. New claim 10 has been added, for which support can be found, e.g., in original claim 2.

These changes are believed to introduce no new matter, and their entry is respectfully requested.

Rejection under 35 U.S.C. §103

The rejection of claims 1 and 3-9 under 35 U.S.C. §103 as allegedly being unpatentable over KIM (US2018/0251741, effectively filed November 30, 2015 according to the Office), is respectfully traversed without Applicant's admission as to the effective date of November 30, 2015 for KIM '741.

Claim 1 is directed to

> [a] method of isolating a botulinum toxin type A macro complex from a botulinum toxin-containing solution, the method comprising: culturing *Clostridium botulinum* in an animal product-free medium to obtain a culture;
>
> removing *Clostridium botulinum* cells from the culture to obtain a botulinum toxin-containing solution;
>
> adding an acid to the botulinum toxin-containing solution to precipitate the botulinum toxin and obtain a precipitate;
>
> dissolving the precipitate in a medium to obtain a solution;

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> filtering the solution to obtain a botulinum toxin-containing solution; and
>
> (a) contacting the botulinum toxin-containing solution with an anion exchange chromatography medium at a pH lower than an isoelectric point (PI) of botulinum toxin; contacting a flow-through portion of the botulinum toxin-containing solution, which is not bound to the anion exchange chromatography medium, with a cation exchange chromatography medium at a pH lower than the PI of botulinum toxin; and separating the botulinum toxin type A macro complex from the cation exchange chromatography medium; or
>
> (b) contacting the botulinum toxin-containing solution with a cation exchange chromatography medium at a pH lower than a PI of botulinum toxin; separating botulinum toxin from the cation exchange chromatography medium; contacting a solution containing the botulinum toxin separated from the cation exchange chromatography medium with an anion exchange chromatography medium at a pH lower than the PI of botulinum toxin; and separating the botulinum toxin type A macro complex from a flow-through portion of the solution containing the botulinum toxin, which is not bound to the anion exchange chromatography medium.

Thus, the anion exchange chromatography (AEX) is performed in a flow-through (FT) mode at a pH lower than an isoelectric point (PI) of botulinum toxin, the cation exchange chromatography (CEX) was performed in a binding mode at a pH lower than an isoelectric point (PI) of botulinum toxin, and the final separated product is a botulinum toxin type A macro complex.

Kim '741 discloses "a method for producing a botulinum toxin, comprising the steps of: (a) treating a culture of a botulinum toxin-producing strain with acid to form a botulinum toxin-containing precipitate; (b) adding a buffer to the botulinum toxin-containing precipitate of step (a), and then clarifying by one or more method selected from the group consisting of depth filtration (DF), microfiltration (MF), ultrafiltration (UF), sterile filtration, membrane chromatography (MC) and centrifugation; (c) subjecting the botulinum toxin-containing solution of step (b) to UF diafiltration, ammonium sulfate precipitation or hydrochloric acid precipitation, and then diluting

Hugel Ex. 1006 - Page 613 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

a retentate resulting from the UF diafiltration in a buffer, or dissolving a precipitate resulting from the ammonium sulfate precipitation or hydrochloric acid precipitate in a buffer; and (d) subjecting the retentate dilution, ammonium sulfate precipitate solution or hydrochloric acid precipitate solution of step (c) to anion-exchange chromatography (AEX), thereby purifying the botulinum toxin."(See paragraphs 25-29 and Example 2 of KIM '741).

In conventional processes for producing botulinum toxin, enzymes such as DNase or RNase were used to remove nucleic acids such as DNA or RNA from the bacteria cells. However, because enzymes such as DNase or RNase are of animal origin, these enyzmes have the potential to contain various disease-causing substances, particularly abnormal prion proteins. See [0015] of KIM '741. Indeed, the object of KIM '741 is to provide a method for producing botulinum toxin, in which an enzymatic treatment step using animal products (APs) is replaced by clarification of the botulinum toxin using at least one technique selected from the group consisting of depth filtration (DF), microfiltration (MF), ultrafiltration (UF), sterile filtration, membrane chromatography (MC) and centrifugation, which are pretreatment processes, followed by performing one process selected from among UF diafiltration, ammonium sulfate precipitation and hydrochloric acid precipitation, followed by further purification using anion/cation-exchange chromatography, thereby ensuring safety with very high yield. See [0024] of KIM '741.

In this regard, KIM '741 does not teach or provide a reason to for "removing *Clostridium botulinum* cells from the culture to obtain a botulinum toxin-containing solution; adding an acid to the botulinum toxin-containing solution to precipitate the botulinum toxin and obtain a precipitate" as claimed. In the claimed method, there is no need to remove or treat nucleic acids from bacteria cells since *Clostridium botulinum* cells from the culture are removed. Further, in

the claimed method, there is no need to include the step of "performing one process selected from among UF diafiltration, ammonium sulfate precipitation and hydrochloric acid precipitation" after the pretreatment processes because *Clostridium botulinum* cells from the culture are removed.

Therefore, it would not have been obvious to one of ordinary skill in the art to modify the method of KIM '741 replace the step of "(a) treating a culture of a botulinum toxin-producing strain with acid to form a botulinum toxin-containing precipitate" with "removing *Clostridium botulinum* cells from the culture to obtain a botulinum toxin-containing solution; adding an acid to the botulinum toxin-containing solution to precipitate the botulinum toxin and obtain a precipitate" from the cell free solution as claimed and remove the step of "performing one process selected from among UF diafiltration, ammonium sulfate precipitation and hydrochloric acid precipitation" after the pretreatment processes as required in KIM '741. Moreover, based on KIM '741, there would have been no reasonable expectation of success with the claimed method to one of skill in the art.

The Office states that "KIM '741 discloses a method for producing and purifying botulinum toxin (p0023) including botulinum toxin type A complex" (p0048)

> whereby after an initial series of clarification and purification steps (steps a-e) of botulinum toxin produced from culture (. . . p0041-p0045), subsequent anion exchange (AEX, step f) and cation exchange (CEX, step h) purification steps are practiced (p0076, p0079-0081). The AEX chromatography step f is performed at a pH of 3-8, and botulinum toxin is collected in a flow-through fraction from the AEX column (. . . p0085 . . .). After diluting in a buffer in step g, the CEX chromatography step h is performed at a pH of 3-8 whereby botulinum toxin is collected as a fraction containing the botulinum toxin bound to the CEX resin . . . . Given the 6.06 isoelectric point (PI) of botulinum toxin type A, the taught pH ranges of 3-8 during AEX and CEX chromatography steps f and h overlap with the claimed ranges of "a pH lower than the PI of botulinum toxin.

Office Action, pg. 4, last ¶, to pg. 5, 1st full ¶.

Ion-exchange chromatography separates ions based on their affinity to the ion exchanger. The two types of ion chromatography are anion-exchange and cation-exchange. Cation-exchange chromatography is used when the molecule of interest is positively charged. The molecule is positively charged because the pH for chromatography is less than the pI. In this type of chromatography, the stationary phase is negatively charged and positively charged molecules are loaded to be attracted to it. Anion-exchange chromatography is when the stationary phase is positively charged and negatively charged molecules (meaning that pH for chromatography is greater than the pI) are loaded to be attracted to it. In this regard, a molecule in a pH lower than an isoelectric point (PI) of botulinum toxin has opposite charge properties compared to the same molecule in a pH higher than an isoelectric point (PI) of botulinum toxin. This means when botulinum toxin is present in a flow-through portion at a pH lower than an isoelectric point (PI) of botulinum toxin in AEX, then the botulinum toxin would not present in a flow-through portion at a pH higher than an isoelectric point (PI) of botulinum toxin in AEX. In this regard, even though KIM '741 discloses that AEX chromatography step f is performed at a pH of 3-8 and the CEX chromatography step h is performed at a pH of 3-8, it would not have been obvious to a person having ordinary skill in the art to select a pH lower than an isoelectric point (PI) of botulinum toxin for the AEX chromatography step f from a pH of 3-8 and a pH lower than an isoelectric point (PI) of botulinum toxin for the CEX chromatography step h from a pH of 3-8.

Further, KIM '741 simply discloses the pH of 3-8, which is simply the largest pH range that can be theoretically performed. Thus, KIM '741 does not disclose or provide a reason that the second AEX and CEX can be performed a pH lower than an isoelectric point (PI) of botulinum

toxin. Indeed, Example 2-3-3 of KIM '741 discloses a process performing TQ purification (anion exchange chromatography flow-through (first AEX, FT)) at a pH higher than the PI, i.e., 6.4-6.6 (See page 8, 0107, and 0109), HQ purification (anion exchange chromatography binding mode (second AEX, binding mode) using the samples purified by TQ at a pH higher than the PI, i.e., 7.7-7.9 (See page 9, 01113, and 0115), and XS purification (cation exchange chromatography binding mode (CEX, binding mode) using the samples purified by HQ at a pH higher than the PI, i.e., 6.9-7.1 (See page 9, 0119, and 0122). Further, KIM '741 discloses that the final purified botulinum toxin is a form of 7S (molecular weight: about 150 kDa), instead of 19S(molecular weight: about 900 kDa)(See page 9, 0125).

In this regard, KIM '741 only discloses a method for purifying 7S form botulinum toxin comprising first AEX in FT mode, second AEX in binding mode, and CEX in binding mode, all performed in a pH higher than an isoelectric point (PI) of botulinum toxin. Thus, KIM '741 does not disclose or provide a reason to use AEX in FT mode and CEX in binding mode using the samples purified from the AEX in FT mode, all performed in a pH lower than an isoelectric point (PI) of botulinum toxin.

The general disclosure that the AEX and CEX can be performed at the pH of 3-8 does not provide a reason for one of ordinary skill in the art to select "a pH lower than the PI of botulinum toxin" in the context of the process of Example 2-3-3 of KIM '741. On the contrary, the pH in the context of the process of Example 2-3-3 of KIM '741 should be "a pH higher than the PI of botulinum toxin." Thus, KIM '741 teaches away from "a pH lower than the PI of botulinum toxin," as claimed.

Therefore, it would not have been obvious to one of ordinary skill in the art to modify the method for purifying 7S form botulinum toxin comprising first AEX in FT mode, second AEX in binding mode, and CEX in binding mode, all performed in a pH higher than an isoelectric point (PI) of botulinum toxin to obtain a method for purifying 19S form botulinum toxin comprising first AEX in FT mode, and CEX in binding mode, all performed in a pH lower than an isoelectric point (PI) of botulinum toxin. Further, there is no reasonable expectation of success to one of ordinary skill for a method for purifying 19S form botulinum toxin comprising first AEX in FT mode, and CEX in binding mode, all performed in a pH lower than an isoelectric point (PI) of botulinum toxin because KIM '741 only discloses a three-step method, i.e., comprising first AEX, second AEX and CEX, and final purified product is 7S form botulinum toxin. The claimed method comprises a two-step method, i.e., AEX and CEX and final purified product is 19S form botulinum toxin. According to the claimed method, 7S form botulinum toxin was not detected. See, Table 1 of the captioned application.

Further, the Office states "the AEX chromatography step f is performed at a pH 3-8, and botulinum toxin in is collected in a flow-through fraction from the AEX column (. . . p0085. . .). The CEX chromatography step h is performed at a pH 3-8 whereby botulinum toxin is collected as a fraction containing the botulinum toxin bound to the CEX resin in (. . . p0087)." See 1st full ¶, pg. 5 of the Office Action. However, paragraph 0087 of KIM '741 states: "and the finally purified botulinum toxin may have a form 7S (molecular weight: 150 kDa)." The disclosure of the reference documents should be considered as a whole, but not mosaicking each claimed element after reading the claimed inventions. Before steps f and g, the method includes "(e) adjusting the pH of the botulinum toxin-containing anion exchange chromatography fraction

upward." This upward adjustment of pH is for purifying a pure botulinum neurotoxin, but 19S macro complex. See [0076] and [0078] of KIM '741. In this regard, the rejection is based improper hindsight.

Applicant now refer to the Declaration signed by a co-inventor, Jung Hyun SONG, submitted July 22, 2021. In order to compare the relative efficiency of botulinum toxin recovery between an AEX using pH lower than the PI of botulinum toxin and an AEX using pH higher than the PI of botulinum toxin, the inventor conducted experiments in which pH 5.5 and pH 6.5 were selected as pH lower than the PI of botulinum toxin and pH higher than the PI of botulinum toxin, respectively.

FIG. 1 refers to a chromatogram for flow through solution and eluted solution from the AEX using pH 5.5, which is a pH lower than the PI of botulinum toxin. As shown in FIG.1, the botulinum toxin containing sample was detected immediately after loading the sample. Please see a peak appearing near 0 ml. On the contrary, no significant peaks were appeared in the eluted solution with buffer B. there was no significant fluctuation of the blue line at retention time between about 30ml to 150ml. that is, there was no significant fluctuation of the absorbance at UVA278 when the buffer B was started to flow. This indicates that the botulinum toxin is contained in the flow through solution.

FIG. 3 refers to a chromatogram for a flow through solution and eluted solution from the AEX using pH 6.5, which is a pH higher than the PI of botulinum toxin. As shown in FIG.3, the botulinum toxin containing sample was detected at about 50 ml retention time and after starting the elution with buffer B after loading the sample. This indicates that the botulinum toxin is not contained in the flow through solution, but contained in the eluted solution with buffer B.

The experiments and results provided in the SONG Declaration show the criticality of the use of "a pH lower than the pI of botulinum toxin."

At least in view of the above, the rejection under 35 U.S.C. §103 should be withdrawn.

Conclusion

All of the stated grounds of objections and rejections have been properly traversed, accommodated, or rendered moot. Applicant therefore respectfully requests that the Examiner reconsider all objections and rejections and that they be withdrawn. Applicant believes that a full and complete reply has been made to the Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Date: December 20, 2021

Respectfully submitted,

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By /Judith U. Kim/
Judith U. Kim
Attorney for Applicant
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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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NOTICE OF ALLOWANCE AND FEE(S) DUE

137713 7590 01/21/2022

Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

EXAMINER
HUANG, RYAN

ART UNIT	PAPER NUMBER
1777	

DATE MAILED: 01/21/2022

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/338,561	04/01/2019	Hyun Ho JUNG	2479-0007US01	3639

TITLE OF INVENTION: METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$0.00	$1200	04/21/2022

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

137713 7590 01/21/2022

Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/338,561	04/01/2019	Hyun Ho JUNG	2479-0007US01	3639

TITLE OF INVENTION: METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$0.00	$1200	04/21/2022

EXAMINER	ART UNIT	CLASS-SUBCLASS
HUANG, RYAN	1777	210-635000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/AIA/122 or PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/AIA/47 or PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑ Issue Fee ❑ Publication Fee (if required) ❑ Advance Order - # of Copies _____

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

❑ Electronic Payment via EFS-Web ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/338,561	04/01/2019	Hyun Ho JUNG	2479-0007US01	3639

137713 7590 01/21/2022

Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

EXAMINER	
HUANG, RYAN	
ART UNIT	PAPER NUMBER
1777	

DATE MAILED: 01/21/2022

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 16/338,561	Applicant(s) JUNG et al.	
	Examiner RYAN B HUANG	Art Unit 1777	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to 20 December 2021.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are 1 and 3-10 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information , please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☑ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☑ All b) ☐ Some* c) ☐ None of the:

   1. ☑ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/Ryan B Huang/
Primary Examiner, Art Unit 1777

DETAILED ACTION

Summary

This is an Allowability Notice in reply to the Request for Continued Examination under 37 CFR 1.114 filed 20 December 2021 for the application filed 01 April 2019. Claims 1 and 3-10 are pending:

- Claim 2 has been canceled;
- Claim 1 has been amended; and
- new Claim 10 has been added.

Notice of Pre-AIA or AIA Status

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Priority

Applicant's claim for the benefit of a prior-filed application (371 of PCT/IB2017/057476, filed 29 November 2017) under 35 U.S.C. 119(e) or under 35 U.S.C. 120, 121, 365(c), or 386(c) is acknowledged.

Acknowledgment is made of applicant's claim for foreign priority (KR10-2016-0127540, filed 04 October 2016) under 35 U.S.C. 119 (a)-(d).

Claim Interpretation

As noted in paragraph 21 of the Specification, the isoelectric point (pI) of botulinum toxin type A is about 6.06.

As noted in paragraph 6 of the Specification, a botulinum toxin macro complex is 900 kDa and includes 150 kDa of botulinum toxin (A-G) and a complex of a variety of non-toxic proteins.

Allowable Subject Matter

Claims 1 and 3-10 are allowed.

The following is an examiner's statement of reasons for allowance:

The claimed invention is directed to a method for isolating a botulinum toxin type A macro complex from a culture of *Clostridium botulinum* utilizing a combination of anion exchange chromatography and cation exchange chromatography at pH levels lower than the isoelectric point of botulinum toxin. While the most relevant prior art of record, KIM et al. (US PGPub 2018/0251741 A1), discloses a method for producing and purifying botulinum toxin, including the desired type A macro complex, using anion exchange and cation exchange chromatography, KIM discloses a broad pH range of 3-8 and provides only embodiments where the pH levels are above the pI of botulinum toxin. As argued by Applicant in the reply filed 20 December 2021, one of ordinary skill in the art would not have been motivated to select a pH lower than the pI of botulinum toxin from the pH range of 3-8 disclosed by KIM because KIM does not disclose or provide any reason that the anion exchange or cation exchange processes can be performed at such a lower pH level. Applicant has also provided a declaration by co-inventor SONG (22 July 2021) comparing the relative efficiency of botulinum toxin recovery from an anion exchange process between using a pH lower than the pI of botulinum toxin and a pH higher than the pI of botulinum toxin, thereby showing the criticality of such a claimed limitation. This showing of criticality was persuasive to overcome the 35 USC 103 rejection of Claim 1 as being unpatentable over KIM.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RYAN B HUANG whose telephone number is (571)270-0327. The examiner can normally be reached 9 am-5 pm EST.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Vickie Kim can be reached on (571)272-0579. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of published or unpublished applications may be obtained from Patent Center. Unpublished application information in Patent Center is available to registered users. To file and manage patent submissions in Patent Center, visit: https://patentcenter.uspto.gov. Visit https://www.uspto.gov/patents/apply/patent-center for more information about Patent Center and https://www.uspto.gov/patents/docx for information about filing in DOCX format. For additional questions, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Ryan B Huang/
Primary Examiner, Art Unit 1777

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/338,561	JUNG et al.
	Examiner	Art Unit
	RYAN B HUANG	1777

CPC - Searched*		
Symbol	**Date**	**Examiner**
B01D15/36$	04/19/2021	RBH
B01D15/36$ and A61K38/4893	05/12/2020	RBH
B01D15/36$	05/12/2020	RBH
B01D15/362 and B01D15/363	05/12/2020	RBH
B01D15/362.cpc. B01J39$.cpc. cation exchange	01/09/2022	RBH
B01D15/363.cpc. B01J41$.cpc. anion exchange	01/09/2022	RBH
B01D15/361 ion exchange	01/09/2022	RBH
B01J47/028.cpc. alternating cation/anion	01/09/2022	RBH

CPC Combination Sets - Searched*		
Symbol	**Date**	**Examiner**

US Classification - Searched*			
Class	**Subclass**	**Date**	**Examiner**

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

/Ryan B Huang/ Primary Examiner, Art Unit 1777	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/338,561	JUNG et al.
	Examiner	Art Unit
	RYAN B HUANG	1777

Search Notes		
Search Notes	**Date**	**Examiner**
Applicants search on EAST and PALM	05/12/2020	RBH
botulinum toxin generic search	05/12/2020	RBH
AEX flow-through, CEX bind-elute search	05/12/2020	RBH
Focused search on AEX flow through	11/03/2020	RBH
Considered PCT search report	11/03/2020	RBH
Applicant is argues KIM teaches away from claimed invention	04/19/2021	RBH
Affidavit/declaration with statistical significance may be sufficient to overcome rejection, allowance	04/19/2021	RBH
Additional CPC/text search	04/19/2021	RBH
Google search for ion exchange principle	08/13/2021	RBH
Updated SEARCH	01/09/2022	RBH
CPC/text SEARCH	01/09/2022	RBH

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**
B01D$.		01/09/2022	RBH
B01J$		01/09/2022	RBH
C07K$		01/09/2022	RBH

/Ryan B Huang/ Primary Examiner, Art Unit 1777	

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/338,561	JUNG et al.
	Examiner	**Art Unit**
	RYAN B HUANG	1777

CPC						
Symbol					**Type**	**Version**
B01D	/	15	/	363	F	2013-01-01
B01D	/	15	/	362	I	2013-01-01
C07K	/	1	/	18	I	2013-01-01
C07K	/	14	/	33	I	2013-01-01

CPC Combination Sets							
Symbol					**Type**	**Set**	**Ranking**	**Version**
	/		/					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	9	
/Ryan B Huang/ Primary Examiner, Art Unit 1777	09 January 2022	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	none

U.S. Patent and Trademark Office — Part of Paper No.: 20220109

Hugel Ex. 1006 - Page 631 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/338,561	JUNG et al.
	Examiner	**Art Unit**
	RYAN B HUANG	1777

INTERNATIONAL CLASSIFICATION				
CLAIMED				
B01D15/36	/	15	/	36
C07K1/18	/	1	/	18
C07K14/33	/	14	/	33

NON-CLAIMED				
B01J	/	47	/	028

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**

CROSS REFERENCES(S)						
CLASS	**SUBCLASS (ONE SUBCLASS PER BLOCK)**					

NONE (Assistant Examiner) (Date)	**Total Claims Allowed:** 9	
/Ryan B Huang/ Primary Examiner, Art Unit 1777 (Primary Examiner) 09 January 2022 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure none

U.S. Patent and Trademark Office Part of Paper No.: 20220109

Hugel Ex. 1006 - Page 632 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/338,561	JUNG et al.
	Examiner	**Art Unit**
	RYAN B HUANG	1777

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	1														
2	3														
3	4														
4	5														
5	6														
6	7														
7	8														
8	9														
9	10														

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 9	
/Ryan B Huang/ Primary Examiner, Art Unit 1777 (Primary Examiner)	09 January 2022 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure none

U.S. Patent and Trademark Office

Part of Paper No.: 20220109

Hugel Ex. 1006 - Page 633 Hugel Inc v. Medytox Inc. IPR2023-00604 - U.S. Patent No. 11,331,598

PE2E SEARCH - Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
L1	153	((("JUNG") near3 ("Hyun") near3 ("Ho")) OR (("YANG") near3 ("Gi") near3 ("Hyeok")) OR (("RHEE") near3 ("Chang") near3 ("Hoon")) OR (("KIM") near3 ("Hack") near3 ("Woo")) OR (("RYU") near3 ("Gi") near3 ("Hyuck")) OR (("HONG") near3 ("Hyung") near3 ("Pyo")) OR (("SONG") near3 ("Jung") near3 ("Hyun")) OR (("KONG") near3 ("Joon") near3 ("Chan"))).INV.	(US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT)	OR	ON	ON	2022/01/09 10:00 AM
L2	4	((("MEDYTOX") near3 ("INC"))).AS,AANM.	(USPAT)	OR	ON	ON	2022/01/09 10:35 AM
L3	4	((("MEDYTOX"))).AS,AANM.	(USPAT)	OR	ON	ON	2022/01/09 10:36 AM
L4	193	((("MEDYTOX"))).AS,AANM.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, CA, CH, CN, DD, DE, EA, EP, ES, FR, GB, JP, KR, OA, RU, SU, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2022/01/09 10:36 AM
L5	31	(clostridium ADJ botulimum)	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, CA, CH, CN, DD, DE, EA, EP, ES, FR, GB, JP, KR, OA, RU, SU, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2022/01/09 01:16 PM
L6	37700	(clostridium ADJ botulinum)	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, CA, CH, CN, DD, DE, EA, EP, ES, FR, GB, JP, KR, OA, RU, SU, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2022/01/09 01:17 PM
L7	149101	anion ADJ exchange AND cation ADJ exchange	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, CA, CH, CN, DD, DE, EA, EP, ES, FR, GB, JP, KR, OA, RU,	OR	ON	ON	2022/01/09 01:19 PM

			SU, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)				
L8	1639	6 AND 7	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, CA, CH, CN, DD, DE, EA, EP, ES, FR, GB, JP, KR, OA, RU, SU, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2022/01/09 01:19 PM
L9	30870	B01D15/362.cpc. B01J39$.cpc.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, CA, CH, CN, DD, DE, EA, EP, ES, FR, GB, JP, KR, OA, RU, SU, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2022/01/09 01:37 PM
L10	24615	B01D15/363.cpc. B01J41$.cpc.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, CA, CH, CN, DD, DE, EA, EP, ES, FR, GB, JP, KR, OA, RU, SU, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2022/01/09 01:38 PM
L11	669	B01J47/028.cpc.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, CA, CH, CN, DD, DE, EA, EP, ES, FR, GB, JP, KR, OA, RU, SU, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2022/01/09 01:38 PM
L12	0	6 AND 11	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, CA, CH, CN, DD, DE, EA, EP, ES, FR, GB, JP, KR, OA, RU, SU, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2022/01/09 01:38 PM
L13	92	6 AND 9 AND 10	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, CA, CH, CN, DD, DE, EA, EP, ES, FR, GB, JP, KR, OA, RU, SU, WO); FPRS; EPO; JPO; DERWENT; IBM_TDB)	OR	ON	ON	2022/01/09 01:39 PM

PE2E SEARCH - Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp

N1	367167	B01D$.cpc. B01J$.cpc.	(US-PGPUB; USPAT)	OR	ON	ON	2022/01/09 04:35 PM
N2	608257	B01D$.cpc. B01J$.cpc. c07K$.cpc.	(US-PGPUB; USPAT)	OR	ON	ON	2022/01/09 04:36 PM
N3	1	((botulinum botox) AND (isoelectric pi) AND macro AND (anion$2) AND (cation$2)).clm.	(US-PGPUB; USPAT)	OR	ON	ON	2022/01/09 04:37 PM
N4	29	((botulinum botox) AND (isoelectric pi) AND (anion$2) AND (cation$2)).clm.	(US-PGPUB; USPAT)	OR	ON	ON	2022/01/09 04:37 PM
N5	1	N2 AND N4	(US-PGPUB; USPAT)	OR	ON	ON	2022/01/09 04:37 PM

Bibliographic Data

Application No: 16/338,561

Foreign Priority claimed: (●) Yes ( ) No

35 USC 119 (a-d) conditions met: (☑) Yes ( ) No | ( ) Met After Allowance

Verified and Acknowledged: /Ryan B Huang/ | Examiner's Signature | Initials

Title: METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
04/01/2019	210	1777	2479-0007US01
RULE			

APPLICANTS

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CONTINUING DATA

This application is a 371 of PCT/IB2017/057476 11/29/2017

FOREIGN APPLICATIONS

KOREA, REPUBLIC OF KR10-2016-0127540 10/04/2016

IF REQUIRED, FOREIGN LICENSE GRANTED**

09/30/2019

STATE OR COUNTRY

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8229 Boone Boulevard
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Vienna, VA 22182

UNITED STATES

FILING FEE RECEIVED

$1,580

PTO/SB/08a (01-22)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16338561
Filing Date	2017-11-29
First Named Inventor	Hyun Ho JUNG
Art Unit	1777
Examiner Name	Ryan HUANG
Attorney Docket Number	2479-0007US01

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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	1	2011074025	JP	A	2011-04-14	Chemo-Sero-Therapeutic Research Institute	English abstract provided	

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16338561
	Filing Date	2017-11-29
	First Named Inventor	Hyun Ho JUNG
	Art Unit	1777
	Examiner Name	Ryan HUANG
	Attorney Docket Number	2479-0007US01

	1	Korean Office Action (Notification of Reason of Refusal) dated February 23, 2022, issued in Korean Application No. 10-2016-0127540, with English translation.	×

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EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16338561
Filing Date	2017-11-29
First Named Inventor	Hyun Ho JUNG
Art Unit	1777
Examiner Name	Ryan HUANG
Attorney Docket Number	2479-0007US01

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☒ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

☒ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Judith U. Kim/	Date (YYYY-MM-DD)	2022-03-09
Name/Print	Judith U. Kim	Registration Number	40679

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

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3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Espacenet

Bibliographic data: JP2011074025 (A) — 2011-04-14

METHOD OF PURIFYING BOTULINUS TOXIN

Inventor(s): NAGANO AKITAKA; NAKAHIRA SHINJI; TORII KYOJI; ITAI SHIHO; HARADA KAORI; OYAMA YUSUKE; GINNAGA AKIHIRO; OZAKI SHUNJI ± (NAGANO AKITAKA, ; NAKAHIRA SHINJI, ; TORII KYOJI, ; ITAI SHIHO, ; HARADA KAORI, ; OYAMA YUSUKE, ; GINNAGA AKIHIRO, ; OZAKI SHUNJI)

Applicant(s): CHEMO SERO THERAPEUT RES INST ± (CHEMO-SERO-THERAPEUTIC RESEARCH INST)

Classification: **- international:** ***A61K38/00; C07K14/33***
- cooperative: Y02A50/30 (EP)

Application number: JP20090228199 20090930 Global Dossier

Priority number(s): JP20090228199 20090930

Abstract of JP2011074025 (A)

PROBLEM TO BE SOLVED: To provide a simpler method of purifying botulinus toxin suited in the purification on an industrial level without requiring a precipitation/centrifuging step. ;SOLUTION: The method of purifying botulinus toxin comprises a combination of a film filtration method, an anion exchange chromatography, and a cation exchange chromatography. According to this purification method, a large quantity of high safety botulinus toxin can be very simply and effectively prepared in a short time from a culture medium of Clostridium botulinum. Further, elimination of the recovering operation of precipitates in the precipitation method/centrifugal separation enables providing complete automation facilities/in-line facilities of a production line. Furthermore, since no operator intervenes and no toxin solution is exposed to the environment, the security of the work safety health environment of operators, GMP conformity, and asepsis can be improved. ;COPYRIGHT: (C)2011,JPO&INPIT

(19)日本国特許庁(JP)　(12)公開特許公報(A)　(11)特許出願公開番号
特開2011-74025
(P2011-74025A)
(43)公開日　平成23年4月14日(2011.4.14)

(51)Int.Cl.		F I		テーマコード（参考）
C07K 14/33	(2006.01)	C07K	14/33	4C084
A61K 38/00	(2006.01)	A61K	37/02	4H045

審査請求 未請求　請求項の数 23　ＯＬ　（全 15 頁）

(21)出願番号　特願2009-228199 (P2009-228199)
(22)出願日　平成21年9月30日 (2009.9.30)

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(54)【発明の名称】ボツリヌス毒素の精製方法

(57)【要約】
【課題】　沈殿・遠心工程を要さない、工業レベルの精製に適したより簡便なボツリヌス毒素の精製方法を提供する。
【解決手段】　膜ろ過法と陰イオン交換クロマトグラフィー法と陽イオン交換クロマトグラフィー法を組み合わせたことを特徴とするボツリヌス毒素の精製方法。本発明の精製方法によれば、ボツリヌス菌培養液から極めて簡便に、短期間にかつ効果的に安定性の高いボツリヌス毒素を大量調製することが可能となる。また、沈殿法・遠心分離における沈殿物の回収作業を排除したことで、製造ラインの完全自動化設備・インライン化設備の提供が可能となる。さらに、作業員の介在がなくなるとともに、毒素溶液が環境に曝露されることがないため、作業者の労働安全衛生環境及びＧＭＰ準拠並びに無菌性確保を向上することが可能となる。
【選択図】　なし

【特許請求の範囲】
【請求項１】
膜ろ過法と陰イオン交換クロマトグラフィーと陽イオン交換クロマトグラフィーを組み合わせることを特徴とするボツリヌス毒素の精製方法。
【請求項２】
以下の工程を含む、請求項１に記載の精製方法。
(a) 膜ろ過法により、ボツリヌス菌培養液からボツリヌス菌体を除去する工程、
(b) 前記(a)工程においてボツリヌス菌体が除去されたボツリヌス毒素溶液を陰イオン交換クロマトグラフィーカラムに展開する工程、
(c) ボツリヌス毒素成分がカラムに吸着するような条件下で、前記(b)工程において得られる陰イオン交換クロマトグラフィーカラムの回収画分を陽イオン交換クロマトグラフィーカラムに展開する工程、及び
(d) 前記陽イオン交換クロマトグラフィーから不純物を洗い流し、次いでボツリヌス毒素を溶出する工程。
【請求項３】
前記膜ろ過法が、精密膜ろ過法である請求項１または２に記載の精製方法。
【請求項４】
前記精密膜ろ過法に用いる膜の孔径が0.22μm以下である請求項３に記載の精製方法。
【請求項５】
前記膜ろ過法において、前記精密膜ろ過を行う前に、前記精密膜ろ過に用いる膜よりも大きな孔径を有する膜で粗膜ろ過を行う工程をさらに含む請求項２に記載の精製方法。
【請求項６】
前記陰イオン交換クロマトグラフィーカラム内の緩衝液のpHが4.5から6.8である、請求項１または２に記載の精製方法。
【請求項７】
前記陰イオン交換クロマトグラフィーカラム内の緩衝液の塩濃度が0から0.3 mol/Lである、請求項１または２に記載の精製方法。
【請求項８】
前記陰イオン交換クロマトグラフィーにおいて、ボツリヌス毒素成分をカラムに吸着させる場合、当該カラムに吸着したボツリヌス毒素を塩濃度が0から2.0 mol/Lの勾配で溶出する、請求項１または２に記載の精製方法。
【請求項９】
前記(b)工程を行う前に、前記(a)工程においてボツリヌス菌体が除去されたボツリヌス毒素溶液を、当該陰イオン交換クロマトグラフィーの塩濃度及びpHの条件に調整する工程をさらに含む請求項２に記載の精製方法。
【請求項１０】
前記陽イオン交換クロマトグラフィーカラム内の緩衝液のpHが4.0から4.5である、請求項１または２に記載の精製方法。
【請求項１１】
前記陽イオン交換クロマトグラフィーカラム内の緩衝液の塩濃度が0から0.2 mol/Lである、請求項１または２に記載の精製方法。
【請求項１２】
前記(c)工程を行う前に、前記(b)工程において得られる陰イオン交換クロマトグラフィーカラムの回収画分を、当該陽イオン交換クロマトグラフィーの塩濃度及びpHの条件に調整する工程をさらに含む請求項２に記載の精製方法。
【請求項１３】
前記(d)工程において、陽イオン交換クロマトグラフィーに吸着したボツリヌス毒素を塩濃度が0.2から0.7 mol/Lの勾配で溶出する、請求項１または２に記載の精製方法。
【請求項１４】
(d)工程で溶出されるボツリヌス毒素がLL毒素、L毒素またはM毒素である請求項２に記載

の精製方法。
【請求項１５】
前記(a)～(d)の工程の後にさらに以下の工程を含む、請求項１または２に記載のボツリヌス毒素の精製方法。
(e) ボツリヌス毒素成分がカラムに吸着し、かつ、ボツリヌス神経毒素（NTX）と無毒非HAタンパク質（NTNH）が解離するような条件下で、前記(d)工程で得られた陽イオン交換クロマトグラフィーの溶出画分を、第二陰イオン交換クロマトグラフィーカラムに展開する工程、及び
(f) 前記第二陰イオン交換クロマトグラフィーから不純物を洗い流し、次いでボツリヌス神経毒素（NTX）を溶出する工程。
【請求項１６】
前記第二陰イオン交換クロマトグラフィーカラム内の緩衝液のpHが6.8から8.0である、請求項１、２または１５のいずれかに記載の精製方法。
【請求項１７】
前記第二陰イオン交換クロマトグラフィーカラム内の緩衝液の塩濃度が0から0.1 mol/Lである、請求項１、２または１５のいずれかに記載の精製方法。
【請求項１８】
前記(f)工程において、第二陰イオン交換クロマトグラフィーに吸着したNTXを塩濃度が0から0.3mol/Lの勾配で溶出する、請求項１、２または１５のいずれかに記載の精製方法。
【請求項１９】
前記(e)工程を行う前に、前記(d)工程において得られる陽イオン交換クロマトグラフィーの溶出画分を、当該第二陰イオン交換クロマトグラフィーの塩濃度及びpHの条件に調整する工程をさらに含む請求項１５に記載の精製方法。
【請求項２０】
当該ボツリヌス毒素がA型、B型、C型、D型、E型、F型及びG型ボツリヌス菌由来のボツリヌス毒素である請求項１から１９のいずれかに記載の精製方法。
【請求項２１】
請求項１から２０のいずれかに記載の精製方法により精製されたボツリヌス毒素。
【請求項２２】
当該ボツリヌス毒素が、LL毒素、L毒素、M毒素、またはNTXのいずれかである請求項２１に記載のボツリヌス毒素。
【請求項２３】
請求項２１または２２に記載のボツリヌス毒素を有効成分として含有する薬剤。
【発明の詳細な説明】
【技術分野】
【０００１】
本発明は、ボツリヌス毒素の精製方法に関する。詳細には、沈殿法・遠心分離を行うことなく、膜ろ過法とクロマトグラフィー法を組み合わせることを特徴とするボツリヌス毒素の精製方法に関する。
【背景技術】
【０００２】
嫌気性のグラム陽性菌であるクロストリジウム・ボツリナム（Clostridium botulinum）が産生するボツリヌス毒素は地球上で最も致死性の高い毒素であり、これまでに血清型A、B、C、D、E、F及びGの7種のボツリヌス菌由来の毒素とその特性が明らかにされている。これらは、それぞれ血清型に特異的な中和抗体で識別される。更に同じ血清型のボツリヌス毒素の中でも、毒素遺伝子の構造の違いによりいくつかの亜型に分類される。ボツリヌス毒素の活性中心蛋白質は、既知のボツリヌス毒素血清型の7種すべてにおいて、分子量約150 KDaのボツリヌス神経毒素（NTX）である。
【０００３】
すべてのボツリヌス毒素はボツリヌス菌から産生される場合、分子量約150 KDaのNTXに

分子量の異なる無毒成分が結合した複合体の形で産生される。複合体毒素は分子量の違いにより、LL毒素（900 KDa）、L毒素（500 KDa）、またはM毒素（300 KDa）の分子形態に分けられる。LL毒素及びL毒素は、NTX、HA蛋白質及びNTNH（無毒非HA蛋白質である蛋白質）より成り、M毒素はこれらのうちHA蛋白質を欠く。LL毒素、L毒素及びM毒素は、ボツリヌス毒素複合体あるいはプロジェニター毒素などと呼ばれている。

【０００４】

A型ボツリヌス菌は、A1型～A5型の亜型に分類される。例えば、A1型ボツリヌス菌は、LL毒素、L毒素及びM毒素を産生するが、A2型ボツリヌス菌は、M毒素しか産生しない。A1型ボツリヌス菌としては、Hall、62A、97A、NCTC887、NCTC7272、NCTC2916及びNCTC11199株等がある。A2型ボツリヌス菌は、1986年に日本で最初に乳児ボツリヌス症に関する患者から同定されている(Sakaguchi G., et al., Int. J. Food Microbiol.,11: 231-242,1990：非特許文献１)。A2型ボツリヌス菌の臨床分離株は、Kyoto-F、Chiba-H、Y-8036、7I03-H、7I05-H及びKZ1828である。その他、A3型ボツリヌス菌としてはLoch Maree株、A4型ボツリヌス菌としては657Ba株、A5型ボツリヌス菌としてはHO 4244 0055、HO 4402 065、HO 4464 107及びHO 4068 0341株が知られている。

【０００５】

その他のボツリヌス菌では、B型、C型及びD型ボツリヌス菌はLL毒素及びM毒素を産生する。E型及びF型ボツリヌス菌はM毒素のみ、G型ボツリヌス菌はL毒素のみを産生する。

【０００６】

ボツリヌス毒素複合体は、アルカリ条件下で、NTX（S毒素とも呼ばれる。）とNTNHとが解離するため、この性質を利用することで、150 KDaのNTXのみを単離することができる。

【０００７】

従来、ボツリヌス毒素の精製方法は、培養液中のボツリヌス毒素複合体及びボツリヌス菌体を沈殿する酸沈殿、共存するDNAやRNA等を除去するプロタミン沈殿、粗精製を行う硫酸アンモニウム塩析等のいくつかの沈殿法を行った後に、イオン交換、金属あるいはゲルろ過等のクロマトグラフィー法を組み合わせる煩雑な方法であった。

【０００８】

例えばNTXの精製方法としては、培養液を硫酸、プロタミン及び硫酸アンモニウムで順次沈殿・抽出した後、遠心分離により得られた沈殿物を溶解し、SP Sephadex C-50（pH3.8）、Sephadex G-200（pH3.8）、DEAE Sephadex（pH8.0）で次々にカラムクロマトグラフィーを実施する阪口らの方法（Infect.Immun.12(6),1262-1270,1975：非特許文献２）がある。阪口らの方法では、A、B、C、D、E、F及びG型ボツリヌス菌由来のLL毒素、L毒素、M毒素あるいはNTXが精製できることが報告されている（Pharmac.Ther.19,165-194,1983：非特許文献３）。

【０００９】

その他、培養液を硫酸、硫酸プロトアミン、硫酸アンモニウムで順次沈殿した後、遠心分離により得られた沈殿物を溶解し、DEAE Sephadex（pH6）、DEAE Sephadex（pH7.9）、SP Sephadex（pH7）で次々にカラムクロマトグラフィーを実施するピガルケ・ハンスらの方法（特表2003-505343：特許文献１）、あるいはボツリヌス毒素複合体をDEAE Sephadex A-50（pH5.5）のカラムから回収し、硫酸アンモニウム塩析の沈殿物を溶解後、DEAE Sephadex A-50（pH7.9）、SP Sephadex C-50（pH7.0）等で次々にカラムクロマトグラフィーを実施するジョンソンらの方法（特表平11-507072：特許文献２）が知られている。これらのいずれの方法もクロマトグラフィーの前処理として、沈殿法・遠心分離を実施する煩雑な方法である。

【００１０】

工業規模で沈殿法を行う場合、1)操作が煩雑である、2)分離が鋭敏でなく低分離性能である、3)工程管理が難しく、少しの条件の違いが分離度に大きく影響する、4)生産規模の変更（スケールアップまたはスケールダウン）が難しい、5)生産性が低い、等の問題がある。

【００１１】

先に示した従来技術での問題点を具体的に述べると、1）酸沈殿によりボツリヌス菌体を全て破壊／除去したことを保証することが難しい、2）核酸除去を主目的とするプロタミン処理では、反応条件の設定が困難なため再現性の良い効率的な核酸の除去ができない、3）残存した核酸がクロマトグラフィー工程の分離度に大きく影響する、4）複数の種類の沈殿法を組み合わせるため製造期間が長い、等の問題がある。

【００１２】

また、沈殿法に伴い実施する遠心分離では、1）投資コスト、2）せん断力による毒素回収率の低下、3）処理時間、4)遠心分離における高速回転によりボツリヌス毒素を含むエアロゾルの発生、5)沈殿物回収作業における作業者の毒素に対する安全性確保、6)完全自動化設備・完全インライン化設備を提供し難い、等の問題がある（Handbook of Process Chromatography Development, Manufacturing, Validation and Economics., Hagel, Elsevier Science & Technology, 2008：非特許文献４）。

【先行技術文献】

【特許文献】

【００１３】

【特許文献１】特表2003-505343号公報

【特許文献２】特表平11-507072号公報

【非特許文献】

【００１４】

【非特許文献１】Sakaguchi G., et al., Int. J. Food Microbiol.,11: 231-242,1990.

【非特許文献２】Sakaguchi G., et al., Infect.Immun.12(6),1262-1270,1975.

【非特許文献３】Sakaguchi G., Pharmac.Ther.19,165-194,1983

【非特許文献４】Handbook of Process Chromatography Development, Manufacturing, Validation and Economics., Hagel, Elsevier Science & Technology, 2008.

【発明の概要】

【発明が解決しようとする課題】

【００１５】

このような状況下、本発明の課題は、従来技術と比較して沈殿・遠心工程を代替する分離技術を見出すことで工程数を短縮した、より簡便なボツリヌス毒素の工業規模での精製方法を提供することにある。

【課題を解決するための手段】

【００１６】

そこで、本発明者等は上述の諸問題に鑑み鋭意検討した結果、沈殿法・遠心分離を代替する分離技術として、膜ろ過法に着目し、ボツリヌス毒素の精製方法を新たに見出し、本発明を完成するに至った。本発明は、ボツリヌス毒素複合体を含有するボツリヌス菌培養液から膜ろ過法とクロマトグラフィー法を組み合わせたことを特徴とするボツリヌス毒素の精製方法を提供するものである。本発明の精製方法を適用した場合、従来技術に比べて極めて簡便に、短期間にかつ効果的に安定性の高いボツリヌス毒素を大量調製することができる。以下に、本発明をさらに詳細に説明する。

（１）膜ろ過法と陰イオン交換クロマトグラフィーと陽イオン交換クロマトグラフィーを組み合わせることを特徴とするボツリヌス毒素の精製方法。

（２）以下の工程を含む、上記（１）に記載の精製方法。

(a) 膜ろ過法により、ボツリヌス菌培養液からボツリヌス菌体を除去する工程、

(b) 前記(a)工程においてボツリヌス菌体が除去されたボツリヌス毒素溶液を陰イオン交換クロマトグラフィーカラムに展開する工程、

(c) ボツリヌス毒素成分がカラムに吸着するような条件下で、前記(b)工程において得られる陰イオン交換クロマトグラフィーカラムの回収画分を陽イオン交換クロマトグラフィーカラムに展開する工程、及び

(d) 前記陽イオン交換クロマトグラフィーから不純物を洗い流し、次いでボツリヌス毒素を溶出する工程。

（３）前記膜ろ過法が、精密膜ろ過法である上記（１）または（２）に記載の精製方法。
（４）前記精密膜ろ過法に用いる膜の孔径が0.22μm以下である上記（３）に記載の精製方法。
（５）前記膜ろ過法において、前記精密膜ろ過を行う前に、前記精密膜ろ過に用いる膜よりも大きな孔径を有する膜で粗膜ろ過を行う工程をさらに含む上記（２）に記載の精製方法。
（６）前記陰イオン交換クロマトグラフィーカラム内の緩衝液のpHが4.5から6.8である、上記（１）または（２）に記載の精製方法。
（７）前記陰イオン交換クロマトグラフィーカラム内の緩衝液の塩濃度が0から0.3 mol/Lである、上記（１）または（２）に記載の精製方法。
（８）前記陰イオン交換クロマトグラフィーにおいて、ボツリヌス毒素成分をカラムに吸着させる場合、当該カラムに吸着したボツリヌス毒素を塩濃度が0から2.0 mol/Lの勾配で溶出する、上記（１）または（２）に記載の精製方法。
（９）前記(b)工程を行う前に、前記(a)工程においてボツリヌス菌体が除去されたボツリヌス毒素溶液を、当該陰イオン交換クロマトグラフィーの塩濃度及びpHの条件に調整する工程をさらに含む上記（２）に記載の精製方法。
（１０）前記陽イオン交換クロマトグラフィーカラム内の緩衝液のpHが4.0から4.5である、上記（１）または（２）に記載の精製方法。
（１１）前記陽イオン交換クロマトグラフィーカラム内の緩衝液の塩濃度が0から0.2 mol/Lである、上記（１）または（２）に記載の精製方法。
（１２）前記(c)工程を行う前に、前記(b)工程において得られる陰イオン交換クロマトグラフィーカラムの回収画分を、当該陽イオン交換クロマトグラフィーの塩濃度及びpHの条件に調整する工程をさらに含む上記（２）に記載の精製方法。
（１３）前記(d)工程において、陽イオン交換クロマトグラフィーに吸着したボツリヌス毒素を塩濃度が0.2から0.7 mol/Lの勾配で溶出する、上記（１）または（２）に記載の精製方法。
（１４）(d)工程で溶出されるボツリヌス毒素がL毒素、LL毒素またはM毒素である上記（２）に記載の精製方法。
（１５）前記(a)～(d)の工程の後にさらに以下の工程を含む、上記（１）または（２）に記載のボツリヌス毒素の精製方法。
(e) ボツリヌス毒素成分がカラムに吸着し、かつ、ボツリヌス神経毒素（NTX）と無毒非HAタンパク質（NTNH）が解離するような条件下で、前記(d)工程で得られた陽イオン交換クロマトグラフィーの溶出画分を、第二陰イオン交換クロマトグラフィーカラムに展開する工程、及び
(f) 前記第二陰イオン交換クロマトグラフィーから不純物を洗い流し、次いでボツリヌス神経毒素（NTX）を溶出する工程。
（１６）前記第二陰イオン交換クロマトグラフィーカラム内の緩衝液のpHが6.8から8.0である、上記（１）、（２）または（１５）のいずれかに記載の精製方法。
（１７）前記第二陰イオン交換クロマトグラフィーカラム内の緩衝液の塩濃度が0から0.1 mol/Lである、上記（１）、（２）または（１５）のいずれかに記載の精製方法。
（１８）前記(f)工程において、第二陰イオン交換クロマトグラフィーに吸着したNTXを塩濃度が0から0.3mol/Lの勾配で溶出する、上記（１）、（２）または（１５）のいずれかに記載の精製方法。
（１９）前記(e)工程を行う前に、前記(d)工程において得られる陽イオン交換クロマトグラフィーの溶出画分を、当該第二陰イオン交換クロマトグラフィーの塩濃度及びpHの条件に調整する工程をさらに含む上記（１５）に記載の精製方法。
（２０）当該ボツリヌス毒素がA型、B型、C型、D型、E型、F型及びG型ボツリヌス菌由来のボツリヌス毒素である上記（１）から（１９）のいずれかに記載の精製方法。
（２１）上記（１）から（２０）のいずれかに記載の精製方法により精製されたボツリヌス毒素。

（２２）当該ボツリヌス毒素が、LL毒素、L毒素、M毒素、またはNTXのいずれかである上記（２１）に記載のボツリヌス毒素。
（２３）上記（２１）または（２２）に記載のボツリヌス毒素を有効成分として含有する薬剤。
【発明の効果】
【００１７】
本発明によれば、沈殿法・遠心分離を伴う従来技術と比較して、膜ろ過法とクロマトグラフィー法を組み合わせることによりボツリヌス菌培養液から極めて簡便に、短期間にかつ効果的に安定性の高いボツリヌス毒素を大量調製することが可能となった。また、沈殿法・遠心分離における沈殿物の回収作業を排除したことで、製造ラインの完全自動化設備・インライン化設備の提供が可能となった。さらに、作業員の介在がなくなるとともに、毒素溶液が環境に曝露されることがないため、作業者の労働安全衛生環境及びＧＭＰ準拠並びに無菌性確保を向上することを可能にした。
【発明を実施するための最良の形態】
【００１８】
本発明の方法が適用されるボツリヌス菌は、A～G型菌である。
【００１９】
本発明の膜ろ過法と陰イオン交換クロマトグラフィーと陽イオン交換クロマトグラフィーを組み合わせたボツリヌス毒素の精製方法は、具体的には以下の工程を含む。
(a) 膜ろ過法により、ボツリヌス菌培養液からボツリヌス菌体を除去する工程、
(b) 前記(a)工程においてボツリヌス菌体が除去されたボツリヌス毒素溶液を陰イオン交換クロマトグラフィーカラムに展開する工程、
(c) ボツリヌス毒素成分がカラムに吸着するような条件下で、前記(b)工程において得られる陰イオン交換クロマトグラフィーカラムの回収画分を陽イオン交換クロマトグラフィーカラムに展開する工程、及び
(d) 前記陽イオン交換クロマトグラフィーから不純物を洗い流し、次いでボツリヌス毒素を溶出する工程。
【００２０】
ボツリヌス菌体、ボツリヌス毒素複合体及びその他の夾雑物を含むボツリヌス菌培養液は、例えば、阪口らの培養方法（Biochemical aspects of botulism: Purification and oral toxicities of Clostridium botulinum progenitor toxins.,21-34,Lewis GE.,1981, Academic Press,New York）に従って調製される。その一例としては、PYG培地で30℃、4日間培養する方法により得られるが、使用される培地、培養条件は目的により適宜選択される。別の培養方法として、Siegel, L.S.ら（Appl.Microbiol.Vol.38, No.4:606-611.,1979）の変法で調製した培養液を使用することもできる。
【００２１】
本発明の精製方法によれば、まず、(a)工程として、得られたボツリヌス菌培養液を精密ろ過用フィルターでろ過することにより、ボツリヌス菌体を除去する。ろ過の様態に特別な制限はなく、例えばデェッドエンドろ過法、タンジェンシャルフロー法等が採用され得る。精密ろ過膜は蛋白低吸着性のもので、例えば、酢酸セルロース、親水性ポリエーテルスルホン、親水性ポリフッ化ビニリデン（PVDF）等の市販のろ過膜を使用することができる。また、精密ろ過膜への負荷を軽減する目的から、精密ろ過の前に比較的膜孔径の大きな膜でろ過してもよい。膜孔径は、特に限定されるものではないが、最終ろ過膜の膜孔径は0.1～0.22 μｍ、好ましくは、0.2μｍであり、フィルターの完全性試験によりボツリヌス菌体の除去を保証できるものでなければならない。
【００２２】
(a)工程で得られたボツリヌス菌体を除去した夾雑物を含むボツリヌス毒素複合体溶液を(b)工程の陰イオン交換クロマトグラフィーカラムに展開する前に、当該ボツリヌス毒素複合体溶液を(b)工程における陰イオン交換クロマトグラフィーの塩濃度、pHの条件に調整する。本調整は、例えば、工業用タンジェンシャルフロー式限外ろ過装置で濃縮及び

バッファー交換を行い、蛋白濃度、塩濃度及びpHを調整する。限外ろ過膜の膜孔径としては、10～100 KDaであればよい。

【００２３】

次に(b)工程として、調製したボツリヌス毒素複合体溶液を、緩衝液等で平衡化した陰イオン交換樹脂に展開し、核酸やその他の不純物を吸着除去する。当該緩衝液としては、塩濃度が0.3 mol/L以下で、pHが4.5～6.8の範囲、好適には塩類濃度が0.1mol/LでpHが6.0であれば特に限定されないが、例えばクエン酸またはクエン酸・リン酸混合緩衝液は好適な一例である。また、核酸が吸着除去できれば、ボツリヌス毒素複合体は吸着・非吸着のいずれでもよい。好適には、核酸は吸着除去し、ボツリヌス毒素複合体は非吸着にする。ボツリヌス毒素複合体をカラムに吸着させる場合は、0から2.0mol/Lの勾配の塩濃度の同じ緩衝液で溶出させる。使用する陰イオン交換樹脂は、DEAE Sepharose Fast Flow（GEヘルスケア）、Q Sepharose Fast Flow（GEヘルスケア）、CAPTO-Q（GEヘルスケア）、DEAEトヨパール（東ソー）、QAEトヨパール（東ソー）等の市販の陰イオン交換樹脂を使用することができる。塩の種類は限定されず、イオン強度が上記濃度の塩化ナトリウムに相当する濃度で使用すればよい。

【００２４】

(b)工程で得られたボツリヌス毒素複合体溶液を(c)工程の陽イオン交換クロマトグラフィーに展開する前に、当該ボツリヌス毒素複合体溶液を当該陽イオン交換クロマトグラフィーの塩濃度、pHの条件に調整する。本調整は、例えば、工業用タンジェンシャルフロー式限外ろ過装置で濃縮及びバッファー交換を行い、蛋白濃度、塩濃度及びpHを調整する。

【００２５】

次に(c)工程として、(b)工程で得られたボツリヌス毒素複合体溶液を陽イオン交換樹脂に展開し、ボツリヌス毒素複合体を吸着させる。当該陽イオン交換樹脂は予め塩濃度が0.2 mol/L以下でpHが4.0～4.5の範囲、好適には塩濃度が0.2 mol/LでpHが4.2の酢酸緩衝液等の緩衝液で平衡化しておく。使用する緩衝液は、塩濃度が0.2 mol/L以下でかつpHが4.0～4.5の範囲であればよく、特に酢酸緩衝液に限定されるものではない。ボツリヌス毒素複合体が吸着できれば、夾雑する蛋白質は吸着・非吸着のいずれでもよい。好適には、ボツリヌス毒素複合体を吸着し、夾雑する蛋白質は非吸着にする。前記緩衝液で洗浄後、塩濃度が0.2～0.7 mol/Lの勾配の同じ緩衝液でリニアグラジェントによりボツリヌス毒素複合体を溶出し分画する。使用する陽イオン交換樹脂は、SP Sepharose Fast Flow（GEヘルスケア）、CM Sepharose Fast Flow（GEヘルスケア）、S Sepharose Fast Flow（GEヘルスケア）、SP トヨパール（東ソー）等の市販の陽イオン交換樹脂を使用することができる。塩の種類は限定されず、イオン強度が上記濃度の塩化ナトリウムに相当する濃度で使用すればよい。

【００２６】

以上の方法により、夾雑する蛋白質が除去され、高度に精製されたボツリヌス毒素複合体（L毒素、LL毒素、M毒素）が得られる。さらに、より純度の高いボツリヌス毒素複合体が要求される場合は、更なるクロマトグラフィー、例えば、イオン交換クロマトグラフィー、ゲルろ過クロマトグラフィー、疎水クロマトグラフィー、アフィニティークロマトグラフィー等の方法、あるいはそれらを組み合わせることにより高度に精製することができる。

【００２７】

得られたボツリヌス毒素複合体溶液からNTXを得るためには、さらに、以下の工程を行い、当該ボツリヌス毒素複合体から無毒性非HA蛋白（NTNH）を解離・除去しなければならない。

(e) ボツリヌス毒素成分がカラムに吸着し、かつ、ボツリヌス神経毒素（NTX）と無毒非HAタンパク質（NTNH）が解離するような条件下で、前記(d)工程で得られた陽イオン交換クロマトグラフィーの溶出画分を、第二陰イオン交換クロマトグラフィーカラムに展開する工程、及び

(f) 前記第二陰イオン交換クロマトグラフィーから不純物を洗い流し、次いでボツリヌス

神経毒素（NTX）を溶出する工程。

【００２８】

(e)工程として、ボツリヌス毒素複合体溶液を陰イオン交換樹脂に展開し、NTXを吸着させる。当該陰イオン交換樹脂は、予め塩濃度が0.1 mol/L以下でpHが6.8～8.0の範囲、好適には塩濃度が0 mol/LでpHが7.5のリン酸またはトリス緩衝液等の緩衝液で平衡化しておく。使用する緩衝液は、塩濃度が0.1 mol/L以下でかつpHが6.8～8.0の範囲であればよく、特にリン酸またはトリス緩衝液に限定されるものではない。NTX及びNTNHが吸着できれば、夾雑する蛋白質は吸着・非吸着のいずれでもよい。好適には、NTX及びNTNHを吸着し、夾雑する蛋白質は非吸着する。

【００２９】

次に(f)工程として、NTX及びNTNHが吸着されたカラムを前記緩衝液で洗浄後、塩濃度が0～0.3 mol/Lの勾配の同じ緩衝液でリニアグラジェントにより高度精製NTXを溶出し分画する。使用する陰イオン交換樹脂は、DEAE Sepharose Fast Flow（GEヘルスケア）、Q Sepharose Fast Flow（GEヘルスケア）、Capto-Q（GEヘルスケア）、DEAE トヨパール（東ソー）、QAE トヨパール（東ソー）等の市販の陰イオン交換樹脂を使用することができる。塩の種類は限定されず、イオン強度が上記濃度の塩化ナトリウムに相当する濃度で使用すればよい。

【００３０】

また、上記(e)工程において、陰イオン交換樹脂に展開する前に、予めNTXとNTNHを解離させても良い。例えば、M毒素から成るボツリヌス毒素複合体溶液を、限外ろ過法により、塩濃度が0～0.1 mol/LでpHが6.8～8.0の範囲、好適には塩濃度が0 mol/LでpH7.5のリン酸またはトリス緩衝液等でバッファー交換することで、NTXとNTNHを解離させる。本限外ろ過工程は、工業用タンジェンシャルフロー式限外ろ過装置等を用いて行うことができる。本工程に使用する限外ろ過膜は特に限定するものではなく、ハイドロザルト、親水性ポリエーテルスルホン等の市販の限外ろ過膜を使用することができる。膜孔径は、10～30 KDaであればよい。

【００３１】

上記の工程により得られた高度精製ボツリヌス毒素を医薬品として使用する場合、薬学的に使用可能な添加剤を使用する。例えば、安定剤としては、アルブミン、アミノ酸、糖類、界面活性剤等を使用することでき、好適なアミノ酸はアルギニン一塩酸塩である。また、当該高度精製ボツリヌス毒素を長期保存する際は、凍結保存することができる。

【００３２】

以下に実施例において本発明を具体的に説明するが、本発明は実施例の記載に何ら限定されるものではない。

【図面の簡単な説明】

【００３３】

【図１】高度精製A2型NTXの液状保存における活性残存率を示す図。

【実施例】

【００３４】

《試験例：試験及び方法》

（1）SDS-PAGE試験

本法では高度精製NTX溶液またはボツリヌス毒素複合体溶液の純度を調べる。Laemmliらの方法（Nature 227:680-685）を一部改変してSDS-PAGE試験を実施した。マーカーはプレシジョンplusブルーステインド（Bio-Rad Laboratories, Inc.）を使用した。毒素溶液をDTT（ナカライテスク）で還元処理した後、10％単一濃度または5%～20%のリニアグラジエントゲル（ATTO）で電気泳動した。電気泳動後は、クマシーブリリアントブルーR250（ナカライ）で染色した。

（2）マウス腹腔内投与LD_{50}試験（i.p.法）

本法ではボツリヌス毒素溶液の毒素活性を調べる。ボツリヌス毒素をマウス腹腔内に投与し、その後4日間の累積致死率から50％致死量（LD_{50}）を推定した。毒素溶液を希釈し

、マウス一匹あたり希釈検体0.5mLを投与した。Probit法によりLD_{50}を算出した。
（3）マウス尾静脈投与試験
本法はボツリヌス毒素の毒素活性測定法として一般的に使用されるマウス腹腔内投与LD_{50}試験（i.p.法）あるいはラットCMAP法（WO 2007/125604 A1）を代替する簡便法である。高濃度の毒素をマウス尾静脈に投与し生存時間を調べ、事前に作成した生存時間（分）と毒素活性濃度（LD_{50}/mL）との回帰直線から、検体の毒素活性濃度を算出した。
（4）蛋白濃度試験（ローリー法）
本法ではボツリヌス毒素溶液の蛋白濃度を調べる。DCプロテインアッセイキット（BIO-RAD）を用いた。検量線用溶液にはウシ血清アルブミン（PIERCE）を用いた。
（5）サイズ排除HPLC分析（SEC-HPLC）
本法では高度精製NTX溶液の純度を調べる。TSK-GEL G3000SW_{XL}（7.8 mm×300 mm、東ソー）を0.2 mol/L塩化ナトリウム及び0.05w/v%アジ化ナトリウムを含む50 mmol/Lリン酸ナトリウム、pH 5.8で平衡化し、流速0.25 mL/minで分析した。全エリア面積に占めるNTXのエリア面積の割合（%）を純度として算出した。
（6）核酸含量試験
本法では高度精製NTX溶液に含まれる核酸の濃度を調べる。CyQUANT Cell Proliferation Assay Kit(Molecular Probes)を用いた。
（7）エンドトキシン試験
本法では高度精製NTX溶液に含まれるエンドトキシンの濃度を調べる。日本薬局方15改正 エンドトキシン試験に準じて試験した。

【００３５】
《比較例１：従来技術によるA2型M毒素の分離・精製》
A2型ボツリヌス菌Chiba-H株を阪口らの方法（Biochemical aspects of botulism: Purification and oral toxicities of Clostridium botulinum progenitor toxins.,21-34,Lewis GE.,1981, Academic Press,New York）で培養した。96時間後の培養液30 LにpH3.5になるまで3 N硫酸を添加し、室温で一晩静置した。沈殿物を0.2 mol/Lリン酸緩衝液、pH6.0に溶解し、37℃で1時間抽出処理した。抽出液に硫酸アンモニウムを添加し、4℃で一晩静置した。遠心分離により回収した沈殿を50 mmol/L酢酸緩衝液、pH4.2で溶解した。溶解液を透析チューブに入れ、前記緩衝液に4℃で2日間透析した。遠心分離により回収した沈殿を0.5 mol/L塩化ナトリウムを含む50 mmol/L酢酸緩衝液、pH4.2で溶解した。この溶解液に等量の0.5 mol/L塩化ナトリウムを含む50 mmol/Lクエン酸緩衝液、pH4.2を加えた後、プロタミン処理した。遠心分離により沈殿（核酸）を除去した上清を、予め0.2 mol/Lを含む50 mmol/L酢酸緩衝液、pH4.2で平衡化したSP Sepharose Fast Flowへ展開し吸着させた。前記緩衝液で洗浄後、0.7 mol/L塩化ナトリウムを含む同じ酢酸緩衝液でリニアグラジエントにより溶出した。溶出画分の第一ピークを分画し、回収した溶液を撹拌式限外ろ過装置（アミコン、YM-30）で濃縮した。この濃縮液を予め0.2 mol/L塩化ナトリウムを含む50 mmol/L酢酸バッファー、pH6.0で平衡化したSephadex G-200へ展開した。溶出第二ピークを分画し、A2型M毒素を回収した。回収したA2型M毒素溶液を撹拌式限外ろ過装置（アミコン、YM-30）で濃縮した。

【００３６】
《比較例２：従来技術によるA2型NTXの分離・精製》
比較例１にて濃縮したA2型M毒素溶液を透析チューブに入れ、10 mmol/Lリン酸緩衝液、pH7.5で4℃、一晩透析した。透析後液を予め10 mmol/Lリン酸緩衝液、pH7.5で平衡化したDEAE Sepharose Fast Flowに展開し吸着させた。前記緩衝液で洗浄後、0.3 mol/L塩化ナトリウムを含む同じ酢酸緩衝液でリニアグラジエントにより溶出した。溶出画分の第一ピークを分画し、回収した溶液を撹拌式限外ろ過装置（アミコン、YM-10）で濃縮した後、10 mmol/Lリン酸緩衝液、pH7.5でバッファー交換した。これを高度精製A2型NTX溶液とした。培養液から高度精製A2型NTX溶液を精製するまでの製造期間は7日間であった。本試験より得られた結果を表２に示す。

【００３７】

《実施例１：0.2 μmフィルターによるボツリヌス菌培養液の除菌ろ過》

A2型ボツリヌス菌培養液30 Lを6,700×g、4℃、20分間で遠心分離し、遠心上清を回収した。この培養上清液をタンジェンシャルフロー式ろ過装置（ザルトリウス）を用いて膜孔径0.2 μmのハイドロザルト（ザルトリウス）で除菌ろ過した。ろ過処理量は、25 L/m^2/hrであった。

【００３８】

《実施例２：粗ろ過及び除菌ろ過に関する検討》

除菌ろ過の処理量を高めることを目的として、事前に比較的膜孔径の大きい膜でろ過する粗ろ過工程の導入について検討した。粗ろ過用膜として、ゼータプラス（CUNO）及びゼータカーボンR53SLP(CUNO)を使用した。ゼータプラスではろ過精度の異なる4種類の膜を使用した。ろ過精度は90LA、60LA、30LA及び05SPの順で高い。また、除菌ろ過用膜として、0.65 μmザルトクリーンCA（ザルトリウス）及び0.2 μmザルトポア2（ザルトリウス）を連結したフィルターを使用した。A2型ボツリヌス菌Chiba-H株の培養液30 Lを各粗ろ過用膜及び除菌濾過用膜で定圧ろ過（0.03 MPa）し、ろ過量を調べた。本試験で得られた結果を表１に示す。粗ろ過フィルターを導入することで、0.2 μmフィルターによる除菌ろ過の処理量は、実施例１（25 L/m^2/hr）に比べて40倍になった。

【００３９】

【表１】

サンプル	フィルター	処理量（L/m^2/hr）
ボツリヌス菌培養液	ゼータプラス60LA	44
	ゼータプラス30LA	177
	ゼータプラス05SP	854
	ゼータカーボンR53SLP	278
ゼータプラス05SPろ過液	ゼータプラス30LA	776
ゼータプラス30LAろ過液	ゼータプラス60LA	865
ゼータプラス60LAろ過液	0.6μmザルトクリーンCA＋0.2μmザルトポア2	1000

【００４０】

《実施例３：本発明によるA2型M毒素の分離・精製》

A2型ボツリヌス菌Chiba-H株をSiegel, L.S.ら（Appl.Microbiol.Vol.38, No.4:606-611.,1979）の方法を一部改変して培養した。48時間後の培養液 30 Lをゼータプラスフィルター（05SP）とゼータプラスフィルターマキシマイザー（30LA+60LA）を連結したフィルターで0.03 MPaの圧力で粗ろ過した。このろ過液を0.65 μmザルトクリーンCA及び0.2 μmザルトポア2を連結したフィルターで0.03 MPaの圧力で除菌ろ過した。ろ過後に0.2 μmフィルターが完全性試験に適合することを確認した。ボツリヌス菌体を除去したろ過液をタンジェンシャルフロー式限外ろ過装置（ザルトリウス、30 KDa）で濃縮した後、0.1 mol/L塩化ナトリウムを含む20 mmol/Lクエン酸・リン酸混合緩衝液、pH6.0でバッファー交換した。この濃縮液を、予め0.1 mol/L塩化ナトリウムを含む20 mmol/Lクエン酸・リン酸混合緩衝液、pH6.0で平衡化したQ Sepharose Fast Flow（GEヘルスケア）に展開し、さらに使用したカラムクロマトグラフィーのゲル量（CV）の約7倍量（7CV）の前記緩衝液で洗浄し、非吸着画分を集めた。本工程で核酸を効果的に除去すると同時に粗精製を達成することができた。集めた非吸着画分をタンジェンシャルフロー式限外ろ過装置（ザルトリウス、30 KDa）で濃縮した後、0.2 mol/L塩化ナトリウムを含む50 mmol/L酢酸緩衝液、pH4.2でバッファー交換した。この濃縮液を、予め0.2 mol/L塩化ナトリウムを含む50 mmol/L酢酸緩衝液、pH4.2で平衡化したSP Sepharose Fast Flow（GEヘルスケア）に展開し吸着させた。次に、約10CVの前記緩衝液で洗浄後、0.7 mol/L塩化ナトリウムを含む同じ酢酸緩衝液でリニアグラジエントにより溶出した。溶出画分の第一ピークを分画し、A2型M毒素を回収した。回収したA2型M毒素溶液をタンジェンシャルフロー式限外ろ過装置（ザルトリウス、30 KDa）で濃縮した後、0.2 mol/L塩化ナトリウムを含む50 mmol/L酢酸緩衝液

、pH6.0でバッファー交換した。得られたA2型M毒素溶液をSDS-PAGE試験した結果、M毒素以外のバンドを認めなかった。

【００４１】

《実施例４：本発明によるA2型NTXの分離・精製》

更に、実施例３にて回収したA2型M毒素溶液をタンジェンシャルフロー式限外ろ過装置（ザルトリウス、30 KDa）で濃縮した後、10 mmol/Lリン酸緩衝液、pH7.5でバッファー交換した。この濃縮液を、予め10 mmol/Lリン酸緩衝液、pH7.5で平衡化したDEAE Sepharose Fast Flow（GEヘルスケア）に展開し、吸着させた。次に、約10CVの前記緩衝液で洗浄後、0.3 mol/L塩化ナトリウムを含む同じリン酸緩衝液でリニアグラジエントにより溶出した。溶出液の第一ピークを分画し、回収した。これを高度精製A2型NTX溶液とした。培養液から高度精製A2型NTX溶液を精製するまでの製造期間は3日間であった。本試験で得られた結果を表２に示す。その結果、従来技術と比較して、極めて少ない工程数で高純度・高比活性のNTXを大量調製できることが示された。作業者による沈殿物の回収作業を排除したことから、製造ラインを完全自動化設備及びインライン化設備の提供が可能であることが示された。作業者が高濃度のボツリヌス菌またはボツリヌス毒素に曝露する機会を排除・低減したことにより、作業者の労働安全性を著しく向上できること及びGMP準拠及び無菌性確保を向上できることが期待された。さらに、従来技術に比べて製造期間を著しく短縮し、効率的に工業規模で高度精製NTXを製造することが可能であることが示された。

【００４２】

《実施例５：本発明によるA1型M毒素の分離・精製》

A1型ボツリヌス菌Hall株をSiegel, L.S.ら（Appl.Microbiol.Vol.38, No.4:606-611.,1979）の方法を一部改変して培養した。48時間後の培養液30 Lを実施例３と同様の方法で菌体除去、濃縮、バッファー交換、Q Sepharose Fast Flowクロマトグラフィー、SP Sepharose Fast Flowクロマトグラフィーの順で実施した。SP Sepharose Fast Flowクロマトグラフィーでは、溶出画分の第三ピークを分画し、A1型M毒素を回収した。回収したA1型M毒素溶液をタンジェンシャルフロー式限外ろ過装置（ザルトリウス、30 KDa）で濃縮した後、0.2 mol/L塩化ナトリウムを含む50 mmol/L酢酸緩衝液、pH6.0でバッファー交換した。得られたA1型M毒素をSDS-PAGE試験した結果、M毒素以外のバンドを認めなかった。

【００４３】

《実施例６：本発明によるA1型NTXの分離・精製》

更に、実施例５にて回収したA1型M毒素溶液をタンジェンシャルフロー式限外ろ過装置（ザルトリウス、30 KDa）で濃縮した後、0.2 mol/L塩化ナトリウムを含む50mmol/L酢酸緩衝液、pH6.0でバッファー交換した。この濃縮液を予め0.2 mol/L塩化ナトリウムを含む50 mmol/L酢酸緩衝液、pH6.0で平衡化したSephacryl S-300 High Resolution（GEヘルスケア）へ展開し、ゲルろ過した。溶出された第一ピークを分画し回収した。以降の操作は、実施例４と同様に、濃縮、pH7.5緩衝液でのバッファー交換、DEAE Sepharose Fast Flowクロマトグラフィーの順で実施することにより、高度精製A1型NTX溶液を得た。培養液から高度精製A1型NTX溶液を精製するまでの製造期間は4日間であった。本試験で得られた結果を表２に示す。その結果、本発明により、高度精製A2型NTXと同様に高純度・高比活性のA1型NTXを精製することが可能であることが示された。このことから、本発明によれば、ボツリヌス毒素複合体の構成成分（LL毒素/L毒素/M毒素）が異なる菌株（B型～G型）からでもNTXを高純度・高比活性で精製できることが期待できる。

【００４４】

《実施例７：本発明によるA1型LL及びL毒素の分離・精製》

実施例５でのSP Sepharose Fast Flowクロマトグラフィーにおいて、溶出画分の第二ピークを分画し、LL毒素及びL毒素を回収した。回収したLL毒素及びL毒素混合溶液をタンジェンシャルフロー式限外ろ過装置（ザルトリウス、30 KDa）で濃縮した後、0.2 mol/L塩化ナトリウムを含む50 mmol/L酢酸緩衝液、pH6.0でバッファー交換した。得られたLL毒素及びL毒素混合溶液をSDS-PAGE試験した結果、LL毒素及びL毒素以外のバンドを認めなかった。

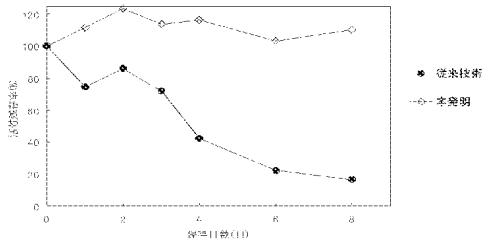
【００４５】
【表２】

項目	試験成績		
	比較例２ （従来技術：A2型NTX）	実施例４ （本発明：A2型NTX）	実施例６ （本発明：A1型NTX）
バッチ数	7	6	1
比活性（$\times 10^{7}$ LD_{50}/mg）	0.6～9.4	12.5～14.6	12.6
純度（%、SEC-HPLC）	93.3	99.69～100.0	100.0
純度（SDS-PAGE）	NTX以外のバンドを認めず	NTX以外のバンドを認めず	NTX以外のバンドを認めず
核酸含量 （$\times 10^{-2}$ pg/100 LD_{50}）	2.5～24.3	＜1.0～＜2.9	N.D.
エンドトキシン含量 （$\times 10^{-7}$ EU/100 LD_{50}）	N.T.	0.7～2.3	N.T.

N.D. ; Not Deteced
N.T. ; Not Tested

【００４６】
《実施例８：高度精製A2型NTXの安定性の比較》
従来技術（比較例２）及び本発明（実施例４）で調製した高度精製A2型NTX溶液の23℃における安定性を比較した。活性残存率の結果を図１に示した。その結果、本発明で調製した高度精製A2型NTXは、従来技術で調製した毒素に比べて毒素活性の低下はなかった。また、SDS-PAGE試験においても本発明で調製した高度精製A2型NTXに分解物は認められなかった。この結果は、本発明で調製した高度精製A2型NTXは従来技術で調製した毒素に比べて安定性が高いことを示すものであった。
【産業上の利用可能性】
【００４７】
本発明によれば、従来技術と比較して、沈殿法・遠心分離を行わずに膜ろ過法とクロマトグラフィー法を組み合わせることによりボツリヌス菌培養液から極めて簡便に、短期間にかつ効果的に安定性の高いボツリヌス毒素を大量調製することが可能となった。また、沈殿法・遠心分離における沈殿物の回収作業を排除したことで、製造ラインの完全自動化設備・インライン化設備の提供が可能となった。さらに、作業員の介在がなくなるとともに、毒素溶液が環境に曝露されることがないため、作業者の労働安全衛生環境及びGMP準拠並びに無菌性確保を向上することを可能にした。

【図１】



フロントページの続き

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First Named Inventor/Applicant Name:	Hyun Ho JUNG
Filer:	Judith Unjin Kim/Jessica LaBossiere
Attorney Docket Number:	2479-0007US01

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Filing Fees for U.S. National Stage under 35 USC 371

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Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
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Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	260	260
		Total in USD ($)		**260**

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First Named Inventor/Applicant Name:	Hyun Ho JUNG
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Attorney Docket Number:	2479-0007US01
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National Stage of an International Application under 35 U.S.C. 371

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New International Application Filed with the USPTO as a Receiving Office

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/338,561	04/01/2019	Hyun Ho JUNG	2479-0007US01	3639

137713 7590 03/23/2022

Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

EXAMINER	
HUANG, RYAN	

ART UNIT	PAPER NUMBER
1777	

NOTIFICATION DATE	DELIVERY MODE
03/23/2022	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

eofficeaction@appcoll.com
patents@potomaclaw.com

Supplemental ***Notice of Allowability***	**Application No.** 16/338,561	**Applicant(s)** JUNG et al.	
	Examiner RYAN B HUANG	**Art Unit** 1777	**AIA (FITF) Status** Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to 09 March 2022.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☐ The allowed claim(s) is/are _____ . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All b) ☐ Some* c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☐ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/Ryan B Huang/
Primary Examiner, Art Unit 1777

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (01-22)
Approved for use through 05/31/2024. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16338561
Filing Date	2017-11-29
First Named Inventor	Hyun Ho JUNG
Art Unit	1777
Examiner Name	Ryan HUANG
Attorney Docket Number	2479-0007US01

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	2011074025	JP	A	2011-04-14	Chemo-Sero-Therapeutic Research Institute	English abstract provided	

If you wish to add additional Foreign Patent Document citation information please click the Add button Add

NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /RBH/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16338561
Filing Date	2017-11-29
First Named Inventor	Hyun Ho JUNG
Art Unit	1777
Examiner Name	Ryan HUANG
Attorney Docket Number	2479-0007US01

	1	Korean Office Action (Notification of Reason of Refusal) dated February 23, 2022, issued in Korean Application No. 10-2016-0127540, with English translation.	X

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature	/Ryan B Huang/	Date Considered	03/16/2022

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /RBH/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16338561
Filing Date	2017-11-29
First Named Inventor	Hyun Ho JUNG
Art Unit	1777
Examiner Name	Ryan HUANG
Attorney Docket Number	2479-0007US01

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

X That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

X The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Judith U. Kim/	Date (YYYY-MM-DD)	2022-03-09
Name/Print	Judith U. Kim	Registration Number	40679

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /RBH/

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /RBH/

Document Description: Issue Fee Payment (PTO-85B)

Issue Fee Transmittal Form

Application Number	Filing Date	First Named Inventor	Atty. Docket No.	Confirmation No.
16338561	01-Apr-2019	Hyun Ho JUNG	2479-0007US01	3639

TITLE OF INVENTION :

METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION

Entity Status	Application Type	Art Unit	Class - Subclass	EXAMINER
Regular Undiscounted	U.S. National Stage under 35 USC 371	1777	635000	

Issue Fee Due	Publication Due	Total Fee(s) Due	Date Due	Prev. Paid Fee
$1200	$0	$1200	21-Apr-2022	$0

1.Change of Correspondence Address and/or Indication Of Fee Address (37 CFR 1.33 & 1.363)

Current Correspondence Address:	Current Indicated Fee Address :
137713 Potomac Law Group, PLLC 8229 Boone Boulevard Suite 430 Vienna VA 22182 UNITED STATES 202-670-3220 eofficeaction@appcoll.com	
☐ Change of correspondence address requested, system generated AIA/122-EFS form attached	☐ Fee Address indication requested, system generated SB/47-EFS form attached

2.Entity Status

Change in Entity Status

○ Applicant certifying micro entity status; system generated Micro Entity certification form attached. See 37 CFR 1.29.
Note: Absent a valid certification of micro entity status, issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment. If this box is checked, you will be prompted to choose a micro entity status on the gross income basis (37 CFR 1.29(a)) or the institution of higher education basis (37 CFR 1.29(d)), and make the applicable certification online.

○ Applicant asserting small entity status. See 37 CFR 1.27.
Note: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

◉ Applicant changing to regular undiscounted fee status.
Note: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

Doc Code: IFEE PTOL/85B-EFS

Document Description: Issue Fee Payment (PTO-85B)

3.The Following Fee(s) Are Submitted:

☒ Issue Fee

☐ I authorize USPTO to apply my previously paid issue fee to the current fees due

☐ Publication Fee

☐ The Director is hereby authorized to apply my previously paid issue fee to the current fee due and to charge deficient fees to Deposit Account Number _____

☐ Advance Order - # of copies _____

☒ If **in addition to** the payment of the issue fee amount submitted with this form, there are any discrepancies in any amount(s) due, the Director is authorized to charge any deficiency or credit any overpayment, to Deposit Account Number 505875 . The **issue fee must be submitted** with this form. **If payment of the issue fee does not accompany this form, checking this box and providing a deposit account number will NOT be effective to satisfy full payment of the fee(s) due.**

4.Firm and/or Attorney Names To Be Printed

NOTE: If no name is listed, no name will be printed
For printing on the patent front page, list to be displayed as entered

1. POTOMAC LAW GROUP, PLLC
2.
3.

5.Assignee Name(s) and Residence Data To Be Printed

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

Name	City	State	Country	Category
MEDYTOX INC.	CHEONGJU-SI		KOREA, REPUBLIC OF	corporation

6.Signature

I certify, in accordance with 37 CFR 1.4(d)(4) that I am an attorney or agent registered to practice before the Patent and Trademark Office who has filed and has been granted power of attorney in this application. I also certify that this Fee(s) Transmittal form is being transmitted to the USPTO via EFS-WEB on the date indicated below.

Signature	/Judith U. Kim/	**Date**	04-18-2022
Name	Judith Unjin Kim	**Registration Number**	40679

Electronic Patent Application Fee Transmittal

Application Number:	16338561
Filing Date:	01-Apr-2019
Title of Invention:	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
First Named Inventor/Applicant Name:	Hyun Ho JUNG
Filer:	Judith Unjin Kim/Jessica LaBossiere
Attorney Docket Number:	2479-0007US01

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY APPL ISSUE FEE	1501	1	1200	1200
PUBL. FEE- EARLY, VOLUNTARY, OR NORMAL	1504	1	0	0
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1200**

Electronic Acknowledgement Receipt

EFS ID:	45488407
Application Number:	16338561
International Application Number:	
Confirmation Number:	3639
Title of Invention:	METHOD OF ISOLATING BOTULINUM TOXIN FROM BOTULINUM TOXIN-CONTAINING SOLUTION
First Named Inventor/Applicant Name:	Hyun Ho JUNG
Customer Number:	137713
Filer:	Judith Unjin Kim/Jessica LaBossiere
Filer Authorized By:	Judith Unjin Kim
Attorney Docket Number:	2479-0007US01
Receipt Date:	18-APR-2022
Filing Date:	01-APR-2019
Time Stamp:	11:10:27
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$1200
RAM confirmation Number	E20224HB10243574
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	Web85b.pdf	62835 f80296a3f6235082959ef8d9439954377cf83c98	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	40691 a60439590aab6479742544f80d5ceece0dc64d7f	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	103526		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/338,561	05/17/2022	11331598	2479-0007US01	3639

137713 7590 04/27/2022

Potomac Law Group, PLLC
8229 Boone Boulevard
Suite 430
Vienna, VA 22182

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Patents Stakeholder Experience (OPSE), Stakeholder Support Division (SSD) at (571)-272-4200.

INVENTOR(s) (Please see PAIR WEB site http://pair.uspto.gov for additional inventors):

Hyun Ho JUNG, Seoul, KOREA, REPUBLIC OF;
Gi Hyeok YANG, Cheonan-si, KOREA, REPUBLIC OF;
Chang Hoon RHEE, Seoul, KOREA, REPUBLIC OF;
Hack Woo KIM, Cheongju-si, KOREA, REPUBLIC OF;
Gi Hyuck RYU, Daejeon, KOREA, REPUBLIC OF;

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

MEDYTOX INC., Cheongju-si, KOREA, REPUBLIC OF

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The USA offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to encourage and facilitate business investment. To learn more about why the USA is the best country in the world to develop technology, manufacture products, and grow your business, visit SelectUSA.gov.