UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO.	: 11,236,318 B2
APPLICATION NO.	: 16/399386
DATED	: February 1, 2022
INVENTOR(S)	: Hikaru Mizumura, Toshio Oda and Shun-ichiro Kawabata

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title Page

(56) References Cited

Page 2, Under OTHER PUBLICATIONS, left column, Line 29, delete "espress" and insert --express-- therefor.

Page 2, Under OTHER PUBLICATIONS, left column, Line 47, delete "[online],Retrived" and insert --[online], Retrieved-- therefor.

Page 2, Under OTHER PUBLICATIONS, right column, Line 23, delete "*Ganssia*" and insert --*Gaussia*-- therefor.

Page 2, Under OTHER PUBLICATIONS, right column, Line 43, delete "Tepidopteran" and insert --lepidopteran-- therefor.

Page 2, Under OTHER PUBLICATIONS, right column, Line 56, delete "Gycobiol" and insert --Glycobiol-- therefor.

In the Specification

Column 29, Line 51, delete "Celfectin" and insert --Cellfectin-- therefor.

Signed and Sealed this
Nineteenth Day of April, 2022

Katherine Kelly Vidal

Katherine Kelly Vidal
Director of the United States Patent and Trademark Office

CRL Exhibit 1040
CRL v. Seikagaku

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Docket No: Q246419
Hikaru MIZUMURA, et al.	Group Art Unit: 1656
Appln. No.: 16/399,386	Examiner: STEADMAN, DAVID J
Confirmation No.: 9293	Patent No.: 11,236,318
Filed: April 30, 2019	Issue Date: February 1, 2022

For: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

REQUEST FOR CERTIFICATE OF CORRECTION

ATTN: Certificate of Correction Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Pursuant to the provisions of 37 C.F.R. § 1.322, please enter the attached Certificate of Correction.

Since some of the errors noted are believed to be the Applicant's responsibility, the statutory fee of $160.00 is being remitted. The USPTO is directed and authorized to charge all required fees, except for the Issue Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

In view of the foregoing, issuance of the Certificate of Correction is respectfully requested.

Respectfully submitted,

/Hui C. Wauters/

Hui C. Wauters
Registration No. 57,426

SUGHRUE MION, PLLC
Telephone: 202.293.7060
Facsimile: 202.293.7860

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

Date: March 7, 2022

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO: 11,236,318

ISSUE DATE: February 1, 2022

INVENTOR(S): Hikaru MIZUMURA, Toshio ODA, Shun-ichiro KAWABATA

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

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In the Specification

Column 29, line 51, delete “Celfectin” and insert --Cellfectin-- therefor.

MAILING ADDRESS OF SENDER:
SUGHRUE MION, PLLC

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

PATENT NO. 11,236,318

No. of additional copies
1

Electronic Patent Application Fee Transmittal

Application Number:	16399386
Filing Date:	30-Apr-2019
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Filer:	Hui Chen Wauters/Sharon Jones
Attorney Docket Number:	Q246419

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

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:				
CERTIFICATE OF CORRECTION	1811	1	160	160

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**160**

Electronic Acknowledgement Receipt	
EFS ID:	45161653
Application Number:	16399386
International Application Number:	
Confirmation Number:	9293
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Customer Number:	23373
Filer:	Hui Chen Wauters/Sharon Jones
Filer Authorized By:	Hui Chen Wauters
Attorney Docket Number:	Q246419
Receipt Date:	07-MAR-2022
Filing Date:	30-APR-2019
Time Stamp:	14:59:19
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$160
RAM confirmation Number	E202237E59422103
Deposit Account	194880
Authorized User	Sharon Jones
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.16 (National application filing, search, and examination fees) 37 CFR 1.17 (Patent application and reexamination processing fees)	

37 CFR 1.19 (Document supply fees)

37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Certificate of Correction	Q246419RequestforCertificateofCorrectionasfiled.pdf	36292 afe8cfa5bcc0809ff385cfd6c6fd163b7382520c	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	38314 0fd02a52555f50e7ab15c8741de57f17c6c9993c	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	74606		

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.
16/399,386	02/01/2022	11236318	Q246419	9293

23373 7590 01/12/2022

SUGHRUE MION, PLLC
2000 PENNSYLVANIA AVENUE, N.W.
SUITE 9000
WASHINGTON, DC 20006

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 Data Management (ODM) at (571)-272-4200.

INVENTOR(s) (Please see PAIR WEB site http://pair.uspto.gov for additional inventors):

Hikaru MIZUMURA, Tokyo, JAPAN;
Toshio ODA, Tokyo, JAPAN;
Shun-ichiro KAWABATA, Fukuoka, JAPAN;

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

SEIKAGAKU CORPORATION, Tokyo, JAPAN;

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.

IR103 (Rev. 10/09)

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)

23373

SUGHRUE MION, PLLC
2000 PENNSYLVANIA AVENUE, NW, SUITE 9000
WASHINGTON, DC 20006-1811

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
16/399,386	04/30/2019	Hikaru MIZUMURA	Q246419	9293

TITLE OF INVENTION: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

APPLN TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	Undiscounted	$1200	$0	$0	$1200	01/26/2022

EXAMINER	ART UNIT	CLASS-SUBCLASS
STEADMAN, DAVID J	1656	435-023000

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/SB/122) attached.
☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 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. Sughrue Mion, PLLC
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: **SEIKAGAKU CORPORATION**

(B) RESIDENCE: (CITY and STATE OR COUNTRY): **Tokyo, Japan**

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☑ Corporation or other private group entity ☐ Government

4a. Fee(s) 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 Number 19-4880

5. Change of 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 as 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 /markboland/ Date December 17, 2021

Typed or Printed Name Mark Boland Registration No. 32,197

Electronic Patent Application Fee Transmittal

Application Number:	16399386
Filing Date:	30-Apr-2019
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Filer:	Ellen Resnick Smith/Sharon Slater
Attorney Docket Number:	Q246419

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

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:				
UTILITY APPL ISSUE FEE	1501	1	1200	1200

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1200**

Electronic Acknowledgement Receipt	
EFS ID:	44546665
Application Number:	16399386
International Application Number:	
Confirmation Number:	9293
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Customer Number:	23373
Filer:	Ellen Resnick Smith/Sharon Slater
Filer Authorized By:	Ellen Resnick Smith
Attorney Docket Number:	Q246419
Receipt Date:	17-DEC-2021
Filing Date:	30-APR-2019
Time Stamp:	12:25:45
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$1200
RAM confirmation Number	E2021BGC26070376
Deposit Account	194880
Authorized User	Sharon Slater
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.20 (Post Issuance fees)	

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)	Q246419IssueFeeTransmittal.pdf	139807 15a51528d6184e759a7ef3696c7ac0fac141568d	no	1
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	38420 c0aaeac8cfe5808134c019372758f79ac19b1bc4	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	178227		

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

NOTICE OF ALLOWANCE AND FEE(S) DUE

23373 7590 10/26/2021

SUGHRUE MION, PLLC
2000 PENNSYLVANIA AVENUE, N.W.
SUITE 9000
WASHINGTON, DC 20006

EXAMINER
STEADMAN, DAVID J

ART UNIT	PAPER NUMBER
1656	

DATE MAILED: 10/26/2021

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/399,386	04/30/2019	Hikaru MIZUMURA	Q246419	9293

TITLE OF INVENTION: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

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	01/26/2022

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 and an extra copy of the form should be submitted. 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)

23373 7590 10/26/2021
SUGHRUE MION, PLLC
2000 PENNSYLVANIA AVENUE, N.W.
SUITE 9000
WASHINGTON, DC 20006

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.
16/399,386	04/30/2019	Hikaru MIZUMURA	Q246419	9293

TITLE OF INVENTION: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

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	01/26/2022

EXAMINER	ART UNIT	CLASS-SUBCLASS
STEADMAN, DAVID J	1656	435-023000

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/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 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. _____

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
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/399,386	04/30/2019	Hikaru MIZUMURA	Q246419	9293

23373 7590 10/26/2021

SUGHRUE MION, PLLC
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SUITE 9000
WASHINGTON, DC 20006

EXAMINER	
STEADMAN, DAVID J	
ART UNIT	**PAPER NUMBER**
1656	

DATE MAILED: 10/26/2021

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. 16/399,386	Applicant(s) MIZUMURA et al.	
	Examiner DAVID J STEADMAN	Art Unit 1656	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 the applicant's response filed on August 24, 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 1-9,12-17 and 19-24 . 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. 14/650,767 .
   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. 20211018.
5. ☑ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/David Steadman/
Primary Examiner, Art Unit 1656

NOTICE OF ALLOWABILITY

Status of the Application

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Applicant's amendment to the claims, filed on August 24, 2021, is acknowledged. This listing of the claims replaces all prior versions and listings of the claims.

Applicant's remarks and declaration under 37 CFR 1.132 filed on August 24, 2021 in response to the non-final rejection mailed on March 9, 2021 have been fully considered.

The text of those sections of Title 35 U.S. Code not included in the instant action can be found in a prior Office action.

Sequence Compliance

In view of the applicant's instant remarks, the requirements for a sequence listing have been perfected.

Examiner's Amendment to the Claims

An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in an interview with Ms. Hui Wauters on October 15, 2021.

Please amend claims 14 and 18 as follows:

14. (Currently Amended) A method for measuring endotoxin in a test specimen, the method comprising

a step of mixing the Factor C protein according to claim 2, Factor B, Pro-clotting enzyme, and t-butoxycarbonyl-leucyl-glycyl-arginyl-pNA (Boc-Leu-Gly-Arg-pNA), and the test specimen, and

a step of measuring the release of pNA, thereby measuring endotoxin in the test specimen~~using an endotoxin assay agent comprising the Factor C protein according to claim 2~~.

18. (Canceled)

Claim Rejections - 35 USC § 103

The rejection of claims 1-9, 12, 13, and 19-23 under 35 U.S.C. 103 as being unpatentable over Ding et al. (US 5,858,706; cited on the IDS filed on April 30, 2019; hereafter "Ding") in view of

Current Protocols in Molecular Biology (1993) 16.23.1-16.23.13, John Wiley and Sons, Inc., 2002 (cited on the IDS filed on June 17, 2020; hereafter "Current Protocols") and

Thomas et al. (*J. Pharm. Tox. Methods* 51:187-200, 2005; cited on the IDS filed on February 3, 2021; hereafter "Thomas"), and

as evidenced by Mizumura et al. (WO 2012/118226 A1; cited on the IDS filed on April 30, 2019; hereafter "Mizumura")

is withdrawn in view of the applicant's amendment to claims 1, 2, and 12, the applicant's declaration under 37 CFR 1.132, and the applicant's convincing argument beginning at p. 9 of the instant remarks.

Election/Restrictions

Claim 2 is directed to an allowable product. Pursuant to the procedures set forth in MPEP § 821.04(b), claims 14-17, directed to the process of making or using the allowable product, previously withdrawn from consideration as a result of a restriction requirement, are hereby rejoined and fully examined for patentability under 37 CFR 1.104. Because a claimed invention previously withdrawn from consideration under 37 CFR 1.142 has been rejoined, the restriction requirement between groups I and II as set forth in the Office action mailed on April 17, 2020 is hereby withdrawn. In view of the withdrawal of the restriction requirement as to the rejoined invention, applicant(s) are advised that if any claim presented in a continuation or divisional application is anticipated by, or includes all the limitations of, a claim that is allowable in the present application, such claim may be subject to provisional statutory and/or nonstatutory double patenting rejections over the claims of the instant application. Once the restriction requirement is withdrawn, the provisions of 35 U.S.C. 121 are no longer applicable. See *In re Ziegler*, 443 F.2d 1211, 1215, 170 USPQ 129, 131-32 (CCPA 1971). See also MPEP § 804.01.

Examiner's Statement of Reasons for Allowance

The following is an examiner's statement of reasons for allowance:

The prior art reference of Koshiba et al. (*J. Biol. Chem.* 282:3962-3967, 2007, cited on the IDS filed on June 17, 2020) discloses truncated fragments of a Factor C recombinantly expressed using a HEK293 cell as an expression host. The truncated fragments, while disclosed as having LPS binding activity, do not have Factor C protease activity. As described in the prior-filed application 14/650,767 (see applicant's remarks at pp. 13-14 of the response filed on March 16, 2017 and Final rejection mailed on April 13, 2017 at the paragraph bridging pp. 7-8), the claims of this application recite "having activity of Factor C". According to the instant specification, "the activity of Factor C refers to such an activity that the Factor C is activated in the presence of endotoxin to convert Factor B into activated Factor B" (specification at p. 36, paragraph [0028]), which is considered to encompass both LPS binding activity and protease activity of Factor C. As such, Koshiba does not disclose a Factor C protein having activity of Factor C as defined according to the specification.

The prior art of record also discloses a Factor C protein recombinantly produced using a CHO cell as an expression host (see, *e.g.,* Ding, *supra*) and discloses CHO DG44 cells and HEK293 cells as expression hosts for recombinant protein production (see, *e.g.,* Current Protocols and Thomas, *supra*). However, the applicant has met the burden of establishing an unexpected result and the applicant's unexpected result is deemed sufficient to rebut a *prima facie* case of obviousness and the Factor C protein and methods of claims 1-9, 12-17, and 19-24 are allowable over the prior art of record.

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."

Information Regarding Quick Path IDS Program

For situations when the applicant needs to file an IDS *after the issue fee has been paid*, The United States Patent and Trademark Office (USPTO) has implemented a Quick Path Information Disclosure Statement (QPIDS) program intended to reduce pendency and applicant costs. This pilot program will permit an examiner to consider an IDS after payment of the issue fee without the need to reopen prosecution, effectively obviating the need to pursue a Request for Continued Examination (RCE). Where the examiner determines that no item of information in the IDS necessitates reopening prosecution, the Office will issue a corrected notice of allowability. In addition to reducing pendency, this program will promote efficiency in the examination process. There will be no fee required to use this program, beyond existing fees, *e.g.*, fees for IDS submission. More information on this and other new initiatives at the Patent Office can be found at http://www.uspto.gov/patents/init_events/patapp-initiatives-timeline.jsp or by calling the examiner or his supervisor.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to DAVID J STEADMAN whose telephone number is (571)272-0942. The examiner can normally be reached on Monday to Friday, 7:30 AM to 4:00 PM.

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, MANJUNATH N. RAO can be reached on 571-272-0939. 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.

/David Steadman/
Primary Examiner, Art Unit 1656

Examiner-Initiated Interview Summary	**Application No.** 16/399,386	**Applicant(s)** MIZUMURA et al.		
	Examiner DAVID J STEADMAN	**Art Unit** 1656	**AIA (First Inventor to File) Status** Yes	**Page** 1 of 1

All Participants (applicant, applicants representative, PTO personnel)	**Title**	**Type**
DAVID J STEADMAN	Primary Examiner	Telephonic
HUI C. WAUTERS	Attorney of Record	

Date of Interview: 15 October 2021

Issues Discussed:

Proposed Amendment(s)

A proposed examiner's amendment to claims 14 and 18 was presented to the applicant on October 14, 2021 and was authorized for entry by the applicant's representative on October 15, 2021.

/David Steadman/ Primary Examiner, Art Unit 1656	

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: It is not necessary for applicant to provide a separate record of the substance of 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.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/399,386	MIZUMURA et al.
	Examiner	Art Unit
	DAVID J STEADMAN	1656

CPC - Searched*		
Symbol	**Date**	**Examiner**
C12N9/6408, See attached EAST search history printout	10/02/2020	/DJS/
C12N9/6408, C12Y304/21084, see attached EAST search history printout	03/04/2021	/DJS/
C12N9/6408, C12Y304/21084, see attached EAST search history printout	10/18/2021	/DJS/

CPC Combination Sets - Searched*		
Symbol	**Date**	**Examiner**
NONE		

US Classification - Searched*			
Class	**Subclass**	**Date**	**Examiner**
NONE			

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/399,386	MIZUMURA et al.
	Examiner	Art Unit
	DAVID J STEADMAN	1656

Search Notes		
Search Notes	**Date**	**Examiner**
PALM inventor name search, all inventors searched	06/24/2020	/DJS/
EAST inventor name and assignee search, all inventors searched	06/24/2020	/DJS/
Reviewed prosecution history for applications 14/650,767, 15/983,725, and 16/399575	06/24/2020	/DJS/
STIC search of SEQ ID NO: 2 and 4 against amino acid and nucleic acid prior and interference databases, see SCORE for search results	06/24/2020	/DJS/
EAST, STN database keyword search, see attached search history printouts for search strategy and databases searched	06/24/2020	/DJS/
PALM inventor name search and EAST inventor name and assignee search, all inventors searched	10/02/2020	/DJS/
PALM inventor name search and EAST inventor name and assignee search, all inventors searched	03/04/2021	/DJS/
Updated EAST, STN database keyword search, see attached search history printouts for search strategy and databases searched	03/04/2021	/DJS/
Updated PALM inventor name search and EAST inventor name and assignee search, all inventors searched	10/18/2021	/DJS/
Updated EAST, STN database keyword search, see attached search history printouts for search strategy and databases searched	10/18/2021	/DJS/
STIC search of SEQ ID NO: 2 against amino acid and nucleic acid prior and interference databases, see SCORE for search results	10/18/2021	/DJS/

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**
	EAST database keyword search, see attached search history printouts for search strategy and databases searched	10/18/2021	/DJS/
	STIC search of SEQ ID NO: 2 against amino acid and nucleic acid interference databases, see SCORE for search results	10/18/2021	/DJS/

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/399,386	MIZUMURA et al.
	Examiner	**Art Unit**
	DAVID J STEADMAN	1656

CPC						
Symbol					**Type**	**Version**
C12N	/	9	/	6408	F	2013-01-01
G01N	/	33	/	579	I	2013-01-01
C12Y	/	304	/	21069	I	2013-01-01
G01N	/	33	/	56911	I	2013-01-01
C12Y	/	304	/	21084	I	2013-01-01
G01N	/	2333	/	96411	A	2013-01-01

CPC Combination Sets							
Symbol					**Type**	**Set**	**Ranking**	**Version**
	/		/					

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	21	
/David Steadman/ Primary Examiner, Art Unit 1656	18 October 2021	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

U.S. Patent and Trademark Office — Part of Paper No.: 20211018

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/399,386	MIZUMURA et al.
	Examiner	**Art Unit**
	DAVID J STEADMAN	1656

INTERNATIONAL CLASSIFICATION				
CLAIMED				
C12N	/	9	/	64
G01N	/	33	/	569
C12Q	/	1	/	37
C12N	/	15	/	85

NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**

CROSS REFERENCES(S)						
CLASS	**SUBCLASS (ONE SUBCLASS PER BLOCK)**					

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 21	
/David Steadman/ Primary Examiner, Art Unit 1656 (Primary Examiner)	18 October 2021 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure NONE

U.S. Patent and Trademark Office — Part of Paper No.: 20211018

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/399,386	MIZUMURA et al.
	Examiner	**Art Unit**
	DAVID J STEADMAN	1656

☑ 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

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 21	
/David Steadman/ Primary Examiner, Art Unit 1656 (Primary Examiner)	18 October 2021 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure NONE

U.S. Patent and Trademark Office — Part of Paper No.: 20211018

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  NEWS  3   AUG  5  Chemical and Technology Databases RAPRA, CEABA,
                    and TEMA Add Numeric Property Search
  NEWS  4   OCT  7  EP Appeal Data from European Patent Register Added to
                    INPADOCDB and INPAFAMDB
  NEWS  5   NOV 15  Enhanced INPADOCDB and INPAFAMDB Files
  NEWS  6   NOV 23  Claims Access in CA/CAplus: WIPO, United States, and China
                    for 1999 - Present.
  NEWS  7   DEC  1  Engineering Flagship Database Inspec Reaches
                    Milestone 20 Million Total Records
  NEWS  8   DEC  1  Full-Text Patent Documents from German Democratic
                    Republic Added to DEFULL
  NEWS  9   FEB  7  Patent Status Indicators in CA/CAplus
  NEWS 10   FEB 16  ReaxysFileSub and companion bibliographic
                    database ReaxysFileBib new on STNext(R)
  NEWS 11   APR 12  New Version of the EPFULL (European Patent Full Text
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  NEWS 12   APR 12  Analytical Abstracts Enhancements Increase Value to Users
  NEWS 13   APR 13  INSPEC Reload Features Numeric Property Searching, Cited
                    References and DOIs
  NEWS 14   MAY 10  May 2021 Update to Emtree(R) Now Available on STN
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                    Property Searching and SLART Improvements
  NEWS 17   SEP 11  September 2021 Update to Emtree(R) Now Available on STN
  NEWS 18   OCT  4  MARPAT(R) Enhanced with Patent Status Data

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* * * * * * * * * * * * * * * STN  Columbus  * * * * * * * * * * * * * * *

FILE 'HOME' ENTERED AT 10:01:33 ON 18 OCT 2021

=> log hold

COST IN U.S. DOLLARS                            SINCE FILE            TOTAL
                                                     ENTRY          SESSION
FULL ESTIMATED COST                                   1.26             1.26

 SESSION WILL BE HELD FOR 120 MINUTES
STN INTERNATIONAL SESSION SUSPENDED AT 10:05:08 ON 18 OCT 2021

 * * * * * * RECONNECTED TO STN INTERNATIONAL * * * * * *
SESSION RESUMED IN FILE 'HOME' AT 10:43:30 ON 18 OCT 2021
FILE 'HOME' ENTERED AT 10:43:30 ON 18 OCT 2021

COST IN U.S. DOLLARS                            SINCE FILE            TOTAL
                                                     ENTRY          SESSION
FULL ESTIMATED COST                                   1.26             1.26

=> file registry

COST IN U.S. DOLLARS                            SINCE FILE            TOTAL
                                                     ENTRY          SESSION
FULL ESTIMATED COST                                   1.47             1.47

FILE 'REGISTRY' ENTERED AT 10:43:51 ON 18 OCT 2021
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STRUCTURE FILE UPDATES:   17 OCT 2021  HIGHEST RN 2713985-90-9
DICTIONARY FILE UPDATES:  17 OCT 2021  HIGHEST RN 2713985-90-9

CAS Information Use Policies apply and are available at:
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http://www.cas.org/legal/infopolicy

TSCA Inventory information is current through August 2021.

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REGISTRY includes numerically searchable data for experimental and
predicted properties as well as tags indicating availability of
experimental property data in the original document. For information
on property searching in REGISTRY, refer to:

http://www.cas.org/training/stn/database-specific

=> s 115743-27-6/rn

L1   1 115743-27-6/RN

=> file .djs scisearch

FILE 'WPIDS' ACCESS NOT AUTHORIZED
COST IN U.S. DOLLARS   SINCE FILE   TOTAL
                                               ENTRY   SESSION
FULL ESTIMATED COST   1.34   2.81

FILE 'MEDLINE' ENTERED AT 10:44:14 ON 18 OCT 2021

FILE 'AGRICOLA' ENTERED AT 10:44:14 ON 18 OCT 2021
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=> s l1

'RN' IS NOT A VALID FIELD CODE
'RN' IS NOT A VALID FIELD CODE
L2   4 L1

=> s (?factor (A) "C")

L3   86767 (?FACTOR (A) "C")

=> s l3 AND (dg44 OR dg-44 OR dg/44)

'44' IS NOT A VALID FIELD CODE
'44' IS NOT A VALID FIELD CODE
'44' IS NOT A VALID FIELD CODE
'44' IS NOT A VALID FIELD CODE
'44' IS NOT A VALID FIELD CODE
'44' IS NOT A VALID FIELD CODE
L4   7 L3 AND (DG44 OR DG-44 OR DG/44)

=> s l3 AND (293 OR ?293)

'?293' NOT LONG ENOUGH FOR LEFT TRUNCATION
You have entered a truncated stem whose length is less than
the minimum allowed for left truncation in the requested
search field. You may increase the length of the stem to
the minimum allowed and try again. The usual minimum stem
length for left truncation is four characters

=> s l3 AND (293 OR HEK293)

L5   623 L3 AND (293 OR HEK293)

=> d his

     (FILE 'HOME' ENTERED AT 10:01:33 ON 18 OCT 2021)

     FILE 'REGISTRY' ENTERED AT 10:43:51 ON 18 OCT 2021
L1   1 S 115743-27-6/RN

     FILE 'MEDLINE, AGRICOLA, CAPLUS, BIOSIS, EMBASE, SCISEARCH' ENTERED AT
     10:44:14 ON 18 OCT 2021
L2   4 S L1
L3   86767 S (?FACTOR (A) "C")

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L4            7 S L3 AND (DG44 OR DG-44 OR DG/44)
L5          623 S L3 AND (293 OR HEK293)

=> s l4 OR l5

L6          627 L4 OR L5

=> s (tridentatus OR polyphemus OR rotundicauda)

L7        13409 (TRIDENTATUS OR POLYPHEMUS OR ROTUNDICAUDA)

=> s l6 and l7

L8            2 L6 AND L7

=> s (tachypleus OR limulus OR Carcinoscorpius)

L9        29182 (TACHYPLEUS OR LIMULUS OR CARCINOSCORPIUS)

=> s l6 and l9

L10           6 L6 AND L9

=> s l8 or l10

L11           6 L8 OR L10

=> dup rem l11

PROCESSING COMPLETED FOR L11
L12           5 DUP REM L11 (1 DUPLICATE REMOVED)

=> d ibib abs 1-
YOU HAVE REQUESTED DATA FROM 5 ANSWERS - CONTINUE? Y/(N):y


L12  ANSWER 1 OF 5     MEDLINE ®  on STN
ACCESSION NUMBER:   2019937754     MEDLINE Full-text
DOCUMENT NUMBER:    PubMed ID: 29866883
TITLE:              Intermolecular autocatalytic activation of serine protease
                    zymogen factor C through an active transition state
                    responding to lipopolysaccharide.
AUTHOR:             Shibata Toshio
CORPORATE SOURCE:   Department of Biology, Faculty of Science, Kyushu
                    University, Fukuoka 819-0395, Japan, Graduate School of
                    Systems Life Sciences, Kyushu University, Fukuoka 819-0395,
                    Japan.
AUTHOR:             Kobayashi Yuki
CORPORATE SOURCE:   Department of Biology, Faculty of Science, Kyushu
                    University, Fukuoka 819-0395, Japan.
AUTHOR:             Ikeda Yuto
CORPORATE SOURCE:   Graduate School of Systems Life Sciences, Kyushu
                    University, Fukuoka 819-0395, Japan.
AUTHOR:             Kawabata Shun-Ichiro
CORPORATE SOURCE:   Department of Biology, Faculty of Science, Kyushu
                    University, Fukuoka 819-0395, Japan, Graduate School of
                    Systems Life Sciences, Kyushu University, Fukuoka 819-0395,
                    Japan. skawascb@kyudai.jp
SOURCE:             The Journal of biological chemistry, (20180720) Vol. 293,
                    No. 29, pp. 11589-11599.  Electronic Publication Date: 4
                    Jun 2018
                    Journal code: 2985121R. E-ISSN: 1083-351X. L-ISSN:
                    0021-9258.
                    Report No.: PMC-PMC6065167.
DIGITAL OBJECT ID:  http://dx.doi.org/10.1074/jbc.RA118.002311
PUB. COUNTRY:       United States
DOCUMENT TYPE:      Journal; Article; (JOURNAL ARTICLE)
                    (RESEARCH SUPPORT, NON-U.S. GOV'T)
LANGUAGE:           English
FILE SEGMENT:       MEDLINE; Priority Journals
FILE SEGMENT:       Print; Electronic
ENTRY MONTH:        201902
ENTRY DATE:         Entered STN: 5 Jun 2018
                    Last Updated on STN: 5 Feb 2019
                    Indexing Added: 5 Feb 2019
                    Entered Medline: 4 Feb 2019
OS.CITING REF COUNT: 5    There are 5 MEDLINE records that cite this record
MEDLINE REFERENCE COUNT:   38    There are 38 cited references available in
                                 MEDLINE for this document.
AB   Horseshoe crab hemolymph coagulation is believed to be triggered by the
     autocatalytic activation of serine protease zymogen factor C to the
     active form, α-factor C, belonging to the trypsin family,
     through an active transition state of factor C responding to bacterial
     lipopolysaccharide (LPS), designated factor C*.  However, the
     existence of factor C* is only speculative, and its proteolytic
     activity has not been validated.  In addition, it remains unclear whether
     the proteolytic cleavage of the Phe(737)-Ile(738) bond (Phe(737) site) of
     factor C required for the conversion to α-factor C occurs
     intramolecularly or intermolecularly between the factor C molecules.
     Here we show that the Phe(737) site of a catalytic Ser-deficient mutant of
```

factor C is LPS-dependently hydrolyzed by a Phe(737) site-uncleavable mutant, clearly indicating the existence of the active transition state of factor C without cleavage of the Phe(737) site. Moreover, we found the following facts using several mutants of factor C: the autocatalytic cleavage of factor C occurs intermolecularly between factor C* molecules on the LPS surface; factor C* does not exhibit intrinsic chymotryptic activity against the Phe(737) site, but it may recognize a three-dimensional structure around the cleavage site; and LPS is required not only to complete the substrate-binding site and oxyanion hole of factor C* by interacting with the N-terminal region but also to allow the Phe(737) site to be cleaved by inducing a conformational change around the Phe(737) site or by acting as a scaffold to induce specific protein-protein interactions between factor C* molecules.
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L12 ANSWER 2 OF 5 MEDLINE ® on STN DUPLICATE 1
ACCESSION NUMBER: 2018386384 MEDLINE Full-text
DOCUMENT NUMBER: PubMed ID: 28317862
TITLE: Biological Activity of Masked Endotoxin.
AUTHOR: Schwarz Harald; Gornicec Jan; Neuper Theresa; Parigiani Maria Alejandra; Wallner Michael; Duschl Albert; Horejs-Hoeck Jutta
CORPORATE SOURCE: Department of Molecular Biology, University of Salzburg, Salzburg, Austria.
SOURCE: Scientific reports, (20170320) Vol. 7, pp. 44750. Electronic Publication Date: 20 Mar 2017
Journal code: 101563288. E-ISSN: 2045-2322. L-ISSN: 2045-2322.
Report No.: PMC-PMC5357793.
DIGITAL OBJECT ID: http://dx.doi.org/10.1038/srep44750
PUB. COUNTRY: England: United Kingdom
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE) (RESEARCH SUPPORT, NON-U.S. GOV'T)
LANGUAGE: English
FILE SEGMENT: MEDLINE; Priority Journals
FILE SEGMENT: Electronic
ENTRY MONTH: 201811
ENTRY DATE: Entered STN: 20 Mar 2017
Last Updated on STN: 11 Nov 2018
Indexing Added: 11 Nov 2018
Entered Medline: 9 Nov 2018
OS.CITING REF COUNT: 15 There are 15 MEDLINE records that cite this record
MEDLINE REFERENCE COUNT: 18 There are 18 cited references available in MEDLINE for this document.

AB Low endotoxin recovery (LER) is a recently discovered phenomenon describing the inability of limulus amebocyte lysate (LAL)-based assays to detect lipopolysaccharide (LPS) because of a "masking effect" caused by chelators or detergents commonly used in buffer formulations for medical products and recombinant proteins. This study investigates the masking capacities of different buffer formulations and whether masked endotoxin is biologically active. We show that both naturally occurring endotoxin as well as control standard endotoxin can be affected by LER. Furthermore, whereas masked endotoxin cannot be detected in Factor C based assays, it is still detectable in a cell-based TLR4-NF-κB-luciferase reporter gene assay. Moreover, in primary human monocytes, masked LPS induces the expression of pro-inflammatory cytokines and surface activation markers even at very low concentrations. We therefore conclude that masked LPS is a potent trigger of immune responses, which emphasizes the potential danger of masked LPS, as it may pose a health threat in pharmaceutical products or compromise experimental results.

L12 Patent | Japanese 3 of 5

Novel recombinant factor C and method for producing same, and method for measuring endotoxin

Full Text

Inventor Name:	Mizumura, Hikaru; Oda, Toshio; Kawabata, Shun-Ichiro
Patent Assignee:	Seikagaku Corporation, Japan
Accession Number:	2014:999086 CAPLUS
Document Number (CA Abstract Number):	161:65883
Source:	PCT Int. Appl., 79pp. CODEN: PIXXD2
Family Accession Number Count:	1

Provided is a method for producing horseshoe crab recombinant factor C. The horseshoe

crab recombinant factor C can be produced by expressing the horseshoe crab factor C using a mammalian cell such as CHO DG44 and HEK293 as a host cell.

Patent Information Table:

PATENT NO.
WO 2014092079
CN 104919046
EP 2930241
EP 2930241
SG 10201704774
CN 108646019
CN 108646019
CN 108642030
EP 3441466
EP 3441466
JP 6511685
ES 2738593
EP 3663400
ES 2784510
US 20150307864
US 10144923
US 20180258414
US 10982202
JP 2019000122
JP 6626174
US 20190256835
US 20190270977

Priority Application Information:

JP 2012-269840
CN 2013-80064649
EP 2013-862466
EP 2018-190686
JP 2014-552048
WO 2013-JP83082
US 2015-14650767
US 2018-15983725

Patent Status Patent Information:

PATENT NO.	KIND	ST
WO 2014092079	A1	De
CN 104919046	A	De
EP 2930241	A1	Ind
EP 2930241	B1	Ind
CN 108646019	A	All
CN 108646019	B	All
CN 108642030	A	All
EP 3441466	A1	Ind
EP 3441466	B1	Ind
JP 6511685	B2	All
ES 2738593	T3	All
EP 3663400	A1	All
ES 2784510	T3	All
US 20150307864	A1	All
US 10144923	B2	All
US 20180258414	A1	All
US 10982202	B2	All
JP 2019000122	A	All
JP 6626174	B2	All
US 20190256835	A1	De
US 20190270977	A1	All

Cited References Count:

8 THERE ARE 8 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

Citing Reference Count:

1 THERE ARE 1 CAPLUS RECORDS THAT CITE THIS RECORD (1 CITINGS)

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT

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L12 ANSWER 4 OF 5 BIOSIS COPYRIGHT (c) 2021 Clarivate Analytics on STN
ACCESSION NUMBER: 2007:224924 BIOSIS Full-text
DOCUMENT NUMBER: PREV200700226988
TITLE: A structural perspective on the interaction between lipopolysaccharide and Factor C, a receptor involved in recognition of Gram-negative bacteria.
AUTHOR(S): Koshiba, Takumi; Hashii, Tornoyuki; Kawabata, Shun-ichiro [Reprint Author]
CORPORATE SOURCE: Kyushu Univ, Fac Sci, Dept Biol, Higashi Ku, 6-10-1 Hakozaki, Fukuoka 8128581, Japan
skawasrc@mbox.nc.kyushu-u.ac.jp
SOURCE: Journal of Biological Chemistry, (FEB 9 2007) Vol. 282, No. 6, pp. 3962-3967. http://www.jbc.org/content/by/year.
E-ISSN: 1083-351X.
DIGITAL OBJECT ID: 10.1074/jbc.M609198200
DOCUMENT TYPE: Article
LANGUAGE: English
ENTRY DATE: Entered STN: 4 Apr 2007
Last Updated on STN: 14 Aug 2019

AB The recognition of broadly conserved microorganism components known as pathogen-associated molecular patterns is an essential step in initiating the innate immune response. In the horseshoe crab, stimulation of hemocytes with lipopolysaccharide (LPS) causes the activation of its innate immune response, and Factor C, a serine protease zymogen, plays an important role in this event. Here, we report that Factor C associates with LPS on the hemocyte surface and directly recognizes Gram-negative bacteria. Structure-function analyses reveal that the LPS binding site is present in the N-terminal cysteine-rich (Cys-rich) region of the molecule and that it contains a tripeptide sequence consisting of an aromatic residue flanked by two basic residues that is conserved in other mammalian LPS-recognizing proteins. Moreover, we have demonstrated that the Cys-rich region specifically binds to LPS on Gram-negative bacteria and that mutations in the tripeptide motif abrogate its association with both LPS and Gram-negative bacteria, underscoring the importance of the tripeptide in LPS interaction. Although the innate immune response to LPS in the horseshoe crab is distinct from that of mammals, it appears to rely on structural features that are conserved among LPS-recognizing proteins from diverse species.

L12 Patent | Japanese **5** of 5

Mouse extracellular matrix proteins, cDNA cloning, and use as cell adhesion agent

Full Text

Inventor Name:	Sekiguchi, Kiyotoshi; Manabe, Riichiro; Hayashizaki, Yoshihide; Kamiya, Mamoru; Furuya, Hiroshi; Tsutsui, Aogu
Patent Assignee:	Japan Science and Technology Corporation, Japan; Institute of Physical and Chemical Research; DNA Form Co., Ltd.
Accession Number:	2004:32343 CAPLUS
Document Number (CA Abstract Number):	140:106529
Source:	Jpn. Kokai Tokkyo Koho, 86 pp. CODEN: JKXXAF
Family Accession Number Count:	1

Novel extracellular matrix proteins of mouse origin, encoding cDNAs, recombinant expression, antibodies against them, and use as cell adhesion agent, are disclosed. Genes coding for those extracellular matrix proteins were designated 33145, 40240, 51140, and 31418. Besides secretion signal sequence, 33145 protein had SCP (sperm-coating glycoprotein) domain, LCCL (Limulus factor C, Coch-5b2 and Lgl1) domain, and a potential N-glycosylation site. Homol. with Cocoa Crisp (cysteine-rich secreted protein), late gestation lung protein, protease inhibitor 15 preproprotein, 25 kDa trypsin inhibitor and glioma pathogenesis-related protein (RTVP-1 protein) was found. 40420 Protein had 2 potential N-glycosylation sites, and showed homol. with human FLJ23191 gene and mouse BB613765, AV243621, AK017759, and AK020942 genes. 51140 Protein has a thrombospondin type 1 repeats (TSP-1) domain, a potential N-glycosylation site, and a cysteine rich region homologous to that of PACE4 protein (Furin-like protease serine protease), and showed homol. with thrombospondin. 31418 Protein has four tandem repeat sequences, and a sequence homologous to bovine prion protein heparin-binding domain, and showed homol. with monkey Taste Bud-specific protein. The proteins were recombinantly expressed as fusion protein with EGFP-His-myc tag in human 293T cells. Localization in extracellular matrix was detected. Cell adhesion and growth activities were found. Prodn. of anti-40240 antibodies is described.

Patent Information Table:

PATENT NO.
JP 2004008021

Priority Application Information:

JP 2002-162211

Patent Status Patent Information:

PATENT NO.	KIND
JP 2004008021	A

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=> d his full

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                D CLUSTER .DJS
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     FILE 'REGISTRY' ENTERED AT 10:43:51 ON 18 OCT 2021
L1              1 SEA 115743-27-6/RN

     FILE 'MEDLINE, AGRICOLA, CAPLUS, BIOSIS, EMBASE, SCISEARCH' ENTERED AT
     10:44:14 ON 18 OCT 2021
L2              4 SEA L1
L3          86767 SEA (?FACTOR (A) "C")
L4              7 SEA L3 AND (DG44 OR DG-44 OR DG/44)
L5            623 SEA L3 AND (293 OR HEK293)
L6            627 SEA L4 OR L5
L7          13409 SEA (TRIDENTATUS OR POLYPHEMUS OR ROTUNDICAUDA)
L8              2 SEA L6 AND L7
L9          29182 SEA (TACHYPLEUS OR LIMULUS OR CARCINOSCORPIUS)
L10             6 SEA L6 AND L9
L11             6 SEA L8 OR L10
L12             5 DUP REM L11 (1 DUPLICATE REMOVED)
                D IBIB ABS 1-
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EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S5	7,687	(horseshoe adj crab) OR limulus	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:46
S6	13,415	factor adj "c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S7	13,415	"factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S8	335	S5 same S6	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S9	212	S8 and recombinant	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S10	89	S8 same recombinant	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S11	26	S10 and mammalian	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:48
S12	9	S10 and ((chinese adj hamster) OR CHO)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:02

EAST Search History (Prior Art)

S13	2	S10 and (hek OR (embryonic adj kidney))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:02
S14	6	S10 and (hek$ OR (embryonic adj kidney))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:03
S15	4	S14 not S13	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:03
S16	2	"6,645,724".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:04
S17	12	"2012118226"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:35
S18	4	"5716834".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 15:58
S19	2,454	seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:23
S20	18,057	(mizumura-h$ OR oda-t$ OR kawabata-s$). in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:23

EAST Search History (Prior Art)

S21	20,466	S19 or S20	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:25
S22	91	S21 and (horseshoe adj crab)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:25
S23	66	S21 and tridentatus	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:25
S24	41	S21 and rotundicauda	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:26
S25	108	S22 or S23 or S24	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:26
S26	40	S25 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:26
S27	23	("5,858,706" OR "5,712,144" OR "5,795,962" OR "6,077,946" OR "20090208995" OR "20030054432" OR "20040235080" OR "20140024062").pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 11:34
S28	4	"5,716,834".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/05 18:17

EAST Search History (Prior Art)

S29	4	"5,858,706".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 11:27
S30	2	"6,645,724".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 15:42
S31	2	"20140249082".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 16:45
S32	2,474	seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:07
S33	18,751	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:07
S34	21,180	S32 or S33	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:07
S35	19	S33 AND (tridentatus OR polyphemus OR rotundicauda OR gigas)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:08
S36	13	S35 and "factor C"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:08

EAST Search History (Prior Art)

S37	2	S36 and (sialic OR sialyl$)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:08
S38	14,664	"factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:53
S39	218	S38 and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:53
S40	7	S38 same (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:54
S41	218	S39 and (cho OR "chinese hamster ovary")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:54
S42	805	"factor c".clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:54
S43	2	S42 and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:55
S44	5,676	(cho OR "chinese hamster ovary") same (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:55

EAST Search History (Prior Art)

S45	211	S44 and S38	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:55
S47	7	S45 and horseshoe	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:56
S48	20	horseshoe and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:45
S49	218	"factor c" and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:47
S50	211	S49 not S48	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:47
S51	0	S50 and horseshoe	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:48
S52	2	"factor c".clm. and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:48
S53	6	"factor c".ti. and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:49

EAST Search History (Prior Art)

S54	7	"factor c".ab. and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:49
S55	1	S54 not S53	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:49
S56	2	"9133495".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:36
S57	19,132	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45
S58	6	S57 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45
S59	2,485	seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45
S60	3	S59 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45
S61	7	S58 or S60	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45

EAST Search History (Prior Art)

S62	19,414	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S63	6	S62 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S64	2,493	seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S65	3	S64 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S66	7	S63 or S65	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S67	19,635	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/08/14 09:19
S68	6	S67 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/08/14 09:19
S80	3	("20080131929"|"20100086967"|"20130295613").PN.	US-PGPUB; USPAT	OR	OFF	2018/09/07 10:58
S81	19,653	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/09/07 11:43

EAST Search History (Prior Art)

S82	6	S81 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/09/07 11:43
S94	6	"10144923".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/10/01 09:23
S96	6	(US-20080131929-$ | US-20100086967-$ | US-20130295613-$).DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/10/01 10:53
S97	20,399	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/10/02 15:15
S98	40	S97 AND (tridentatus OR polyphemus OR rotundicauda OR gigas)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/10/02 15:15
S99	23,093	(mizumura-h$ OR oda-t$ OR kawabata-s$).in. OR seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/02/07 10:15
S100	95	S99 AND (tridentatus OR polyphemus OR rotundicauda OR gigas)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/02/07 10:16
S101	54	S100 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/02/07 10:16

EAST Search History (Prior Art)

S102	24	S101 and ("chinese hamster" OR CHO OR human OR HEK)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/02/07 10:16
S103	627	tridentatus OR rotundicauda	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S104	389	S103 and endotoxin	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S105	263	S104 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S106	224	S103 same "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S107	216	S105 and S106	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S108	140	S107 and "37"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:42
S109	1,050,017	"2 hours"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:42

EAST Search History (Prior Art)

S110	114	S108 and S109	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:42
S113	349,173	cascade	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:44
S114	169	S107 and S113	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:45
S115	468	limulus adj test	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 15:30
S116	1	S115 same cation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 15:31
S117	3	"6,645,724".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 12:19
S118	13	("20030054432"|"20040235080"|"20080131929"|"20090208995"|"20100086967"|"20130295613"|"20140024062"|"20140249082"|"5712144"|"5716834"|"5795962"|"5858706"|"6077946").PN.	US-PGPUB; USPAT	OR	OFF	2020/04/22 14:32
S119	23,218	(mizumura-h$ OR oda-t$ OR kawabata-s$).in. OR seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 14:37
S120	85	S119 AND (tridentatus OR rotundicauda)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 14:37

EAST Search History (Prior Art)

S121	54	S120 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 14:38
S122	33	S121 and endotoxin.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 14:39
S123	23,351	(mizumura-h$ OR oda-t$ OR kawabata-s$).in. OR seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:10
S124	95	S123 AND (tridentatus OR rotundicauda OR polyphemus)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:10
S125	55	S124 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:11
S126	10	S125 and (cho OR "chinese hamster")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:11
S127	8	S125 and (hek OR (human adj embryo$))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:12
S128	10	S126 or S127	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:12

EAST Search History (Prior Art)

S129	23,458	(mizumura-h$ OR oda-t$ OR kawabata-s$). in. OR seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
S130	98	S129 AND (tridentatus OR rotundicauda OR polyphemus)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
S131	57	S130 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
S132	11	S131 and (cho OR "chinese hamster")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
S133	9	S131 and (hek OR (human adj embryo$))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
S134	11	S132 or S133	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
S135	753	c12n9/6408.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:52
S136	44	S135 and (tridentatus OR polyphemus OR rotundicauda)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:53

EAST Search History (Prior Art)

S137	829,430	(cho OR "chinese hamster")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:53
S138	104,016	(hek OR (human adj embryo$))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:53
S139	14	S136 and (S137 OR S138)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:53
S140	4	"6645724".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 04:49
S141	787	c12n9/6408.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 04:49
S142	46	S141 and (tridentatus OR polyphemus OR rotundicauda)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 04:49
S143	854,970	(cho OR "chinese hamster")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 04:49
S144	107,501	(hek OR (human adj embryo$))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 04:49

EAST Search History (Prior Art)

S145	787	c12n9/6408.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 06:20
S146	46	S145 and (tridentatus OR polyphemus OR rotundicauda)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 06:20
S147	854,970	(cho OR "chinese hamster")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 06:20
S148	107,501	(hek OR (human adj embryo$))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 06:20
S149	15	S146 and (S147 OR S148)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 06:20
S150	64	C12Y304/21084.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 06:26
S151	24	S150 and (S147 OR S148)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 06:26
S152	886,720	(cho OR "chinese hamster")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 07:59

EAST Search History (Prior Art)

S153	112,690	(hek OR (human adj embryo$))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 07:59
S154	69	C12Y304/21084.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 07:59
S155	19	S154 and (S152 OR S153)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 07:59
S156	812	c12n9/6408.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 08:00
S157	50	S156 and (tridentatus OR polyphemus OR rotundicauda)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 08:00
S158	16	S157 and (S152 OR S153)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 08:00
S159	40	S156 and (tridentatus OR polyphemus)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 08:30
S160	13	(S152 OR S153) same (tridentatus OR polyphemus)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 08:31

EAST Search History (Prior Art)

S161	204	(S152 OR S153) same "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 08:40
S162	13	S160 and (tridentatus OR polyphemus)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 08:41
S163	4,875	(hek OR hek293 OR (human adj embryo$)).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 08:48
S164	8	S157 and hek293	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 08:48
S165	14	S154 and hek293	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 08:49
S166	6	S165 not S164	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 08:49
S167	8	hek293 same (tridentatus OR polyphemus)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 08:50
S168	24,311	(mizumura-h$ OR oda-t$ OR kawabata-s$).in. OR seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 08:51

EAST Search History (Prior Art)

S169	103	S168 AND (tridentatus OR polyphemus)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 08:51
S170	63	S169 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 08:51
S171	15	S170 and (S152 or S153 OR hek293)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/10/18 08:52

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S69	2,075	(dg44 OR dg-44 OR chodg44 OR cho-dg44)	USPAT	OR	OFF	2018/08/14 09:27
S70	5,851	"factor c"	USPAT	OR	OFF	2018/08/14 09:27
S71	92	S69 and S70	USPAT	OR	OFF	2018/08/14 09:27
S72	17,574	horseshoe OR crab	USPAT	OR	OFF	2018/08/14 09:28
S73	2	S71 and S72	USPAT	OR	OFF	2018/08/14 09:28
S74	40	(dg44 OR dg-44 OR chodg44 OR cho-dg44).clm.	USPAT	OR	OFF	2018/08/14 09:30
S75	380	"factor c".clm.	USPAT	OR	OFF	2018/08/14 09:30
S76	0	S74 and S75	USPAT	OR	OFF	2018/08/14 09:30
S77	2,254	(horseshoe OR crab).clm.	USPAT	OR	OFF	2018/08/14 09:31
S78	0	S74 and S77	USPAT	OR	OFF	2018/08/14 09:31
S79	0	(dg44 OR dg-44 OR chodg44 OR cho-dg44) same "factor c"	USPAT	OR	OFF	2018/08/14 09:31
S83	2,086	(dg44 OR dg-44 OR chodg44 OR cho-dg44)	USPAT	OR	OFF	2018/09/07 11:44
S84	5,873	"factor c"	USPAT	OR	OFF	2018/09/07 11:44
S85	92	S83 and S84	USPAT	OR	OFF	2018/09/07 11:44
S86	17,635	horseshoe OR crab	USPAT	OR	OFF	2018/09/07 11:44
S87	2	S85 and S86	USPAT	OR	OFF	2018/09/07 11:44
S88	41	(dg44 OR dg-44 OR chodg44 OR cho-dg44).clm.	USPAT	OR	OFF	2018/09/07 11:44
S89	383	"factor c".clm.	USPAT	OR	OFF	2018/09/07 11:44

EAST Search History (Interference)

S90	0	S88 and S89	USPAT	OR	OFF	2018/09/07 11:44
S91	2,260	(horseshoe OR crab).clm.	USPAT	OR	OFF	2018/09/07 11:44
S92	0	S88 and S91	USPAT	OR	OFF	2018/09/07 11:44
S93	0	(dg44 OR dg-44 OR chodg44 OR cho-dg44) same "factor c"	USPAT	OR	OFF	2018/09/07 11:44
S173	45	(tridentatus OR polyphemus).clm.	USPAT	OR	OFF	2021/10/18 08:47
S174	455	"factor c".clm.	USPAT	OR	OFF	2021/10/18 08:47
S175	9,209	(cho OR "chinese hamster").clm.	USPAT	OR	OFF	2021/10/18 08:47
S176	43,441	(hek OR hek293 OR (human adj embryo$))	USPAT	OR	OFF	2021/10/18 08:54
S177	51,060	S175 or S176	USPAT	OR	OFF	2021/10/18 08:55
S178	2	S173 and S174 and S177	USPAT	OR	OFF	2021/10/18 08:55
S179	23	S174 and S177	USPAT	OR	OFF	2021/10/18 08:56
S180	21	S179 not S178	USPAT	OR	OFF	2021/10/18 08:56
S181	1,502	(hek OR hek293 OR (human adj embryo$)).clm.	USPAT	OR	OFF	2021/10/18 08:58
S182	10,237	S175 or S181	USPAT	OR	OFF	2021/10/18 08:58
S183	4	S174 and S182	USPAT	OR	OFF	2021/10/18 08:58
S184	2	S173 and S182	USPAT	OR	OFF	2021/10/18 08:59
S185	4	S183 or S184	USPAT	OR	OFF	2021/10/18 08:59
S186	16	rotundicauda.clm.	USPAT	OR	OFF	2021/10/18 10:40
S187	9,209	(cho OR "chinese hamster").clm.	USPAT	OR	OFF	2021/10/18 10:41
S188	43,441	(hek OR hek293 OR (human adj embryo$))	USPAT	OR	OFF	2021/10/18 10:41
S189	51,060	S187 or S188	USPAT	OR	OFF	2021/10/18 10:41
S190	2	S186 and S189	USPAT	OR	OFF	2021/10/18 10:41
S191	247,408	(cho OR "chinese hamster")	USPAT	OR	OFF	2021/10/18 10:42
S192	247,408	S187 or S191	USPAT	OR	OFF	2021/10/18 10:42

S193	2	S186 and S192	USPAT	OR	OFF	2021/10/18 10:42

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16/399,386	04/30/2019 RULE	435	1656	Q246419

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Shun-ichiro KAWABATA, Fukuoka, JAPAN;

**** CONTINUING DATA ***************************

This application is a CON of 15/983,725 05/18/2018 PAT 10982202
which is a CON of 14/650,767 06/09/2015 PAT 10144923
which is a 371 of PCT/JP2013/083082 12/10/2013

**** FOREIGN APPLICATIONS ***************************

JAPAN 2012-269840 12/10/2012

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05/10/2019

Foreign Priority claimed ☑ Yes ☐ No 35 USC 119(a-d) conditions met ☑ Yes ☐ No Verified and Acknowledged /DAVID J STEADMAN/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		JAPAN	10	18	2

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TITLE

NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

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 NEWS  1  JUN 26  COMPENDEX - New Fields for Author Identifier and URL
 NEWS  2  JUN 30  Numeric Property Search Added to USPATFULL and USPAT2
 NEWS  3  AUG  5  Chemical and Technology Databases RAPRA, CEABA,
                  and TEMA Add Numeric Property Search
 NEWS  4  OCT  7  EP Appeal Data from European Patent Register Added to
                  INPADOCDB and INPAFAMDB
 NEWS  5  NOV 15  Enhanced INPADOCDB and INPAFAMDB Files
 NEWS  6  NOV 23  Claims Access in CA/CAplus: WIPO, United States, and China
                  for 1999 - Present.
 NEWS  7  DEC  1  Engineering Flagship Database Inspec Reaches
                  Milestone 20 Million Total Records
 NEWS  8  DEC  1  Full-Text Patent Documents from German Democratic
                  Republic Added to DEFULL
 NEWS  9  FEB  7  Patent Status Indicators in CA/CAplus
 NEWS 10  FEB 16  ReaxysFileSub and companion bibliographic
                  database ReaxysFileBib new on STNext(R)
 NEWS 11  APR 12  New Version of the EPFULL (European Patent Full Text
                  Database) Launched on STN
 NEWS 12  APR 12  Analytical Abstracts Enhancements Increase Value to Users
 NEWS 13  APR 13  INSPEC Reload Features Numeric Property Searching, Cited
                  References and DOIs
 NEWS 14  MAY 10  May 2021 Update to Emtree(R) Now Available on STN
 NEWS 15  SEP  6  New Russian Patent Full Text Database RUFULL Launched on STN
 NEWS 16  SEP  6  NTIS Reload on STNext Resumes Updating; Introduces Numeric
                  Property Searching and SLART Improvements
 NEWS 17  SEP 11  September 2021 Update to Emtree(R) Now Available on STN
 NEWS 18  OCT  4  MARPAT(R) Enhanced with Patent Status Data

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=> s 115743-27-6/rn

L1           1 115743-27-6/RN

=> d l1


L1   ANSWER 1 OF 1  REGISTRY  COPYRIGHT 2021 ACS on STN
RN   115743-27-6  REGISTRY
ED   Entered STN:  13 Aug 1988
CN   Hemolymph-coagulation factor, C, activated (Tachypleus tridentatus
     hemocyte A-chain reduced) (9CI)  (CA INDEX NAME)
OTHER NAMES:
CN   Hemolymph-coagulation factor C (Carcinoscorpius rotundicauda clone CrFC21
     light chain A)
CN   Hemolymph-coagulation factor C (Carcinoscorpius rotundicauda clone CrFC26
     light chain A)
FS   PROTEIN SEQUENCE
MF   C335 H527 N105 O108 S4
CI   PMS, MAN
SR   CA
LC   STN Files:   CA, CAPLUS, CHEMCATS, TOXCENTER


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=> s "FACTOR C"

L2       83416 "FACTOR C"

=> S (cho OR "chinese hamster" OR hek OR hek293 or (human adj embryo?))

L3      621251 (CHO OR "CHINESE HAMSTER" OR HEK OR HEK293 OR (HUMAN ADJ EMBRYO?
               ))

=> S L2 and L3

L4         699 L2 AND L3

=> S (LIMULUS or tridentatus OR TACHYPLEUS or polyphemus)

L5       33326 (LIMULUS OR TRIDENTATUS OR TACHYPLEUS OR POLYPHEMUS)

=> S L4 AND L5

L6           6 L4 AND L5

=> DUP REM L6

PROCESSING COMPLETED FOR L6
L7             5 DUP REM L6 (1 DUPLICATE REMOVED)

=> D IBIB ABS 1-

YOU HAVE REQUESTED DATA FROM 5 ANSWERS - CONTINUE? Y/(N):Y


L7   ANSWER 1 OF 5     MEDLINE ®  on STN
ACCESSION NUMBER:   2019937754     MEDLINE Full-text
DOCUMENT NUMBER:    PubMed ID: 29866883
TITLE:              Intermolecular autocatalytic activation of serine protease
                    zymogen factor C through an active transition state
                    responding to lipopolysaccharide.
AUTHOR:             Shibata Toshio
CORPORATE SOURCE:   Department of Biology, Faculty of Science, Kyushu
                    University, Fukuoka 819-0395, Japan, Graduate School of
                    Systems Life Sciences, Kyushu University, Fukuoka 819-0395,
                    Japan.
AUTHOR:             Kobayashi Yuki
CORPORATE SOURCE:   Department of Biology, Faculty of Science, Kyushu
                    University, Fukuoka 819-0395, Japan.
AUTHOR:             Ikeda Yuto
CORPORATE SOURCE:   Graduate School of Systems Life Sciences, Kyushu
                    University, Fukuoka 819-0395, Japan.
AUTHOR:             Kawabata Shun-Ichiro
CORPORATE SOURCE:   Department of Biology, Faculty of Science, Kyushu
                    University, Fukuoka 819-0395, Japan, Graduate School of
                    Systems Life Sciences, Kyushu University, Fukuoka 819-0395,
                    Japan. skawascb@kyudai.jp
SOURCE:             The Journal of biological chemistry, (20180720) Vol. 293,
                    No. 29, pp. 11589-11599.  Electronic Publication Date: 4
                    Jun 2018
                    Journal code: 2985121R. E-ISSN: 1083-351X. L-ISSN:
                    0021-9258.
                    Report No.: PMC-PMC6065167.
DIGITAL OBJECT ID:  http://dx.doi.org/10.1074/jbc.RA118.002311
PUB. COUNTRY:       United States
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DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LANGUAGE: English
FILE SEGMENT: MEDLINE; Priority Journals
FILE SEGMENT: Print; Electronic
ENTRY MONTH: 201902
ENTRY DATE: Entered STN: 5 Jun 2018
Last Updated on STN: 5 Feb 2019
Indexing Added: 5 Feb 2019
Entered Medline: 4 Feb 2019
OS.CITING REF COUNT: 5 There are 5 MEDLINE records that cite this record
MEDLINE REFERENCE COUNT: 38 There are 38 cited references available in MEDLINE for this document.

AB Horseshoe crab hemolymph coagulation is believed to be triggered by the autocatalytic activation of serine protease zymogen factor C to the active form, α-factor C, belonging to the trypsin family, through an active transition state of factor C responding to bacterial lipopolysaccharide (LPS), designated factor C*. However, the existence of factor C* is only speculative, and its proteolytic activity has not been validated. In addition, it remains unclear whether the proteolytic cleavage of the Phe(737)-Ile(738) bond (Phe(737) site) of factor C required for the conversion to α-factor C occurs intramolecularly or intermolecularly between the factor C molecules. Here we show that the Phe(737) site of a catalytic Ser-deficient mutant of factor C is LPS-dependently hydrolyzed by a Phe(737) site-uncleavable mutant, clearly indicating the existence of the active transition state of factor C without cleavage of the Phe(737) site. Moreover, we found the following facts using several mutants of factor C: the autocatalytic cleavage of factor C occurs intermolecularly between factor C* molecules on the LPS surface; factor C* does not exhibit intrinsic chymotryptic activity against the Phe(737) site, but it may recognize a three-dimensional structure around the cleavage site; and LPS is required not only to complete the substrate-binding site and oxyanion hole of factor C* by interacting with the N-terminal region but also to allow the Phe(737) site to be cleaved by inducing a conformational change around the Phe(737) site or by acting as a scaffold to induce specific protein-protein interactions between factor C* molecules.
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L7 ANSWER 2 OF 5 MEDLINE ® on STN DUPLICATE 1
ACCESSION NUMBER: 2018386384 MEDLINE Full-text
DOCUMENT NUMBER: PubMed ID: 28317862
TITLE: Biological Activity of Masked Endotoxin.
AUTHOR: Schwarz Harald; Gornicec Jan; Neuper Theresa; Parigiani Maria Alejandra; Wallner Michael; Duschl Albert; Horejs-Hoeck Jutta
CORPORATE SOURCE: Department of Molecular Biology, University of Salzburg, Salzburg, Austria.
SOURCE: Scientific reports, (20170320) Vol. 7, pp. 44750.
Electronic Publication Date: 20 Mar 2017
Journal code: 101563288. E-ISSN: 2045-2322. L-ISSN: 2045-2322.
Report No.: PMC-PMC5357793.
DIGITAL OBJECT ID: http://dx.doi.org/10.1038/srep44750
PUB. COUNTRY: England: United Kingdom
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LANGUAGE: English
FILE SEGMENT: MEDLINE; Priority Journals
FILE SEGMENT: Electronic
ENTRY MONTH: 201811
ENTRY DATE: Entered STN: 20 Mar 2017
Last Updated on STN: 11 Nov 2018
Indexing Added: 11 Nov 2018
Entered Medline: 9 Nov 2018
OS.CITING REF COUNT: 15 There are 15 MEDLINE records that cite this record
MEDLINE REFERENCE COUNT: 18 There are 18 cited references available in MEDLINE for this document.

AB Low endotoxin recovery (LER) is a recently discovered phenomenon describing the inability of limulus amebocyte lysate (LAL)-based assays to detect lipopolysaccharide (LPS) because of a "masking effect" caused by

chelators or detergents commonly used in buffer formulations for medical products and recombinant proteins. This study investigates the masking capacities of different buffer formulations and whether masked endotoxin is biologically active. We show that both naturally occurring endotoxin as well as control standard endotoxin can be affected by LER. Furthermore, whereas masked endotoxin cannot be detected in Factor C based assays, it is still detectable in a cell-based TLR4-NF-.kappa.B-luciferase reporter gene assay. Moreover, in primary human monocytes, masked LPS induces the expression of pro-inflammatory cytokines and surface activation markers even at very low concentrations. We therefore conclude that masked LPS is a potent trigger of immune responses, which emphasizes the potential danger of masked LPS, as it may pose a health threat in pharmaceutical products or compromise experimental results.

L7 ANSWER 3 OF 5 CAPLUS COPYRIGHT 2021 ACS on STN
ACCESSION NUMBER: 2014:999086 CAPLUS Full-text
DOCUMENT NUMBER: 161:65883
TITLE: Novel recombinant factor C and method for producing same, and method for measuring endotoxin
INVENTOR(S): Mizumura, Hikaru; Oda, Toshio; Kawabata, Shun-Ichiro
PATENT ASSIGNEE(S): Seikagaku Corporation, Japan
SOURCE: PCT Int. Appl., 79pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: Japanese
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2014092079	A1	20140619	WO 2013-JP83082	20131210
CN 104919046	A	20150916	CN 2013-80064649	20131210
EP 2930241	A1	20151014	EP 2013-862466	20131210
EP 2930241	B1	20190626		
SG 10201704774	A1	20170728	SG 2017-10201704774	20131210
CN 108646019	A	20181012	CN 2018-10481773	20131210
CN 108646019	B	20210625		
CN 108642030	A	20181012	CN 2018-10483329	20131210
EP 3441466	A1	20190213	EP 2018-190686	20131210
EP 3441466	B1	20200205		
JP 6511685	B2	20190515	JP 2014-552048	20131210
ES 2738593	T3	20200123	ES 2013-862466	20131210
EP 3663400	A1	20200610	EP 2019-216609	20131210
ES 2784510	T3	20200928	ES 2018-190686	20131210
US 20150307864	A1	20151029	US 2015-14650767	20150609
US 10144923	B2	20181204		
US 20180258414	A1	20180913	US 2018-15983725	20180518
US 10982202	B2	20210420		
JP 2019000122	A	20190110	JP 2018-183630	20180928
JP 6626174	B2	20191225		
US 20190256835	A1	20190822	US 2019-16399575	20190430
US 20190270977	A1	20190905	US 2019-16399386	20190430

PRIORITY APPLN. INFO.:

JP 2012-269840	A	20121210
CN 2013-80064649	A3	20131210
EP 2013-862466	A3	20131210
EP 2018-190686	A3	20131210
JP 2014-552048	A3	20131210
WO 2013-JP83082	W	20131210
US 2015-14650767	A1	20150609
US 2018-15983725	A1	20180518

PATENT STATUS PATENT INFORMATION:

PATENT NO.	KIND	STATUS	STATUS DATE
WO 2014092079	A1	Dead	20201201
CN 104919046	A	Dead	20201120
EP 2930241	A1	Indeterminate	20201107
EP 2930241	B1	Indeterminate	20201106
CN 108646019	A	Alive	20201120
CN 108646019	B	Alive	20210708
CN 108642030	A	Alive	20201120
EP 3441466	A1	Indeterminate	20201106

EP 3441466	B1	Indeterminate	20201106
JP 6511685	B2	Alive	20201121
ES 2738593	T3	Alive	20201121
EP 3663400	A1	Alive	20201202
ES 2784510	T3	Alive	20201121
US 20150307864	A1	Alive	20201120
US 10144923	B2	Alive	20201120
US 20180258414	A1	Alive	20201121
US 10982202	B2	Alive	20210430
JP 2019000122	A	Alive	20201121
JP 6626174	B2	Alive	20201121
US 20190256835	A1	Dead	20210805
US 20190270977	A1	Alive	20201120

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT

AB Provided is a method for producing horseshoe crab recombinant factor C. The horseshoe crab recombinant factor C can be produced by expressing the horseshoe crab factor C using a mammalian cell such as CHO DG44 and HEK293 as a host cell.

OS.CITING REF COUNT: 1 THERE ARE 1 CAPLUS RECORDS THAT CITE THIS RECORD (1 CITINGS)

REFERENCE COUNT: 8 THERE ARE 8 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L7 ANSWER 4 OF 5 BIOSIS COPYRIGHT (c) 2021 Clarivate Analytics on STN

ACCESSION NUMBER: 2007:224924 BIOSIS Full-text

DOCUMENT NUMBER: PREV200700226988

TITLE: A structural perspective on the interaction between lipopolysaccharide and Factor C, a receptor involved in recognition of Gram-negative bacteria.

AUTHOR(S): Koshiba, Takumi; Hashii, Tornoyuki; Kawabata, Shun-ichiro [Reprint Author]

CORPORATE SOURCE: Kyushu Univ, Fac Sci, Dept Biol, Higashi Ku, 6-10-1 Hakozaki, Fukuoka 8128581, Japan skawascb@mbox.nc.kyushu-u.ac.jp

SOURCE: Journal of Biological Chemistry, (FEB 9 2007) Vol. 282, No. 6, pp. 3962-3967. http://www.jbc.org/content/by/year. E-ISSN: 1083-351X.

DIGITAL OBJECT ID: 10.1074/jbc.M609198200

DOCUMENT TYPE: Article

LANGUAGE: English

ENTRY DATE: Entered STN: 4 Apr 2007
Last Updated on STN: 14 Aug 2019

AB The recognition of broadly conserved microorganism components known as pathogen-associated molecular patterns is an essential step in initiating the innate immune response. In the horseshoe crab, stimulation of hemocytes with lipopolysaccharide (LPS) causes the activation of its innate immune response, and Factor C, a serine protease zymogen, plays an important role in this event. Here, we report that Factor C associates with LPS on the hemocyte surface and directly recognizes Gram-negative bacteria. Structure-function analyses reveal that the LPS binding site is present in the N-terminal cysteine-rich (Cys-rich) region of the molecule and that it contains a tripeptide sequence consisting of an aromatic residue flanked by two basic residues that is conserved in other mammalian LPS-recognizing proteins. Moreover, we have demonstrated that the Cys-rich region specifically binds to LPS on Gram-negative bacteria and that mutations in the tripeptide motif abrogate its association with both LPS and Gram-negative bacteria, underscoring the importance of the tripeptide in LPS interaction. Although the innate immune response to LPS in the horseshoe crab is distinct from that of mammals, it appears to rely on structural features that are conserved among LPS-recognizing proteins from diverse species.

L7 ANSWER 5 OF 5 CAPLUS COPYRIGHT 2021 ACS on STN

ACCESSION NUMBER: 2004:32343 CAPLUS Full-text

DOCUMENT NUMBER: 140:106529

TITLE: Mouse extracellular matrix proteins, cDNA cloning, and use as cell adhesion agent

INVENTOR(S): Sekiguchi, Kiyotoshi; Manabe, Riichiro; Hayashizaki, Yoshihide; Kamiya, Mamoru; Furuya, Hiroshi; Tsutsui, Aogu

PATENT ASSIGNEE(S): Japan Science and Technology Corporation, Japan; Institute of Physical and Chemical Research; DNA Form Co., Ltd.
SOURCE: Jpn. Kokai Tokkyo Koho, 86 pp.
CODEN: JKXXAF
DOCUMENT TYPE: Patent
LANGUAGE: Japanese
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
JP 2004008021	A	20040115	JP 2002-162211	20020603

PRIORITY APPLN. INFO.: JP 2002-162211 20020603
PATENT STATUS PATENT INFORMATION:

PATENT NO.	KIND	STATUS	STATUS DATE
JP 2004008021	A	Dead	20201121

AB Novel extracellular matrix proteins of mouse origin, encoding cDNAs, recombinant expression, antibodies against them, and use as cell adhesion agent, are disclosed. Genes coding for those extracellular matrix proteins were designated 33145, 40240, 51140, and 31418. Besides secretion signal sequence, 33145 protein had SCP (sperm-coating glycoprotein) domain, LCCL (Limulus factor C, Coch-5b2 and Lgl1) domain, and a potential N-glycosylation site. Homol. with Cocoa Crisp (cysteine-rich secreted protein), late gestation lung protein, protease inhibitor 15 preproprotein, 25 kDa trypsin inhibitor and glioma pathogenesis-related protein (RTVP-1 protein) was found. 40420 Protein had 2 potential N-glycosylation sites, and showed homol. with human FLJ23191 gene and mouse BB613765, AV243621, AK017759, and AK020942 genes. 51140 Protein has a thrombospondin type 1 repeats (TSP-1) domain, a potential N-glycosylation site, and a cysteine rich region homologous to that of PACE4 protein (Furin-like protease serine protease), and showed homol. with thrombospondin. 31418 Protein has four tandem repeat sequences, and a sequence homologous to bovine prion protein heparin-binding domain, and showed homol. with monkey Taste Bud-specific protein. The proteins were recombinantly expressed as fusion protein with EGFP-His-myc tag in human 293T cells. Localization in extracellular matrix was detected. Cell adhesion and growth activities were found. Prodn. of anti-40240 antibodies is described.

=>

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Docket No: Q246419
Hikaru MIZUMURA, et al.	
Appln. No.: 16/399,386	Group Art Unit: 1656
Confirmation No.: 9293	Examiner: STEADMAN, DAVID J
Filed: April 30, 2019	

For: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

AMENDMENT UNDER 37 C.F.R. § 1.111

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

Sir:

In response to the Office Action dated March 9, 2021, please amend the above-identified application as follows on the accompanying pages. A Petition and payment for a three month extension of time are being filed herewith, making this Amendment due on or before September 9, 2021.

TABLE OF CONTENTS

AMENDMENTS TO THE CLAIMS 2

REMARKS 8

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions and listings of claims in the application:

LISTING OF CLAIMS:

1. (currently amended): A horseshoe crab Factor C protein having activity of Factor C,

wherein the horseshoe crab is selected from the group consisting of *Tachypleus tridentatus,* and *Limulus polyphemus, ~~and Carcinoscorpius rotundicauda,~~* and wherein the horseshoe crab Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or Human Embryonic Kidney (HEK) 293 cell, and

wherein the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a corresponding native horseshoe crab Factor C and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell.

2. (currently amended): A Factor C protein having activity of Factor C,

wherein the Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or Human Embryonic Kidney (HEK) 293 cell, and

wherein the expressed Factor C protein comprises an amino acid sequence selected from the group consisting of:

(A) the amino acid sequence of SEQ ID NO: 2, and the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor

C of *Tachypleus tridentatus* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell; and

(B) an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:2, and having activity of Factor C, and the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Tachypleus tridentatus* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell; and

~~(C) the amino acid sequence of SEQ ID NO: 4, and the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Carcinoscorpius rotundicauda* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell; and~~

~~(D) an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:4, and having activity of Factor C, and the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Carcinoscorpius rotundicauda* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell, and~~

wherein the Factor C protein exhibits a residual activity of at least one selected from the group consisting of:

(1) 10% or higher in the presence of 21 mM sodium citrate; and

(2) 15% or higher in the presence of 16 mM magnesium sulfate.

3. (original): The Factor C protein according to claim 2, which is water soluble.

4. (original): The Factor C protein according to claim 2, which has a molecular weight of 115 kDa to 140 kDa as measured by SDS-PAGE under non-reducing condition.

5. (original): The Factor C protein according to claim 4, which has a molecular weight of 127 kDa±5 kDa.

6. (previously presented): The Factor C protein according to claim 2, which exhibits a residual Factor C activity of 35% or higher in the presence of 52 mM sodium hydrogen carbonate.

7. (previously presented): The Factor C protein according to claim 2, which exhibits a residual Factor C activity of 35% or higher in the presence of 214 mM sodium chloride.

8. (previously presented): The Factor C protein according to claim 2, which exhibits a residual Factor C activity of 25% or higher in the presence of 16 mM magnesium sulfate.

9. (original): The Factor C protein according to claim 2, which exhibits a residual Factor C activity of 35% or higher in the presence of 2.5 mM calcium chloride.

10. (canceled).

11. (canceled).

12. (currently amended): A method for producing the Factor C protein of claim 2, the method comprising culturing a Chinese Hamster Ovary (CHO) DG44 cell or Human Embryonic Kidney (HEK) 293 cell recombinantly expressing said Factor C protein under conditions suitable for the expression of the Factor C protein to produce the Factor C protein.

13. (original): An endotoxin assay kit comprising the Factor C protein according to claim 2 and one or more elements.

14. (withdrawn): A method for measuring endotoxin in a test specimen using an endotoxin assay agent comprising the Factor C protein according to claim 2.

15. (withdrawn): The method according to claim 14, wherein the test specimen comprises at least one of:

(A) an ion;

(B) a cation;

(C) a metal ion;

(D) an alkali metal ion or an alkaline earth metal ion; and

(E) one or more kinds of ions selected from the group consisting of sodium ion, potassium ion, calcium ion, and magnesium ion.

16. (withdrawn): The method according to claim 15, wherein the test specimen comprises a cation and the final concentration of the cation after mixing the test specimen with the assay agent is 1 mM or higher.

17. (withdrawn): The method according to claim 14, wherein the test specimen is an injection.

18. (withdrawn): A product which has been subjected to an endotoxin assay using an endotoxin assay agent comprising the Factor C protein according to claim 2,

wherein the product is selected from the group consisting of water for medical use, a pharmaceutical product, an infusion solution, a blood product, a medical apparatus, a medical instrument, a biological specimen, a native protein, a recombinant protein, a nucleic acid, and a saccharide.

19. (previously presented): The Factor C protein according to claim 2, which exhibits a residual activity of 20% or higher in the presence of 21 mM sodium citrate.

20. (previously presented): The Factor C protein according to claim 2, which exhibits a residual activity of 25% or higher in the presence of 52 mM sodium hydrogen carbonate.

21. (previously presented): The Factor C protein according to claim 2, which exhibits a residual activity of 25% or higher in the presence of 214 mM sodium chloride.

22. (previously presented): The Factor C protein according to claim 1, wherein the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared

with a corresponding native horseshoe crab Factor C and a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell.

23. (previously presented): The Factor C protein according to claim 2, wherein the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a corresponding native horseshoe crab Factor C and a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell.

24. (new): A horseshoe crab Factor C protein having activity of Factor C,

wherein the horseshoe crab is *Limulus polyphemus*, and wherein the horseshoe crab Factor C protein is produced through being recombinantly expressed from a Human Embryonic Kidney (HEK) 293 cell, and

wherein the Factor C protein exhibits a residual activity of at least one selected from the group consisting of:

(1) 10% or higher in the presence of 21 mM sodium citrate; and

(2) 15% or higher in the presence of 16 mM magnesium sulfate.

REMARKS

In the present Amendment, claims 1, 2 and 12 have been amended to recite "Human Embryonic Kidney (HEK) 293 cell". Support is found, for example, in paragraph [0038] of the specification. In addition, claim 1 has been amended to delete "*Carcinoscorpius rotundicauda*". Claim 2 has been amended to delete amino acid sequence (C) and amino acid sequence (D). New claim 24 has been added. Support for claim 24 is found, for example, in paragraphs [0019], [0020], [0035] and [0038] of the specification. No new matter has been added, and entry of the Amendment is respectfully requested.

Upon entry of the Amendment, claims 1-9 and 12-24 will be pending, of which claims 14-18 are withdrawn from consideration.

Sequence Compliance

At page 3 of the Office Action, the Examiner states that in order to perfect the requirements for a sequence listing, the specification must be amended to contain a statement in a separate paragraph that incorporates by reference the material in the ASCII text file of the sequence listing filed on April 30, 2019, identifying the name of the ASCII text file, the date of creation, and the size of the ASCII text file in bytes.

Applicant respectfully disagrees.

The above-mentioned statement is required only when there is no PDF file of the sequence listing filed. However, in the present application, a PDF file of the sequence listing was filed with the application on April 30, 2019.

Further, a Request for Transfer of a Computer Readable Form under 37 CFR 1.821(e) was filed on April 30, 2019. The Request contains the statement that the PDF file of the Sequence Listing submitted in this application in accordance with 37 CFR 1.821(c) is identical to

the sequence information in the computer readable form and that the PDF file of the Sequence Listing contains no new matter.

Accordingly, the requirements for a sequence listing have been perfected and complied with.

Response to § 103 Rejection

Claims 1-9, 12, 13, and 19-23 are rejected under 35 U.S.C. § 103 as being unpatentable over Ding et al. (US 5,858,706; cited on the IDS filed on April 30, 2019; hereafter "Ding") in view of

Current Protocols in Molecular Biology (1993) 16.23.1-16.23.13, John Wiley and Sons, Inc., 2002 (cited on the IDS filed on June 17, 2020; hereafter "Current Protocols") and

Thomas et al. (J. Pharm. Tox. Methods 51:187-200, 2005; cited on the IDS filed on February 3, 2021; hereafter "Thomas"), and

as evidenced by Mizumura et al. (WO 2012/118226 A1; cited on the IDS filed on April 30, 2019; hereafter "Mizumura").

This rejection should be withdrawn because Ding, Current Protocols and Thomas do not disclose or render obvious the claimed invention, either alone or in combination.

In response to the statement at page 5 of the Office Action that the unexpected result of the Mizumura declaration is based on *T. tridentatus* Factor C polypeptides recombinantly expressed from CHO DG44 cells and HEK293 cells, the term "HEK cell" in the claims is amended to "HEK 293 cell".

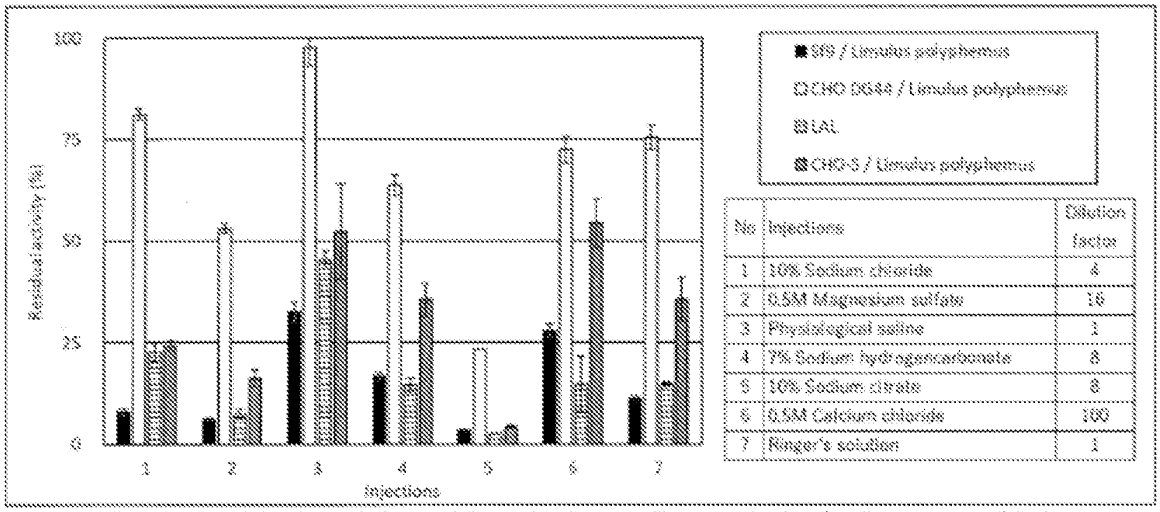
In addition, Applicant submits herewith a Declaration Under 37 C.F.R. § 1.132 executed by Dr. Hikaru MIZUMURA, the first-named inventor of the present application, to demonstrate

that the claimed *Limulus Polyphemus* Factor C, which is produced by being expressed in CHO DG44 cells, has an unexpectedly superior property of being *less* susceptible to inhibition by ions (i.e., superior salt tolerance) compared to a Factor C, which is produced by being expressed in CHO-S cells (and other cell lines such as Sf9).

Dr. Mizumura explained in the Declaration that as shown in Fig. 7 of the specification, *T. tridentatus* Factor C recombinantly produced by using CHO cell or HEK cell as a host has superior salt tolerance compared to native *T. tridentatus* Factor C (TAL) or *T. tridentatus* Factor C recombinantly produced by using Sf9 cell as a host.

Additional experimental data were prepared in the Declaration, which shows that the same is true in the case of *L. polyphemus* Factor C recombinantly produced by using CHO cell.

Specifically, residual activity of *L. polyphemus* Factor Cs in the presence of salt was shown in Fig. 1 at page 4 of the Declaration (also shown below).



No	Injections	Dilution factor
1	10% Sodium chloride	4
2	0.5M Magnesium sulfate	16
3	Physiological saline	1
4	7% Sodium hydrogencarbonate	8
5	10% Sodium citrate	8
6	0.5M Calcium chloride	100
7	Ringer's solution	1

Fig. 1. Residual Activity of *L. polyphemus* Factor Cs in the Presence of Salt

According to Dr. Mizumura, as shown above, *L. polyphemus* Factor C recombinantly produced by using CHO cell as a host has superior salt tolerance compared to native *L.*

polyphemus Factor C (LAL) and *L. polyphemus* Factor C recombinantly produced by using Sf9 cell as a host. Especially, *L. polyphemus* Factor C recombinantly produced by using CHO DG44 cell has superior salt tolerance even compared to *L. polyphemus* Factor C recombinantly produced by using CHO-S cell as a host. Under presence of salts, residual activity of LAL or *L. polyphemus* Factor C recombinantly produced by using Sf9 cell as a host is remarkably decreased, as same as in the case of *T. tridentatus* Factor C.

Also, Dr. Mizumura explained that the "Inhibition data" (citation 15 of the IDS filed on February 3, 2021) shows evaluation results for salt tolerance by using *Limulus* Factor C recombinantly produced by using HEK 293 cell as a host, which is not the cell disclosed in Ding. Taking into consideration of the results of Fig. 1 shown above, it is understood that salt tolerance of *Limulus* Factor C produced by HEK 293 cell is superior to LAL and *L. polyphemus* Factor C recombinantly produced by using Sf9 cell as a host.

Furthermore, Dr. Mizumura explained that the "Inhibition data" shows that such superior salt tolerance can be reproduced irrespective of presence of (α-2,3)-linked terminal sialic acid, at least for *Limulus* Factor C recombinantly produced by using HEK 293 cell as a host. That is, the "Inhibition data" shows that salt tolerance of *Limulus* Factor C produced by HEK 293 cell is increased not dependent on presence or absence of (α-2,3)-linked terminal sialic acid.

In other words, as to HEK293, according to "Inhibition data", one skilled in the art would understand that (i) it is possible to produce *Limulus* Factor C with excellent salt tolerance; and (ii) it is possible to obtain *Limulus* Factor C with excellent salt tolerance by using HEK293 as a host cell regardless of presence or absence of (α-2,3)-linked terminal sialic acid.

Paragraph [0154] of the specification describes that "The results of the above experiments support that the difference in mode of N-type sugar chain modification after

translation is mainly attributed to the difference in molecular weight of Factor C." However, according to Dr. Mizumura, such description does not exclude a possibility that increase of salt tolerance depends on factors other than (α-2,3)-linked terminal sialic acid.

Accordingly, Dr. Mizumura explained that one of ordinary skill in the art would understand that Factor C of the amended claims provides unexpected superior effects in salt tolerance over the cited references.

In view of the above, the present claims, including new claim 24, are not obvious and are patentable over Ding, Current Protocols and Thomas, either alone or in combination. Reconsideration and withdrawal of the §103 rejection based on Ding, Current Protocols and Thomas are respectfully requested.

Allowance is respectfully requested. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,

/Hui C. Wauters/

Hui C. Wauters
Registration No. 57,426

SUGHRUE MION, PLLC
Telephone: 202.293.7060
Facsimile: 202.293.7860

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

Date: August 24, 2021
Attachment: Declaration Under 37 C.F.R. § 1.132 executed by Dr. Hikaru MIZUMURA.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)		Docket Number Q246419 Confirmation Number 9293	
Application Number	16/399,386	Filed	April 30, 2019
For	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN		
Art Unit	1656	Examiner	STEADMAN, DAVID J

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))	$220.00	$110.00	$55.00	
☐	Two month (37 CFR 1.17(a)(2))	$640.00	$320.00	$160.00	
☑	Three month (37 CFR 1.17(a)(3))	$1480.00	$740.00	$370.00	$1,480.00
☐	Four month (37 CFR 1.17(a)(4))	$2320.00	$1160.00	$580.00	
☐	Five month (37 CFR 1.17(a)(5))	$3160.00	$1580.00	$790.00	

☐ Previous Payment Amount _____ Date Submitted _____

☐ 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 attached or have been submitted previously.

☐ A check in the amount of the fee is attached.

☑ Payment by credit card.

☐ 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, **except for the Issue Fee and the Publication Fee**, or credit any overpayment, to Deposit Account Number 19-4880.

☑ Payment made via EFS-Web.

I am the ☐ applicant.

☑ attorney or agent of record. Registration Number 57,426

☐ attorney or agent under 37 CFR 1.34. Registration number _____

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

/Hui C. Wauters/	August 24, 2021
Signature	Date
Hui C. Wauters	202.293.7060
Typed or printed name	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 1 form is submitted.

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Docket No: Q246419
Hikaru MIZUMURA, et al.	
Appln. No.: 16/399,386	Group Art Unit: 1656
Confirmation No.: 9293	Examiner: STEADMAN, DAVID J
Filed: April 30, 2019	

For: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

STATEMENT OF SUBSTANCE OF INTERVIEW

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

Sir:

Please review and enter the following remarks summarizing the interview conducted on June 15, 2021:

REMARKS

An Examiner's Interview Summary Record (PTO-413) was dated June 18, 2021.

As an initial matter, Applicant's representative would like to thank Primary Examiner Steadman for the courtesies extended during the telephone interview conducted June 15, 2021.

The Interview Summary mailed June 18, 2021 provides an accurate summary and statement of the substance of the interview.

It is respectfully submitted that the instant STATEMENT OF SUBSTANCE OF INTERVIEW complies with the requirements of 37 C.F.R. §§1.2 and 1.133 and MPEP §713.04.

It is believed that no petition or fee is required. However, if the USPTO deems otherwise, Applicant hereby petitions for any extension of time which may be required to

maintain the pendency of this case, and any required fee, except for the Issue Fee, for such extension is to be charged to Deposit Account No. 19-4880.

Respectfully submitted,

/Hui C. Wauters/

Hui C. Wauters
Registration No. 57,426

SUGHRUE MION, PLLC
Telephone: 202.293.7060
Facsimile: 202.293.7860

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

Date: August 24, 2021

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Docket No: Q246419
Hikaru MIZUMURA, et al.	
Appln. No.: 16/399,386	Group Art Unit: 1656
Confirmation No.: 9293	Examiner: STEADMAN, DAVID J
Filed: April 30, 2019	

For: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

DECLARATION UNDER 37 C.F.R. § 1.132

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

Sir:

I, Dr. Hikaru MIZUMURA, hereby declare and state that I am the same Dr. Hikaru MIZUMURA who executed the Declarations under 37 C.F.R. § 1.132 submitted September 17, 2020 and February 3, 2021; and that my education and experience are as stated in my previous Declarations.

I am the first-named inventor of the above-identified application; I am familiar with the subject matter of the above-identified application and the Office Action mailed March 9, 2021 in the above-identified application.

This Declaration is submitted to further explain and demonstrate that our invention provides unexpected superior results over Ding et al. (US 5,858,706; hereafter "Ding") in view of Current Protocols in Molecular Biology (1993) 16.23.1-16.23.13, John Wiley and Sons, Inc., 2002 (hereafter "Current Protocols") and Thomas et al. (J. Pharm. Tox. Methods 51:187-200,

2005; hereafter "Thomas"), and as evidenced by Mizumura et al. (WO 2012/118226 A1; hereafter "Mizumura"), and thus patentability of our invention over the cited references.

The following additional experiments were conducted by me or under my direct supervision.

As shown in Fig. 7 of the specification, *T. tridentatus* Factor C recombinantly produced by using CHO cell or HEK cell as a host has superior salt tolerance compared to native *T. tridentatus* Factor C (TAL) or *T. tridentatus* Factor C recombinantly produced by using Sf9 cell as a host.

The following additional experimental data shows that the same is true in the case of *L. polyphemus* Factor C recombinantly produced by using CHO cell.

L. polyphemus Factor Cs were prepared in the following manners.

(1) Recombinant *L. polyphemus* Factor C Produced by Using Sf9 cell

cDNA encoding *L. polyphemus* Factor C (SEQ ID NO:1 in US2019/0241629A1) was inserted between EcoRI recognition site and XhoI recognition site of pIZ-V5 vector. Thus obtained plasmid was introduced into cultured Sf9 cells so that *L. polyphemus* Factor C was recombinantly produced, as described in paragraphs [0103] to [0105] of the specification as filed.

(2) Recombinant *L. polyphemus* Factor C Produced by Using CHO DG44 cell

cDNA encoding *L. polyphemus* Factor C (SEQ ID NO:1 in US2019/0241629A1) optimized for expression in CHO cell was synthesized and inserted between MluI recognition

site and XbaI recognition site of pCI-neo vector. Thus obtained plasmid was introduced into cultured CHO DG44 cells so that *L. polyphemus* Factor C was recombinantly produced, as described in paragraphs [0109] to [0112] of the specification as filed.

(3) Native *L. polyphemus* Factor C from Amebocyte Lysate

Native *L. polyphemus* Factor C was purified from the amebocyte lysate of *L. polyphemus* by the cation exchange chromatography. Absence of Factor B and Proclotting enzyme in a purified sample was checked by western blot and lack of the protease activity for each protein.

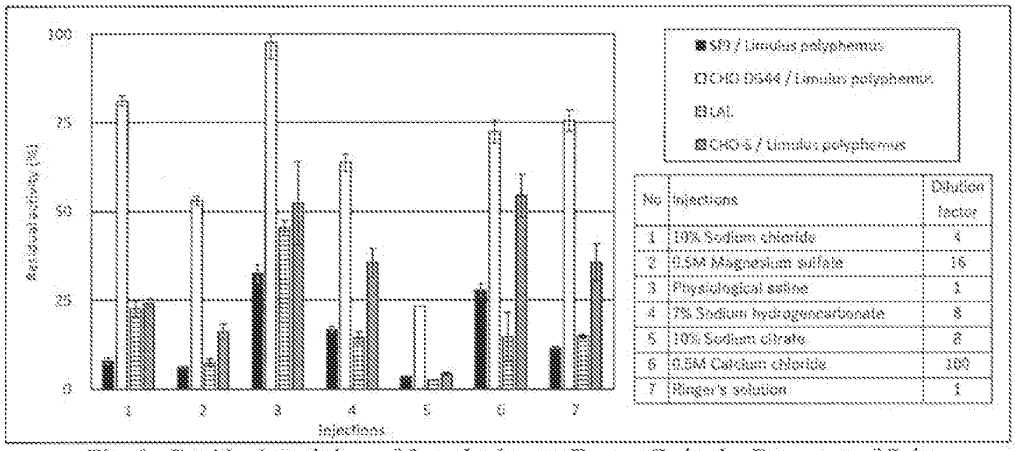
(4) Recombinant *L. polyphemus* Factor C Produced by Using CHO-S cell

The expression plasmid of *L. polyphemus* Factor C was prepared as described in "(2) Recombinant *L. polyphemus* Factor C Produced by Using CHO DG44 cell". The expression plasmid was transfected into CHO-S cell (ExpiCHO™ Expression System; Thermo Fisher Scientific) and recombinant *L. polyphemus* Factor C was collected as culture supernatant fraction in accordance with a manufacturer's instruction.

(5) Residual Activity in the Presence of Salt

The above *L. polyphemus* Factor Cs were used for endotoxin assay in the presence of salt. The assay was conducted under the same condition and materials in Fig.7 and paragraphs [0134] to [0136] of the specification as filed, except that *L. polyphemus* Factor Cs were used instead of *T. tridentatus* Factor Cs.

The results are shown in the following Fig. 1.



No	Injections	Dilution factor
1	10% Sodium chloride	4
2	0.5M Magnesium sulfate	16
3	Physiological saline	1
4	7% Sodium hydrogencarbonate	8
5	10% Sodium citrate	2
6	0.5M Calcium chloride	100
7	Ringer's solution	1

Fig. 1. Residual Activity of *L. polyphemus* Factor Cs in the Presence of Salt

As shown above, *L. polyphemus* Factor C recombinantly produced by using CHO cells as a host have superior salt tolerance compared to native *L. polyphemus* Factor C (LAL) and *L. polyphemus* Factor C recombinantly produced by using Sf9 cell as a host. Especially, *L. polyphemus* Factor C recombinantly produced by using CHO DG44 cell has superior salt tolerance even compared to *L. polyphemus* Factor C recombinantly produced by using CHO-S cell as a host. Under presence of salts, residual activity of LAL and *L. polyphemus* Factor C recombinantly produced by using Sf9 cell as a host is remarkably decreased, as same as in the case of *T. tridentatus* Factor C.

Also, the "Inhibition data" (citation 15 of the IDS filed on February 3, 2021) shows evaluation results for salt tolerance by using *Limulus* Factor C recombinantly produced by using HEK 293 cell as a host, which is not the cell disclosed in Ding. Taking into consideration of the results of Fig. 1 shown above, it is understood that salt tolerance of *Limulus* Factor C produced

by HEK 293 cell is superior to LAL and *L. polyphemus* Factor C recombinantly produced by using Sf9 cell as a host.

Furthermore, the "Inhibition data" shows that such superior salt tolerance can be reproduced irrespective of presence of (α-2,3)-linked terminal sialic acid, at least for *Limulus* Factor C recombinantly produced by using HEK 293 cell as a host. That is, the "Inhibition data" shows that salt tolerance of *Limulus* Factor C produced by HEK 293 cell is increased not dependent on presence or absence of (α-2,3)-linked terminal sialic acid.

Paragraph [0154] of the specification describes that "The results of the above experiments support that the difference in mode of N-type sugar chain modification after translation is mainly attributed to the difference in molecular weight of Factor C." However, such description does not exclude a possibility that increase of salt tolerance depends on factors other than (α-2,3)-linked terminal sialic acid.

Accordingly, one of ordinary skill in the art would understand that Factor C of the amended claims provides unexpected superior effects in salt tolerance.

I declare further that all statements made herein of my 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 wilful 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 the application or any patent issuing thereon.

Date: 11. AUG. 2021

Hikaru Mizumura
Hikaru MIZUMURA

Electronic Patent Application Fee Transmittal

Application Number:	16399386
Filing Date:	30-Apr-2019
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Filer:	Hui Chen Wauters/Sharon Jones
Attorney Docket Number:	Q246419

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

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 - 3 months with $0 paid	1253	1	1480	1480
Miscellaneous:				
		Total in USD ($)		**1480**

Electronic Acknowledgement Receipt

EFS ID:	43589232
Application Number:	16399386
International Application Number:	
Confirmation Number:	9293
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Customer Number:	23373
Filer:	Hui Chen Wauters/Sharon Jones
Filer Authorized By:	Hui Chen Wauters
Attorney Docket Number:	Q246419
Receipt Date:	24-AUG-2021
Filing Date:	30-APR-2019
Time Stamp:	15:41:14
Application Type:	Utility under 35 USC 111(a)

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		Q246419Amendment111asfiled.pdf	192419 a91499f06d416b01bbeca03c1720ce13672567b1	yes	12

	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Amendment/Req. Reconsideration-After Non-Final Reject		1	1	
	Claims		2	7	
	Applicant Arguments/Remarks Made in an Amendment		8	12	
Warnings:					
Information:					
2	Extension of Time	Q246419EOTasfiled.pdf	40150 fe9985bf6f2d3e5721c41de411c96bb0607aaa2d	no	1
Warnings:					
Information:					
3	Applicant summary of interview with examiner	Q246419StatementofSubstanceofInterviewasfiled.pdf	34124 0ee29b8fc1a96e82481e6b00d0f908d12d574ba9	no	2
Warnings:					
Information:					
4	Affidavit-traversing rejectns or objectns rule 132	Q246419ExecutedDeclaration132asfiled.pdf	903521 50b5548c1002394e9ea1ea8669d147496bcd9bec	no	6
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	38982 e1e68db3a0ea322b24335cbba86e0df026c9f4cf	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	1209196		

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.

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
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 16/399,386	Filing Date 04/30/2019	☐ 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	08/24/2021	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 22	Minus	** 22	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 2	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".	/DEIDRE C KING-WOODWARD/
*** 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.	

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/399,386	04/30/2019	Hikaru MIZUMURA	Q246419	9293

23373 7590 06/18/2021

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WASHINGTON, DC 20006

EXAMINER
STEADMAN, DAVID J

ART UNIT	PAPER NUMBER
1656	

NOTIFICATION DATE	DELIVERY MODE
06/18/2021	ELECTRONIC

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Applicant-Initiated Interview Summary	**Application No.** 16/399,386	**Applicant(s)** MIZUMURA et al.		
	Examiner DAVID J STEADMAN	**Art Unit** 1656	**AIA (First Inventor to File) Status** Yes	**Page** 1 of 1

All Participants (applicant, applicants representative, PTO personnel)	**Title**	**Type**
DAVID J STEADMAN	Primary Examiner	Telephonic
HUI C. WAUTERS	Attorney of Record	

Date of Interview: 15 June 2021

Issues Discussed:

35 U.S.C. 103

The applicant's representative provided an interview agenda for discussion purposes only (see attachment). The applicant's proposed arguments 1-3 in the interview agenda were discussed. Regarding the applicant's proposed argument 1, the examiner noted that amending "HEK" to recite " HEK293" would closer align the unexpected result for T. tridentatus Factor C protein with the claimed invention. Regarding the applicant's proposed arguments 2 and 3, to establish an unexpected result for the L. polyphemus and C. rotundicauda Factor C proteins, the examiner suggested providing experimental evidence similar to Figure S1 of the declaration filed on February 3, 2021 for each of the L. polyphemus and C. rotundicauda Factor C proteins. No agreement was reached as to the patentability of the claims.

☑ Attachment

/David Steadman/ Primary Examiner, Art Unit 1656	

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.

Primary Examiner David J. Steadman
U.S. Patent and Trademark Office
Group Art Unit: 1656
Email: david.steadman@uspto.gov

Re: **Interview Agenda (FOR DISCUSSION PURPOSES ONLY)**
U.S. Application No.: 16/399,386
Inventor: Hikaru MIZUMURA, et al.
Assignee: SEIKAGAKU CORPORATION
Attorney Docket No.: Q246419

Dear Primary Examiner Steadman:

The following are proposals for our telephone interview scheduled for Tuesday, June 15, 2021 at 10 am, which are for your consideration and **for discussion purposes only**.

Proposed Amendment

Claims 1, 2 and 12 might be amended to recite "Human Embryonic Kidney (HEK) 293 cell". Support is found, for example, in paragraph [0038] of the specification.

New claim 24 shown below might be added.

> 24. (new): A horseshoe crab Factor C protein having activity of Factor C,
>
> wherein the horseshoe crab is *Limulus polyphemus*, and wherein the horseshoe crab Factor C protein is produced through being recombinantly expressed from a Human Embryonic Kidney (HEK) 293 cell, and
>
> wherein the Factor C protein exhibits a residual activity of at least one selected from the group consisting of:
>
> (1) 10% or higher in the presence of 21 mM sodium citrate; and
>
> (2) 15% or higher in the presence of 16 mM magnesium sulfate.

Proposed Arguments

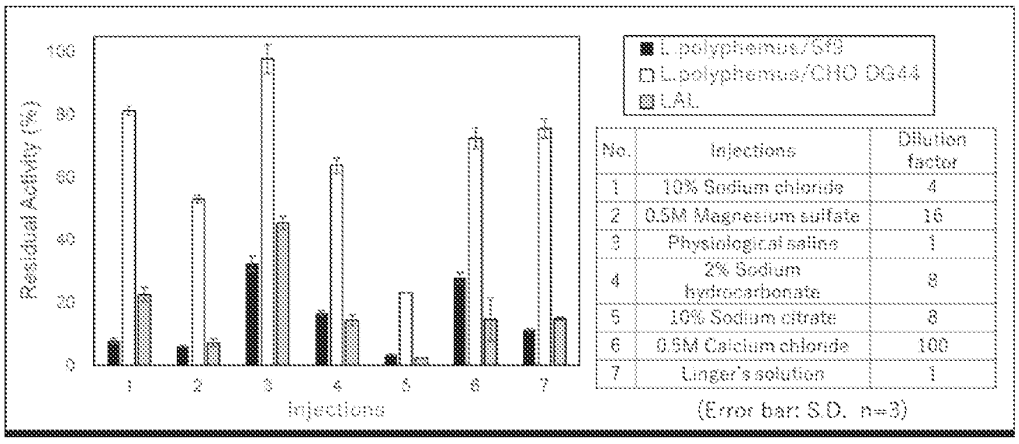
1. In response to the statement at page 5 of the Office Action that the unexpected result of the Mizumura declaration is based on *T. tridentatus* Factor C polypeptides recombinantly expressed from CHO DG44 cells and HEK293 cells, the term "HEK cell" in the claims is amended to "HEK 293 cell".

2. It is stated at pages 15-16 of the Office Action:

"Also, the "Inhibition data" (citation 15 of the IDS filed on February 3, 2021) shows that a Limulus Factor C recombinantly expressed using a HEK293 cell line has (α-2,3)-linked terminal sialic acid (p. 3, Figure 2) but does not exhibit increased salt tolerance (p. 4, Figure 4; p. 5, Figure 6). According to "Inhibition data", the presence of (α-2,3)-linked terminal sialic acid has no effect on the residual activity of Limulus Factor C (p. 6). Thus, one would not expect the applicant's result achieved with *T. tridentatus* Factor C polypeptide recombinantly expressed using CHO DG44 and HEK293 cell to extend beyond *T. tridentatus* Factor C polypeptide to other Factor C polypeptide as recited in the claims."

In response, firstly, Fig. 7 of the specification shows that *T. tridentatus* Factor C recombinantly produced by using CHO cell or HEK cell as a host has superior salt tolerance compared to native *T. tridentatus* Factor C (TAL) or *T. tridentatus* Factor C recombinantly produced by using Sf9 cell as a host.

In addition, Applicant plans to provide additional experimental data as shown in Fig. 1 shown below in a Declaration under 37 C.F.R. § 1.132, which shows that the same is true in the case of *L. polyphemus* Factor C recombinantly produced by using CHO cell.



No.	Injections	Dilution factor
1	10% Sodium chloride	4
2	0.5M Magnesium sulfate	16
3	Physiological saline	1
4	2% Sodium hydrocarbonate	8
5	10% Sodium citrate	8
6	0.5M Calcium chloride	100
7	Linger's solution	1

Fig. 1 Salt tolerance of *L. polyphemus* Factor C

As shown above, *L. polyphemus* Factor C recombinantly produced by using CHO cell as a host has superior salt tolerance compared to native *L. polyphemus* Factor C (LAL) or *L. polyphemus* Factor C recombinantly produced by using Sf9 cell as a host. Under presence of salts, residual activity of LAL or *L. polyphemus* Factor C recombinantly produced by using Sf9 cell as a host is remarkably decreased, as same as in the case of *T. tridentatus* Factor C.

Secondly, the "Inhibition data" (citation 15 of the IDS filed on February 3, 2021) shows evaluation results for salt tolerance by using *Limulus* Factor C recombinantly produced by using HEK 293 cell as a host. Taking into consideration of the results of Fig. 1 shown above, it is understood that salt tolerance of *Limulus* Factor C produced by HEK 293 cell is superior to LAL or *Limulus* Factor C recombinantly produced by using Sf9 cell as a host, not due to presence or absence of (α-2,3)-linked terminal sialic acid. That is, the "Inhibition data" shows that salt tolerance of *Limulus* Factor C produced by HEK 293 cell is increased not dependent on presence or absence of (α-2,3)-linked terminal sialic acid.

Paragraph [0154] of the specification describes that "The results of the above experiments support that the difference in mode of N-type sugar chain modification after translation is mainly attributed to the difference in molecular weight of Factor C." However, such description does not exclude a possibility that increase of salt tolerance depends on factors other than (α-2,3)-linked terminal sialic acid.

3. As shown in the Declaration under 37 C.F.R. § 1.132 submitted September 17, 2020, *C. rotundicauda* Factor C has 90% or more of identity with *T. tridentatus* Factor C or *L. polyphemus* Factor C, and has Factor C activity. Therefore, one skilled in the art would naturally understand that salt tolerance increasing effect confirmed in *T. tridentatus* Factor C or *L. polyphemus* Factor C is also provided in *C. rotundicauda* Factor C.

Accordingly, one of ordinary skill in the art would understand that Factor C of the amended claims provides unexpected superior effects in salt tolerance.

Hui C. Wauters (Reg. No. 57,426)

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/399,386	04/30/2019	Hikaru MIZUMURA	Q246419	9293

23373 7590 03/09/2021

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EXAMINER	
STEADMAN, DAVID J	

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Office Action Summary	Application No. 16/399,386	Applicant(s) MIZUMURA et al.	
	Examiner DAVID J STEADMAN	Art Unit 1656	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 03 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 and 12-23 is/are pending in the application.

5a) Of the above claim(s) 14-18 is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-9,12-13 and 19-23 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 CORRESPONDENCE

Status of the Application

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 February 3, 2021 has been entered.

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Claims 1-9 and 12-23 are pending in the application.

Applicant's amendment to the claims, filed on February 3, 2021, is acknowledged. This listing of the claims replaces all prior versions and listings of the claims.

Applicant's remarks and declaration under 37 CFR 1.132 filed on February 3, 2021 (hereafter "Mizumura declaration") in response to the final rejection mailed on October 8, 2020 have been fully considered.

The text of those sections of Title 35 U.S. Code not included in the instant action can be found in a prior Office action.

Rejections not reiterated from a previous Office action are hereby withdrawn.

Election/Restriction

Claims 14-18 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to nonelected inventions, there being no allowable generic or linking claim. Election was made without traverse in the reply filed on June 17, 2020.

Claims 1-9, 12, 13, and 19-23 are being examined on the merits.

Information Disclosure Statement

The information disclosure statement (IDS) submitted on February 3, 2021 is in compliance with the provisions of 37 CFR 1.97. Accordingly, the IDS is being considered by the examiner.

Sequence Compliance

In order to perfect the requirements for a sequence listing, the specification must be amended to contain a statement in a separate paragraph that incorporates by reference the material in the ASCII text file of the sequence listing filed on April 30, 2019, identifying the name of the ASCII text file, the date of creation, and the size of the ASCII text file in bytes. See MPEP 2422.03.I and see MPEP 2422.03(a) for additional information pertaining to EFS-Web submission of sequence listings.

Claim Rejections - 35 USC § 103

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.

Claims 1-9, 12, 13, and 19-23 are rejected under 35 U.S.C. 103 as being unpatentable over Ding et al. (US 5,858,706; cited on the IDS filed on April 30, 2019; hereafter "Ding") in view of

Current Protocols in Molecular Biology (1993) 16.23.1-16.23.13, John Wiley and Sons, Inc., 2002 (cited on the IDS filed on June 17, 2020; hereafter "Current Protocols") and

Thomas et al. (*J. Pharm. Tox. Methods* 51:187-200, 2005; cited on the IDS filed on February 3, 2021; hereafter "Thomas"), and

as evidenced by Mizumura et al. (WO 2012/118226 A1; cited on the IDS filed on April 30, 2019; hereafter "Mizumura").

As amended, claims 1 and 22 are drawn to a horseshoe crab Factor C protein having activity of Factor C, wherein the horseshoe crab is selected from the group consisting of *Tachypleus tridentatus, Limulus polyphemus*, and *Carcinoscorpius rotundicauda*, and wherein the horseshoe crab Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or Human Embryonic Kidney (HEK) cell, and

wherein the Factor C protein contains (α-2,3) linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell.

As amended, claims 2-9, 19-21, and 23 are drawn to a Factor C protein having activity of Factor C,

wherein the Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or Human Embryonic Kidney (HEK) cell, and

wherein the expressed Factor C protein comprises an amino acid sequence selected from the group consisting of:

(A) the amino acid sequence of SEQ ID NO: 2, and the Factor C protein contains (α-2,3) linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Tachypleus tridentatus* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell;

(B) an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:2, and having activity of Factor C, and the Factor C protein contains (α-2,3) linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Tachypleus tridentatus* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell;

(C) the amino acid sequence of SEQ ID NO: 4, and the Factor C protein contains (α-2,3) linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Carcinoscorpinus rotundicauda* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell; and

(D) an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:4, and having activity of Factor C, and the Factor C protein contains (α-2,3) linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Carcinoscorpinus rotundicauda* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell, and

wherein the Factor C protein exhibits a residual activity of at least one selected from the group consisting of:

(1) 10% or higher in the presence of 21 mM sodium citrate; and

(2) 15% or higher in the presence of 16 mM magnesium sulfate.

Claim 12 is drawn to a method for producing the Factor C protein of claim 2, the method comprising culturing a Chinese Hamster Ovary (CHO) DG44 cell or Human Embryonic Kidney (HEK) cell recombinantly expressing said Factor C protein under conditions suitable for the expression of the Factor C protein to produce the Factor C protein.

Claim 13 is drawn to an endotoxin assay kit comprising the Factor C protein according to claim 2 and one or more elements.

Regarding claims 1, 2, and 12, the reference of Ding discloses a mammalian host cell, including a CHO cell, as a host cell for recombinant expression of a cDNA encoding a Factor C (column 9, Example 1). Ding discloses the cDNAs for expressing the horseshoe crab Factor C polypeptide include the representative example of *T. tridentatus* Factor C polypeptide (column 3, lines 14-20). Ding discloses a method for producing the Factor C polypeptide by culturing the host cell and recovering the Factor C protein (column 9, Example 1). Ding discloses activation of the Factor C by endotoxin (column 1, lines 44-47).

Regarding claim 2, Ding does not disclose the amino acid sequence of *T. tridentatus* factor C has the amino acid sequence of SEQ ID NO: 2. However, the evidentiary reference of Mizumura is cited to show that the amino acid sequence of *T. tridentatus* factor C is identical to SEQ ID NO: 2 of this application.

Regarding claim 3, Ding discloses solubilization of the factor C (column 7, lines 456-53) and thus Ding's Factor C is considered to be water-soluble.

Regarding claim 13, Ding discloses the recombinant Factor C for use in an assay for endotoxin detection and teaches elements for conducting such an assay (column 8, lines 50-60), which is considered to be an endotoxin assay kit as encompassed by claim 13.

The differences between Ding and the claimed invention is that Ding does not disclose CHO DG44 or HEK cells as an expression host as recited in claims 1, 2, and 12, does not explicitly disclose the a greater amount of (α-2,3) linked terminal sialic acid as recited in claims 1, 2, 22, and 23, and does not explicitly disclose the residual activity and molecular weight limitations as recited in claims 2, 4-9, and 19-21.

The reference of Current Protocols generally teaches a method for using CHO cells as an expression host for amplifying and expressing a desired gene (see whole document). Current Protocols teaches CHO DG44 as a cell line that greatly increases the ease of gene amplification (p. 16.23.11).

The reference of Thomas teaches that the HEK293 cell line has been extensively used as an expression tool for recombinant proteins since it was generated over 25 years ago and that its biochemical machinery is capable of carrying out most of the post-translational folding and processing required to generate functional, mature protein from non-mammalian nucleic acids (p. 187, Abstract).

It would have been obvious to one of ordinary skill in the art before the effective filing date to combine the teachings of Ding, Current Protocols, and Thomas to modify Ding to use a CHO DG44 cell line of Current Protocols or a HEK293 cell line of Thomas

as an expression host. One would have been motivated to do this because Ding discloses a mammalian cell as an expression host, specifically CHO cells, and Current Protocols and Thomas each teaches a mammalian cell line that is routinely used for recombinant expression of proteins. One would have had a reasonable expectation of success to modify Ding to use a CHO DG44 cell line of Current Protocols or a HEK293 cell line of Thomas as an expression host because Ding teaches cDNAs for expressing a horseshoe crab Factor C polypeptide using a mammalian cell and each of Current Protocols and Thomas teaches a method for expressing a desired gene using a CHO DG44 cell line or a HEK293 cell line, respectively.

Regarding claims 2 and 19, the combination of Ding, Current Protocols, and Thomas does not teach or suggest activated horseshoe crab Factor C polypeptides recombinantly expressed and recovered from CHO DG44 cells and HEK293 cells exhibit a residual activity of 10% or 20% or higher in the presence of 21 mM sodium citrate. However, Figure 7 of this application shows that horseshoe crab Factor C polypeptides recombinantly expressed and recovered from CHO DG44 cells and HEK293 cells exhibit a residual activity of 20% or higher in the presence of 10% sodium citrate with a dilution factor of 8, which, according to the specification is about 21 mM sodium citrate (p. 88). As such, since horseshoe crab Factor C proteins recombinantly expressed and recovered from CHO DG44 cells and HEK293 cells exhibits a residual activity of 20% or higher in the presence of 21 mM sodium citrate, it follows that Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells necessarily exhibits a residual activity of 20% or higher in the presence of 21 mM sodium citrate.

Regarding claims 4 and 5, the combination of Ding, Current Protocols, and Thomas does not teach or suggest activated horseshoe crab Factor C polypeptides recombinantly expressed and recovered from CHO DG44 cells and HEK293 cells has a molecular weight as recited in the claims. However, the applicant's specification discloses that *T. tridentatus* factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells has a molecular weight of 128 kDa by SDS-PAGE under non-reducing conditions and *T. tridentatus* factor C polypeptide recombinantly expressed and recovered from HEK293 cells has a molecular weight of 124 kDa by SDS-PAGE under non-reducing conditions (p. 92, paragraph [0144] and p. 94, Table 1). As such, Ding's activated Factor C polypeptide expressed from CHO DG44 cells or HEK293 cells necessarily has a molecular weight of as recited in claims 4 and 5.

Regarding claims 6 and 20, the combination of Ding, Current Protocols, and Thomas does not teach or suggest activated horseshoe crab Factor C polypeptides recombinantly expressed and recovered from CHO DG44 cells and HEK293 cells exhibit a residual activity of 25% or 35% or higher in the presence of 52 mM sodium hydrogen carbonate. However, Figure 7 of this application shows that horseshoe crab Factor C polypeptides recombinantly expressed and recovered from CHO DG44 cells and HEK293 cells exhibit a residual activity of 35% or higher in the presence of about 7% sodium citrate with a dilution factor of 8, which, according to the specification is about 52 mM sodium hydrogen carbonate (specification at p. 88). As such, Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells or HEK293 cells necessarily exhibits a residual activity of 35% or higher in the presence of 52 mM sodium hydrogen carbonate.

Regarding claims 7 and 21, the combination of Ding, Current Protocols, and Thomas does not teach or suggest activated horseshoe crab Factor C polypeptides recombinantly expressed and recovered from CHO DG44 cells and HEK293 cells exhibit a residual activity of 25% or 35% or higher in the presence of 214 mM sodium chloride. However, Figure 7 of this application shows that horseshoe crab Factor C polypeptides recombinantly expressed and recovered from CHO DG44 cells and HEK293 cells exhibit a residual activity of 35% or higher in the presence of about 10% sodium citrate with a dilution factor of 4, which, according to the specification is about 214 mM sodium chloride (specification at p. 88). As such, Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells or HEK293 cells necessarily exhibits a residual activity of 35% or higher in the presence of 214 mM sodium chloride.

Regarding claims 2 and 8, the combination of Ding, Current Protocols, and Thomas does not explicitly teach activated horseshoe crab Factor C polypeptides recombinantly expressed and recovered from CHO DG44 cells and HEK293 cells exhibit a residual activity of 15% or 25% or higher in the presence of 16 mM magnesium sulfate. However, Figure 7 of this application shows that horseshoe crab Factor C polypeptides recombinantly expressed and recovered from CHO DG44 cells and HEK293 cells exhibits a residual activity of 25% or higher in the presence of about 0.5 M magnesium sulfate with a dilution factor of 16, which, according to the specification is about 16 mM magnesium sulfate (specification at p. 89). As such, Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from

CHO DG44 cells or HEK293 cells necessarily exhibits a residual activity of 25% or higher in the presence of 16 mM magnesium sulfate.

Regarding claim 9, the combination of Ding, Current Protocols, and Thomas does not teach or suggest activated horseshoe crab Factor C polypeptides recombinantly expressed and recovered from CHO DG44 cells and HEK293 cells exhibits a residual activity of 35% or higher in the presence of 2.5 mM calcium chloride. However, Figure 7 of this application shows that horseshoe crab Factor C polypeptides recombinantly expressed and recovered from CHO DG44 cells and HEK293 cells exhibit a residual activity of 35% or higher in the presence of 0.5 M calcium chloride with a dilution factor of 100, which, according to the specification is about 2.5 mM calcium chloride (specification at p. 89). As such, Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells or HEK293 cells necessarily exhibits a residual activity of 35% or higher in the presence of 2.5 mM calcium chloride.

Regarding claims 2 and 10, the combination of Ding, Current Protocols, and Thomas does not teach or suggest activated horseshoe crab Factor C polypeptides recombinantly expressed and recovered from CHO DG44 cells and HEK293 cells has (α-2,3) linked terminal sialic acid in a greater amount as compared with a native horseshoe crab Factor C and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell. However, the applicant's specification discloses that horseshoe crab Factor C polypeptides recombinantly expressed and recovered from CHO DG44 cells and HEK293 cells have (α-2,3) linked terminal sialic acid in a greater amount as compared with a native *T. tridentatus* horseshoe crab Factor C and/or a

corresponding *T. tridentatus* Factor C protein recombinantly expressed using Sf9 as a host cell (see Example 9, beginning at p. 94 of the specification). As such, Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells or HEK293 cells necessarily has a greater amount of (α-2,3)-linked terminal sialic acid than a native *T. tridentatus* horseshoe crab Factor C and/or a corresponding *T. tridentatus* Factor C protein recombinantly expressed using Sf9 as a host cell.

Therefore, the invention of claims 1-9, 12, 13, and 19-23 would have been obvious to one of ordinary skill in the art at the time of the invention.

RESPONSE TO REMARKS: The applicant argues that the claimed Factor C protein exhibits the unexpected property of increased salt resistance and improved endotoxin sensitivity as compared to the native Factor C. According to the applicant, the examiner's statement regarding inherent characteristics improperly requires a comparison of the claimed invention with a recombinant Factor C suggested by the combination of Ding and Current Protocols.

The applicant's argument is not found persuasive. Contrary to the applicant's position, the examiner has not required (and is not requiring) the applicant to compare the salt resistance and endotoxin sensitivity of the claimed invention and a recombinant Factor C suggested by the combination of Ding and Current Protocols. Rather, the examiner's statement regarding inherent characteristics is to address limitations of the claimed invention that are not explicitly taught by the cited prior art (see MPEP 2143.03).

The applicant further argues the cited prior art does not teach or suggest using CHO DG44 cells to produce Factor C. According to the applicant, the cited prior art does not teach or remotely suggest using CHO DG44 cells to produce Factor C.

The applicant's argument is not found persuasive. As noted above, Ding discloses a CHO cell as a host cell for recombinant expression of *T. tridentatus* and *C. rotundicauda* Factor C polypeptides and Current Protocols is cited to show that CHO DG44 was a well-known CHO cell line for recombinant protein expression. Thus, contrary to the applicant's position, the cited prior art does suggests using CHO DG44 cells to produce recombinant Factor C.

The applicant further argues that the Mizumura declaration demonstrates that the claimed Factor C has an unexpected property of being less susceptible to inhibition by ions in an injection and is structurally different as compared to recombinant Factor C produced in CHO-S cells. The applicant argues that the cited prior art does not teach or suggest the unexpected property of being less susceptible to inhibition by ions in an injection.

The applicant's argument is not found persuasive. In view of the evidence of the Mizumura declaration, the examiner acknowledges unexpected results for *T. tridentatus* Factor C polypeptides recombinantly expressed using CHO DG44 cells and HEK293 cells. However, as explained in detail below, the applicant's evidence of unexpected results does not rebut a *prima facie* case of obviousness because the applicant's results are not commensurate in scope with the claimed invention (see MPEP 716.02(d)).

Citing to Figure 7 of the specification and to the Mizumura declaration, the applicant further argues that while recombinant Factor C polypeptides produced in CHO

DG44 cells and HEK cells but not Sf9 and CHO-S cells exhibit 10% or higher residual activity in the presence of 21 mM sodium citrate and 15% or higher residual activity in the presence of 16 mM magnesium sulfate.

The examiner acknowledges the applicant's noted results, however, the rejection is not based on recombinant Factor C polypeptides produced in Sf9 and CHO-S cells. Rather the rejection is based on the Factor C of Ding recombinantly produced in CHO DG44 cells of Current Protocols and HEK293 cells of Thomas.

The applicant further argues that the examiner's understanding of motivation to use CHO DG44 cells for recombinant protein production is not correct because maximum expression for CHO DG44 cells, which use a DHFR system, requires "time and labor" and one would have instead selected a CHO cell line using a GS system. The applicant argues that even assuming *arguendo* gene amplification for CHO DG44 is easy, one would not have expected the improvement in salt tolerance because gene amplification or amount of recombinant protein does not relate to salt tolerance.

The applicant's argument is not found persuasive. MPEP 2123 states that "[a] reference may be relied upon for all that it would have reasonably suggested to one having ordinary skill the art" and while Current Protocols acknowledges that the DHFR process is "lengthy", Current Protocols teaches that the DHFR system "is probably more widely used, and results in very high level of amplification…and expression" (p. 16.23.1, top). Given that Current Protocols teaches that the DHFR system "is probably more widely used, and results in very high level of amplification…and expression", one would have been motivated to use CHO DG44 cells as the mammalian host cell in Ding.

Regarding the improved salt tolerance, MPEP 2112 states that the inherent disclosures of a prior art reference may be relied upon in the rejection of claims under 35 U.S.C. 103. While it is acknowledged that the cited prior art does not teach or suggest that Factor C of Ding recombinantly produced in CHO DG44 cells of Current Protocols and HEK293 cells of Thomas exhibits an improvement in salt tolerance, as noted above, these properties are inherent to Factor C produced in CHO DG44 cells and HEK293 cells.

The applicant further argues the unexpected results are commensurate with the amended claims.

The applicant's argument is not found persuasive. As noted above, in view of the evidence of the Mizumura declaration, the examiner acknowledges an unexpected result for *T. tridentatus* Factor C polypeptides recombinantly expressed using CHO DG44 cells and HEK293 cells. However, the unexpected result does not rebut a *prima facie* case of obviousness because the applicant's result is not commensurate in scope with the claimed invention. The unexpected result of the Mizumura declaration is based on *T. tridentatus* Factor C polypeptides recombinantly expressed CHO DG44 cells and *HEK293* cells, however, the claims generally recite a Human Embryonic Kidney cell. Given the specification's disclosure that "Examples of HEK include HEK293" (p. 45, middle), the recitation of "Human Embryonic Kidney (HEK)" is interpreted as encompassing *any* HEK cell. As supported by the Mizumura declaration's differing results obtained with CHO DG44 cells and CHO-S cells, one would not expect the applicant's result achieved with a *T. tridentatus* Factor C polypeptide recombinantly expressed using a HEK293 cell to extend to all HEK cells. Also, the "Inhibition data"

(citation 15 of the IDS filed on February 3, 2021) shows that a *Limulus* Factor C recombinantly expressed using a HEK293 cell line has (α-2,3)-linked terminal sialic acid (p. 3, Figure 2) but does not exhibit increased salt tolerance (p. 4, Figure 4; p. 5, Figure 6). According to "Inhibition data", the presence of (α-2,3)-linked terminal sialic acid has no effect on the residual activity of *Limulus* Factor C (p. 6). Thus, one would not expect the applicant's result achieved with *T. tridentatus* Factor C polypeptides recombinantly expressed using CHO DG44 and HEK293 cells to extend beyond *T. tridentatus* Factor C polypeptide to other Factor C polypeptides as recited in the claims.

For these reasons, it is the examiner's position that the claimed invention would have been *prima facie* obvious to one of ordinary skill in the art before the effective filing date.

Conclusion

Status of the claims:

Claims 1-9 and 12-23 are pending.

Claims 14-18 are withdrawn from consideration.

Claims 1-9, 12, 13, and 19-23 are rejected.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to DAVID J STEADMAN whose telephone number is (571)272-0942. The examiner can normally be reached on Monday to Friday, 7:30 AM to 4:00 PM.

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, MANJUNATH N. RAO can be reached on 571-272-0939. 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.

/David Steadman/
Primary Examiner, Art Unit 1656

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/399,386	MIZUMURA et al.
	Examiner	Art Unit
	DAVID J STEADMAN	1656

CPC - Searched*		
Symbol	**Date**	**Examiner**
C12N9/6408, See attached EAST search history printout	10/02/2020	/DJS/
C12N9/6408, C12Y304/21084, see attached EAST search history printout	03/04/2021	/DJS/

CPC Combination Sets - Searched*		
Symbol	**Date**	**Examiner**
NONE		

US Classification - Searched*			
Class	**Subclass**	**Date**	**Examiner**
NONE			

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/399,386	MIZUMURA et al.
	Examiner	Art Unit
	DAVID J STEADMAN	1656

Search Notes		
Search Notes	**Date**	**Examiner**
PALM inventor name search, all inventors searched	06/24/2020	/DJS/
EAST inventor name and assignee search, all inventors searched	06/24/2020	/DJS/
Reviewed prosecution history for applications 14/650,767, 15/983,725, and 16/399575	06/24/2020	/DJS/
STIC search of SEQ ID NO: 2 and 4 against amino acid and nucleic acid prior and interference databases, see SCORE for search results	06/24/2020	/DJS/
EAST, STN database keyword search, see attached search history printouts for search strategy and databases searched	06/24/2020	/DJS/
PALM inventor name search and EAST inventor name and assignee search, all inventors searched	10/02/2020	/DJS/
PALM inventor name search and EAST inventor name and assignee search, all inventors searched	03/04/2021	/DJS/
Updated EAST, STN database keyword search, see attached search history printouts for search strategy and databases searched	03/04/2021	/DJS/

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S5	7,687	(horseshoe adj crab) OR limulus	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:46
S6	13,415	factor adj "c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S7	13,415	"factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S8	335	S5 same S6	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S9	212	S8 and recombinant	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S10	89	S8 same recombinant	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S11	26	S10 and mammalian	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:48
S12	9	S10 and ((chinese adj hamster) OR CHO)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:02

EAST Search History (Prior Art)

S13	2	S10 and (hek OR (embryonic adj kidney))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:02
S14	6	S10 and (hek$ OR (embryonic adj kidney))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:03
S15	4	S14 not S13	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:03
S16	2	"6,645,724".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:04
S17	12	"2012118226"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:35
S18	4	"5716834".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 15:58
S19	2,454	seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:23
S20	18,057	(mizumura-h$ OR oda-t$ OR kawabata-s$). in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:23

EAST Search History (Prior Art)

S21	20,466	S19 or S20	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:25
S22	91	S21 and (horseshoe adj crab)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:25
S23	66	S21 and tridentatus	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:25
S24	41	S21 and rotundicauda	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:26
S25	108	S22 or S23 or S24	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:26
S26	40	S25 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:26
S27	23	("5,858,706" OR "5,712,144" OR "5,795,962" OR "6,077,946" OR "20090208995" OR "20030054432" OR "20040235080" OR "20140024062").pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 11:34
S28	4	"5,716,834".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/05 18:17

EAST Search History (Prior Art)

S29	4	"5,858,706".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 11:27
S30	2	"6,645,724".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 15:42
S31	2	"20140249082".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 16:45
S32	2,474	seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:07
S33	18,751	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:07
S34	21,180	S32 or S33	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:07
S35	19	S33 AND (tridentatus OR polyphemus OR rotundicauda OR gigas)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:08
S36	13	S35 and "factor C"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:08

EAST Search History (Prior Art)

S37	2	S36 and (sialic OR sialyl$)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:08
S38	14,664	"factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:53
S39	218	S38 and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:53
S40	7	S38 same (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:54
S41	218	S39 and (cho OR "chinese hamster ovary")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:54
S42	805	"factor c".clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:54
S43	2	S42 and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:55
S44	5,676	(cho OR "chinese hamster ovary") same (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:55

EAST Search History (Prior Art)

S45	211	S44 and S38	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:55
S47	7	S45 and horseshoe	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:56
S48	20	horseshoe and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:45
S49	218	"factor c" and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:47
S50	211	S49 not S48	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:47
S51	0	S50 and horseshoe	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:48
S52	2	"factor c".clm. and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:48
S53	6	"factor c".ti. and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:49

EAST Search History (Prior Art)

S54	7	"factor c".ab. and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:49
S55	1	S54 not S53	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:49
S56	2	"9133495".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:36
S57	19,132	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45
S58	6	S57 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45
S59	2,485	seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45
S60	3	S59 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45
S61	7	S58 or S60	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45

EAST Search History (Prior Art)

S62	19,414	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S63	6	S62 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S64	2,493	seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S65	3	S64 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S66	7	S63 or S65	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S67	19,635	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/08/14 09:19
S68	6	S67 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/08/14 09:19
S80	3	("20080131929"|"20100086967"|"20130295613").PN.	US-PGPUB; USPAT	OR	OFF	2018/09/07 10:58
S81	19,653	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/09/07 11:43

EAST Search History (Prior Art)

S82	6	S81 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/09/07 11:43
S94	6	"10144923".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/10/01 09:23
S96	6	(US-20080131929-$ | US-20100086967-$ | US-20130295613-$).DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/10/01 10:53
S97	20,399	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/10/02 15:15
S98	40	S97 AND (tridentatus OR polyphemus OR rotundicauda OR gigas)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/10/02 15:15
S99	23,093	(mizumura-h$ OR oda-t$ OR kawabata-s$).in. OR seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/02/07 10:15
S100	95	S99 AND (tridentatus OR polyphemus OR rotundicauda OR gigas)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/02/07 10:16
S101	54	S100 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/02/07 10:16

EAST Search History (Prior Art)

S102	24	S101 and ("chinese hamster" OR CHO OR human OR HEK)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/02/07 10:16
S103	627	tridentatus OR rotundicauda	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S104	389	S103 and endotoxin	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S105	263	S104 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S106	224	S103 same "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S107	216	S105 and S106	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S108	140	S107 and "37"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:42
S109	1,050,017	"2 hours"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:42

EAST Search History (Prior Art)

S110	114	S108 and S109	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:42
S113	349,173	cascade	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:44
S114	169	S107 and S113	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:45
S115	468	limulus adj test	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 15:30
S116	1	S115 same cation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 15:31
S117	3	"6,645,724".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 12:19
S118	13	("20030054432"|"20040235080"|"20080131929"|"20090208995"|"20100086967"|"20130295613"|"20140024062"|"20140249082"|"5712144"|"5716834"|"5795962"|"5858706"|"6077946").PN.	US-PGPUB; USPAT	OR	OFF	2020/04/22 14:32
S119	23,218	(mizumura-h$ OR oda-t$ OR kawabata-s$).in. OR seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 14:37
S120	85	S119 AND (tridentatus OR rotundicauda)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 14:37

EAST Search History (Prior Art)

S121	54	S120 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 14:38
S122	33	S121 and endotoxin.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 14:39
S123	23,351	(mizumura-h$ OR oda-t$ OR kawabata-s$).in. OR seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:10
S124	95	S123 AND (tridentatus OR rotundicauda OR polyphemus)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:10
S125	55	S124 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:11
S126	10	S125 and (cho OR "chinese hamster")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:11
S127	8	S125 and (hek OR (human adj embryo$))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:12
S128	10	S126 or S127	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:12

EAST Search History (Prior Art)

S129	23,458	(mizumura-h$ OR oda-t$ OR kawabata-s$). in. OR seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
S130	98	S129 AND (tridentatus OR rotundicauda OR polyphemus)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
S131	57	S130 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
S132	11	S131 and (cho OR "chinese hamster")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
S133	9	S131 and (hek OR (human adj embryo$))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
S134	11	S132 or S133	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
S135	753	c12n9/6408.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:52
S136	44	S135 and (tridentatus OR polyphemus OR rotundicauda)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:53

EAST Search History (Prior Art)

S137	829,430	(cho OR "chinese hamster")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:53
S138	104,016	(hek OR (human adj embryo$))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:53
S139	14	S136 and (S137 OR S138)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:53
S140	4	"6645724".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 04:49
S141	787	c12n9/6408.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 04:49
S142	46	S141 and (tridentatus OR polyphemus OR rotundicauda)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 04:49
S143	854,970	(cho OR "chinese hamster")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 04:49
S144	107,501	(hek OR (human adj embryo$))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 04:49

EAST Search History (Prior Art)

S145	787	c12n9/6408.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 06:20
S146	46	S145 and (tridentatus OR polyphemus OR rotundicauda)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 06:20
S147	854,970	(cho OR "chinese hamster")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 06:20
S148	107,501	(hek OR (human adj embryo$))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 06:20
S149	15	S146 and (S147 OR S148)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 06:20
S150	64	C12Y304/21084.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 06:26
S151	24	S150 and (S147 OR S148)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2021/03/04 06:26

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S69	2,075	(dg44 OR dg-44 OR chodg44 OR cho-dg44)	USPAT	OR	OFF	2018/08/14 09:27
S70	5,851	"factor c"	USPAT	OR	OFF	2018/08/14 09:27

EAST Search History (Interference)

S71	92	S69 and S70	USPAT	OR	OFF	2018/08/14 09:27
S72	17,574	horseshoe OR crab	USPAT	OR	OFF	2018/08/14 09:28
S73	2	S71 and S72	USPAT	OR	OFF	2018/08/14 09:28
S74	40	(dg44 OR dg-44 OR chodg44 OR cho-dg44).clm.	USPAT	OR	OFF	2018/08/14 09:30
S75	380	"factor c".clm.	USPAT	OR	OFF	2018/08/14 09:30
S76	0	S74 and S75	USPAT	OR	OFF	2018/08/14 09:30
S77	2,254	(horseshoe OR crab).clm.	USPAT	OR	OFF	2018/08/14 09:31
S78	0	S74 and S77	USPAT	OR	OFF	2018/08/14 09:31
S79	0	(dg44 OR dg-44 OR chodg44 OR cho-dg44) same "factor c"	USPAT	OR	OFF	2018/08/14 09:31
S83	2,086	(dg44 OR dg-44 OR chodg44 OR cho-dg44)	USPAT	OR	OFF	2018/09/07 11:44
S84	5,873	"factor c"	USPAT	OR	OFF	2018/09/07 11:44
S85	92	S83 and S84	USPAT	OR	OFF	2018/09/07 11:44
S86	17,635	horseshoe OR crab	USPAT	OR	OFF	2018/09/07 11:44
S87	2	S85 and S86	USPAT	OR	OFF	2018/09/07 11:44
S88	41	(dg44 OR dg-44 OR chodg44 OR cho-dg44).clm.	USPAT	OR	OFF	2018/09/07 11:44
S89	383	"factor c".clm.	USPAT	OR	OFF	2018/09/07 11:44
S90	0	S88 and S89	USPAT	OR	OFF	2018/09/07 11:44
S91	2,260	(horseshoe OR crab).clm.	USPAT	OR	OFF	2018/09/07 11:44
S92	0	S88 and S91	USPAT	OR	OFF	2018/09/07 11:44
S93	0	(dg44 OR dg-44 OR chodg44 OR cho-dg44) same "factor c"	USPAT	OR	OFF	2018/09/07 11:44

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                D CLUSTER
                D CLUSTER .DJS
                D SET

     FILE 'REGISTRY' ENTERED AT 07:06:03 ON 04 MAR 2021
L1              1 SEA 115743-27-6/RN
                D L1

     FILE 'MEDLINE, AGRICOLA, CAPLUS, BIOSIS, EMBASE, SCISEARCH' ENTERED AT
     07:06:35 ON 04 MAR 2021
L2              4 SEA L1
L3          80292 SEA "FACTOR C"
L4          13174 SEA (TRIDENTATUS OR POLYPHEMUS OR ROTUNDICAUDA)
L5          28800 SEA (TACHYPLEUS OR LIMULUS OR CARCINOSCORPIUS)
L6          33232 SEA L4 OR L5
L7          80292 SEA L2 OR L3
L8            660 SEA L6 AND L7
L9         731383 SEA CHO OR "CHINESE HAMSTER" OR HEK OR HEK293 OR "HUMAN
                  EMBRYONIC"
L10             6 SEA L8 AND L9
L11             5 DUP REM L10 (1 DUPLICATE REMOVED)
                D IBIB ABS 1-
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                    and TEMA Add Numeric Property Search
  NEWS  4   OCT  7  EP Appeal Data from European Patent Register Added to
                    INPADOCDB and INPAFAMDB
  NEWS  5   NOV 15  Enhanced INPADOCDB and INPAFAMDB Files
  NEWS  6   NOV 23  Claims Access in CA/CAplus: WIPO, United States, and China
                    for 1999 - Present.
  NEWS  7   DEC  1  Engineering Flagship Database Inspec Reaches
                    Milestone 20 Million Total Records
  NEWS  8   DEC  1  Full-Text Patent Documents from German Democratic
                    Republic Added to DEFULL
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=> s 115743-27-6/rn

L1           1 115743-27-6/RN

=> d l1
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L1 1 of 1

115743-27-6

CAS Registry Number: 115743-27-6 REGISTRY

Entered STN: 13 Aug 1988

CAS Registry Number Locator: STN Files: CA, CAPLUS, CHEMCATS, TOXCENTER

*** STRUCTURE DIAGRAM IS NOT AVAILABLE ***

*** USE 'SQD' OR 'SQIDE' FORMATS TO DISPLAY SEQUENCE ***

Chemical Name:	**Hemolymph-coagulation factor, C, activated (Tachypleus tridentatus hemocyte A-chain reduced) (9CI) (CA INDEX NAME)**	Hemolymph-coagulation factor C (Carcinoscorpius rotundicauda clone CrFC21 light chain A)
	OTHER NAMES:	Hemolymph-coagulation factor C (Carcinoscorpius rotundicauda clone CrFC26 light chain A)
Class Identifier:	PMS, MAN	
File Segment:	PROTEIN SEQUENCE	
Molecular Formula:	C335 H527 N105 O108 S4	
Source of Registration:	CA	

4 REFERENCES IN FILE CA (1907 TO DATE)

4 REFERENCES IN FILE CAPLUS (1907 TO DATE)

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=> file .djs scisearch

FILE 'WPIDS' ACCESS NOT AUTHORIZED
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                                              ENTRY           SESSION
FULL ESTIMATED COST                              6.59             6.80

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=> s l1

'RN' IS NOT A VALID FIELD CODE
'RN' IS NOT A VALID FIELD CODE
L2            4 L1

=> s "factor c"

L3        80292 "FACTOR C"

=> s (tridentatus OR polyphemus OR rotundicauda)

L4        13174 (TRIDENTATUS OR POLYPHEMUS OR ROTUNDICAUDA)

=> s (Tachypleus OR Limulus OR Carcinoscorpius)

L5        28800 (TACHYPLEUS OR LIMULUS OR CARCINOSCORPIUS)
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=> d his

     (FILE 'HOME' ENTERED AT 07:05:51 ON 04 MAR 2021)

     FILE 'REGISTRY' ENTERED AT 07:06:03 ON 04 MAR 2021
L1             1 S 115743-27-6/RN

     FILE 'MEDLINE, AGRICOLA, CAPLUS, BIOSIS, EMBASE, SCISEARCH' ENTERED AT
     07:06:35 ON 04 MAR 2021
L2             4 S L1
L3         80292 S "FACTOR C"
L4         13174 S (TRIDENTATUS OR POLYPHEMUS OR ROTUNDICAUDA)
L5         28800 S (TACHYPLEUS OR LIMULUS OR CARCINOSCORPIUS)

=> s l4 or l5

L6         33232 L4 OR L5

=> s l2 or l3

L7         80292 L2 OR L3

=> s l6 and l7

L8           660 L6 AND L7

=> s cho OR "chinese hamster" OR HEK OR HEK293 OR "human embryonic"

L9        731383 CHO OR "CHINESE HAMSTER" OR HEK OR HEK293 OR "HUMAN EMBRYONIC"

=> s l8 and l9

L10            6 L8 AND L9

=> dup rem l10

PROCESSING COMPLETED FOR L10
L11            5 DUP REM L10 (1 DUPLICATE REMOVED)

=> d ibib abs 1-
YOU HAVE REQUESTED DATA FROM 5 ANSWERS - CONTINUE? Y/(N):y


L11  ANSWER 1 OF 5     MEDLINE ®  on STN
ACCESSION NUMBER:    2019937754      MEDLINE Full-text
DOCUMENT NUMBER:     PubMed ID: 29866883
TITLE:               Intermolecular autocatalytic activation of serine protease
                     zymogen factor C through an active transition state
                     responding to lipopolysaccharide.
AUTHOR:              Shibata Toshio; Kobayashi Yuki; Kawabata Shun-Ichiro
AUTHOR IDENTIFIERS:  ORCID: https://orcid.org/0000-0003-0211-5525
CORPORATE SOURCE:    From the Department of Biology, Faculty of Science and.
                     skawascb@kyudai.jp
AUTHOR:              Shibata Toshio; Ikeda Yuto; Kawabata Shun-Ichiro
AUTHOR IDENTIFIERS:  ORCID: https://orcid.org/0000-0003-0211-5525
CORPORATE SOURCE:    the Graduate School of Systems Life Sciences, Kyushu
                     University, Fukuoka 819-0395, Japan. skawascb@kyudai.jp
SOURCE:              The Journal of biological chemistry, (20180720) Vol. 293,
                     No. 29, pp. 11589-11599.  Electronic Publication Date: 4
                     Jun 2018
                     Journal code: 2985121R. E-ISSN: 1083-351X. L-ISSN:
                     0021-9258.
                     Report No.: PMC-PMC6065167.
DIGITAL OBJECT ID:   http://dx.doi.org/10.1074/jbc.RA118.002311
PUB. COUNTRY:        United States
DOCUMENT TYPE:       Journal; Article; (JOURNAL ARTICLE)
                     (RESEARCH SUPPORT, NON-U.S. GOV'T)
LANGUAGE:            English
FILE SEGMENT:        MEDLINE; Priority Journals
FILE SEGMENT:        Print; Electronic
ENTRY MONTH:         201902
ENTRY DATE:          Entered STN: 5 Jun 2018
                     Last Updated on STN: 5 Feb 2019
                     Indexing Added: 5 Feb 2019
                     Entered Medline: 4 Feb 2019
OS.CITING REF COUNT: 5    There are 5 MEDLINE records that cite this record
MEDLINE REFERENCE COUNT:   38    There are 38 cited references available in
                                 MEDLINE for this document.
AB   Horseshoe crab hemolymph coagulation is believed to be triggered by the
     autocatalytic activation of serine protease zymogen factor C to the
     active form, α-factor C, belonging to the trypsin family,
     through an active transition state of factor C responding to bacterial
     lipopolysaccharide (LPS), designated factor C*.  However, the
     existence of factor C* is only speculative, and its proteolytic
     activity has not been validated.  In addition, it remains unclear whether
     the proteolytic cleavage of the Phe(737)-Ile(738) bond (Phe(737) site) of
     factor C required for the conversion to α-factor C occurs
     intramolecularly or intermolecularly between the factor C molecules.
     Here we show that the Phe(737) site of a catalytic Ser-deficient mutant of
```

factor C is LPS-dependently hydrolyzed by a Phe(737) site-uncleavable mutant, clearly indicating the existence of the active transition state of factor C without cleavage of the Phe(737) site. Moreover, we found the following facts using several mutants of factor C: the autocatalytic cleavage of factor C occurs intermolecularly between factor C* molecules on the LPS surface; factor C* does not exhibit intrinsic chymotryptic activity against the Phe(737) site, but it may recognize a three-dimensional structure around the cleavage site; and LPS is required not only to complete the substrate-binding site and oxyanion hole of factor C* by interacting with the N-terminal region but also to allow the Phe(737) site to be cleaved by inducing a conformational change around the Phe(737) site or by acting as a scaffold to induce specific protein-protein interactions between factor C* molecules.
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L11 ANSWER 2 OF 5 MEDLINE ® on STN DUPLICATE 1
ACCESSION NUMBER: 2018386384 MEDLINE Full-text
DOCUMENT NUMBER: PubMed ID: 28317862
TITLE: Biological Activity of Masked Endotoxin.
AUTHOR: Schwarz Harald; Gornicec Jan; Neuper Theresa; Parigiani Maria Alejandra; Wallner Michael; Duschl Albert; Horejs-Hoeck Jutta
CORPORATE SOURCE: Department of Molecular Biology, University of Salzburg, Salzburg, Austria.
SOURCE: Scientific reports, (20170320) Vol. 7, pp. 44750. Electronic Publication Date: 20 Mar 2017 Journal code: 101563288. E-ISSN: 2045-2322. L-ISSN: 2045-2322. Report No.: PMC-PMC5357793.
DIGITAL OBJECT ID: http://dx.doi.org/10.1038/srep44750
PUB. COUNTRY: England: United Kingdom
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE) (RESEARCH SUPPORT, NON-U.S. GOV'T)
LANGUAGE: English
FILE SEGMENT: MEDLINE; Priority Journals
FILE SEGMENT: Electronic
ENTRY MONTH: 201811
ENTRY DATE: Entered STN: 20 Mar 2017 Last Updated on STN: 11 Nov 2018 Indexing Added: 11 Nov 2018 Entered Medline: 9 Nov 2018
OS.CITING REF COUNT: 11 There are 11 MEDLINE records that cite this record
MEDLINE REFERENCE COUNT: 18 There are 18 cited references available in MEDLINE for this document.

AB Low endotoxin recovery (LER) is a recently discovered phenomenon describing the inability of limulus amebocyte lysate (LAL)-based assays to detect lipopolysaccharide (LPS) because of a "masking effect" caused by chelators or detergents commonly used in buffer formulations for medical products and recombinant proteins. This study investigates the masking capacities of different buffer formulations and whether masked endotoxin is biologically active. We show that both naturally occurring endotoxin as well as control standard endotoxin can be affected by LER. Furthermore, whereas masked endotoxin cannot be detected in Factor C based assays, it is still detectable in a cell-based TLR4-NF-κB-luciferase reporter gene assay. Moreover, in primary human monocytes, masked LPS induces the expression of pro-inflammatory cytokines and surface activation markers even at very low concentrations. We therefore conclude that masked LPS is a potent trigger of immune responses, which emphasizes the potential danger of masked LPS, as it may pose a health threat in pharmaceutical products or compromise experimental results.

L11 Patent | Japanese 3 of 5

Novel recombinant factor C and method for producing same, and method for measuring endotoxin

Full Text

Inventor Name:	Mizumura, Hikaru; Oda, Toshio; Kawabata, Shun-Ichiro
Patent Assignee:	Seikagaku Corporation, Japan
Accession Number:	2014:999086 CAPLUS
Document Number (CA Abstract Number):	161:65883
Source:	PCT Int. Appl., 79pp. CODEN: PIXXD2
Family Accession Number Count:	1

Provided is a method for producing horseshoe crab recombinant factor C. The horseshoe

crab recombinant factor C can be produced by expressing the horseshoe crab factor C using a mammalian cell such as CHO DG44 and HEK293 as a host cell.

Patent Information Table:

PATENT NO.
WO 2014092079
CN 104919046
EP 2930241
EP 2930241
SG 10201704774
CN 108646019
CN 108642030
EP 3441466
EP 3441466
JP 6511685
ES 2738593
EP 3663400
ES 2784510
US 20150307864
US 10144923
US 20180258414
JP 2019000122
JP 6626174
US 20190256835
US 20190270977

Priority Application Information:

JP 2012-269840
CN 2013-80064649
EP 2013-862466
EP 2018-190686
JP 2014-552048
WO 2013-JP83082
US 2015-14650767
US 2018-15983725

Patent Status Patent Information:

PATENT NO.	KIND
WO 2014092079	A1

PATENT NO.	KIND	
CN 104919046	A	
EP 2930241	A1	
EP 2930241	B1	
CN 108646019	A	
CN 108642030	A	
EP 3441466	A1	
EP 3441466	B1	
JP 6511685	B2	
ES 2738593	T3	
EP 3663400	A1	
ES 2784510	T3	
US 20150307864	A1	
US 10144923	B2	
US 20180258414	A1	
JP 2019000122	A	
JP 6626174	B2	
US 20190256835	A1	
US 20190270977	A1	

Cited References Count:

8 THERE ARE 8 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

Citing Reference Count:

1 THERE ARE 1 CAPLUS RECORDS THAT CITE THIS RECORD (1 CITINGS)

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT

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L11 ANSWER 4 OF 5 BIOSIS COPYRIGHT (c) 2021 Clarivate Analytics on STN
ACCESSION NUMBER: 2007:224924 BIOSIS Full-text
DOCUMENT NUMBER: PREV200700226988
TITLE: A structural perspective on the interaction between lipopolysaccharide and Factor C, a receptor involved in recognition of Gram-negative bacteria.
AUTHOR(S): Koshiba, Takumi; Hashii, Tornoyuki; Kawabata, Shun-ichiro [Reprint Author]
CORPORATE SOURCE: Kyushu Univ, Fac Sci, Dept Biol, Higashi Ku, 6-10-1 Hakozaki, Fukuoka 8128581, Japan
skawascb@mbox.nc.kyushu-u.ac.jp
SOURCE: Journal of Biological Chemistry, (FEB 9 2007) Vol. 282, No. 6, pp. 3962-3967. http://www.jbc.org/content/by/year.
E-ISSN: 1083-351X.
DIGITAL OBJECT ID: 10.1074/jbc.M609198200
DOCUMENT TYPE: Article
LANGUAGE: English
ENTRY DATE: Entered STN: 4 Apr 2007
Last Updated on STN: 14 Aug 2019

AB The recognition of broadly conserved microorganism components known as pathogen-associated molecular patterns is an essential step in initiating the innate immune response. In the horseshoe crab, stimulation of hemocytes with lipopolysaccharide (LPS) causes the activation of its innate immune response, and Factor C, a serine protease zymogen, plays an important role in this event. Here, we report that Factor C associates with LPS on the hemocyte surface and directly recognizes

Gram-negative bacteria. Structure-function analyses reveal that the LPS binding site is present in the N-terminal cysteine-rich (Cys-rich) region of the molecule and that it contains a tripeptide sequence consisting of an aromatic residue flanked by two basic residues that is conserved in other mammalian LPS-recognizing proteins. Moreover, we have demonstrated that the Cys-rich region specifically binds to LPS on Gram-negative bacteria and that mutations in the tripeptide motif abrogate its association with both LPS and Gram-negative bacteria, underscoring the importance of the tripeptide in LPS interaction. Although the innate immune response to LPS in the horseshoe crab is distinct from that of mammals, it appears to rely on structural features that are conserved among LPS-recognizing proteins from diverse species.

L11 Patent | Japanese **5** of 5

Mouse extracellular matrix proteins, cDNA cloning, and use as cell adhesion agent

Full Text

Inventor Name:	Sekiguchi, Kiyotoshi; Manabe, Riichiro; Hayashizaki, Yoshihide; Kamiya, Mamoru; Furuya, Hiroshi; Tsutsui, Aogu
Patent Assignee:	Japan Science and Technology Corporation, Japan; Institute of Physical and Chemical Research; DNA Form Co., Ltd.
Accession Number:	2004:32343 CAPLUS
Document Number (CA Abstract Number):	140:106529
Source:	Jpn. Kokai Tokkyo Koho, 86 pp. CODEN: JKXXAF
Family Accession Number Count:	1

Novel extracellular matrix proteins of mouse origin, encoding cDNAs, recombinant expression, antibodies against them, and use as cell adhesion agent, are disclosed. Genes coding for those extracellular matrix proteins were designated 33145, 40240, 51140, and 31418. Besides secretion signal sequence, 33145 protein had SCP (sperm-coating glycoprotein) domain, LCCL (Limulus factor C, Coch-5b2 and Lgl1) domain, and a potential N-glycosylation site. Homol. with Cocoa Crisp (cysteine-rich secreted protein), late gestation lung protein, protease inhibitor 15 preproprotein, 25 kDa trypsin inhibitor and glioma pathogenesis-related protein (RTVP-1 protein) was found. 40420 Protein had 2 potential N-glycosylation sites, and showed homol. with human FLJ23191 gene and mouse BB613765, AV243621, AK017759, and AK020942 genes. 51140 Protein has a thrombospondin type 1 repeats (TSP-1) domain, a potential N-glycosylation site, and a cysteine rich region homologous to that of PACE4 protein (Furin-like protease serine protease), and showed homol. with thrombospondin. 31418 Protein has four tandem repeat sequences, and a sequence homologous to bovine prion protein heparin-binding domain, and showed homol. with monkey Taste Bud-specific protein. The proteins were recombinantly expressed as fusion protein with EGFP-His-myc tag in human 293T cells. Localization in extracellular matrix was detected. Cell adhesion and growth activities were found. Prodn. of anti-40240 antibodies is described.

Patent Information Table:

PATENT NO.
JP 2004008021

Priority Application Information:

JP 2002-162211

Patent Status Patent Information:

PATENT NO.	KIND
JP 2004008021	A

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16/399,386
Confirmation Number	9293
Filing Date	April 30, 2019
First Named Inventor	Hikaru MIZUMURA
Art Unit	1656
Examiner Name	STEADMAN, DAVID J
Attorney Docket Number	Q246419

U.S. PATENTS

Examiner Initials*	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.	6645724	B1	2003-11-11	Ding et al.	

U.S. PATENT APPLICATION PUBLICATIONS

Examiner Initials*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No	Foreign Document Number[3]	Country Code[2]	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]

NON-PATENT LITERATURE DOCUMENTS

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, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	2.	Communication of Notice of Opposition dated November 11, 2020, issued by the European Patent Office in EP Application no. 18190686.8	
	3.	A certified translation of the priority document JP 2012269840 filed December 10, 2012	
	4.	CARLESSO, E. et al., "The rule regulating pH changes during crystalloid infusion", Intensive Care Med., 2011, 37(3): 461-468	
	5.	The Wikipedia site relating to "Blood Plasma" as of Nov. 19, 2012, provided by the WayBack Machine	
	6.	The Wikipedia site relating to "Horseshoe Crab" as of Nov. 23, 2011, provided by the WayBack Machine	
	7.	HARADA-SUZUKI, T. et al., "Further Studies on the Chromogenic Substrate Assay Method for Bacterial Endotoxins Using Horseshoe Crab (Tachypleus tridentatus) Hemocyte Lysate", J. Biochem., 1982, 92(3): 793-800	
	8.	TOMIYA, N. et al., "Comparing N-glycan processing in mammalian cell lines to native and engineered lepidopteran insect cell lines", Glycoconjugate Journal, 2004, 21:343-360	
	9.	NETTLESHIP, JOANNE E., "Structural Biology of Glycoproteins", Glycosylation, 2012, p. 41-62. Available from: https://www.intechopen.com/books/glycosylation/structural- biologyof- glycoproteins	
	10.	THOMAS, P. AND SMART, T.G., "HEK293 cell line: A vehicle for the expression of recombinant proteins", J. Pharmacol. And Toxicol. Methods, 2005, 51: 187-200	
	11.	Declaration by Dr. Hikaru Mizumura submitted to the USPTO, signed Oct. 11, 2017 in US14/650767	
	12.	Excerpt from Essential Cell Biology (2nd Edition), Alberts et al., Taylor and Francis Group, New York and London, 2004 (page 209)	
	13.	HOSSLER, P. et al., "Optimal and consistent protein glycosylation in mammalian cell culture", Gycobiol. 19(9): 936-949, 2009	
	14.	Declaration by Dr. Hikaru Mizumura submitted to the USPTO, signed Feb. 22, 2018 in US14/650767	

*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. /DJS/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16/399,386
	Confirmation Number	9293
	Filing Date	April 30, 2019
	First Named Inventor	Hikaru MIZUMURA
	Art Unit	1656
	Examiner Name	STEADMAN, DAVID J
	Attorney Docket Number	Q246419

NON-PATENT LITERATURE DOCUMENTS			
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, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	15.	Inhibition data performed using a Limulus Factor C expressed in HEK 293 cells and HEK 293 GnTI- cells filed with Opposition against EP3441466B dated November 5, 2020	

EXAMINER SIGNATURE			
Examiner Signature	/David Steadman/	Date Considered	03/04/2021

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	Art Unit	1656
	Examiner Name	STEADMAN, DAVID J
	Attorney Docket Number	Q246419

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.

☑ The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

☑ A certification statement is not submitted herewith.

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23373
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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	/Hui C. Wauters/	Date (YYYY-MM-DD)	2021-02-03
Name/Print	Hui C. Wauters	Registration Number	57,426

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[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. /DJS/



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Sale Item Reference Number	Effective Date
16399386	02/03/2021

Document Number	Fee Code	Fee Code Description	Amount Paid	Payment Method
I202124G07511552	1202	CLAIMS IN EXCESS OF 20	$100.00	Deposit Account

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 16/399,386	Filing Date 04/30/2019	☐ 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

AMENDMENT	02/03/2021	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 21	Minus	** 20	= 1		x $100 =	100
	Independent (37 CFR 1.16(h))	* 2	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	100

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) 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".	/CAROL A BARNES/
*** 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.

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Docket No: Q246419
Hikaru MIZUMURA, et al.	
Appln. No.: 16/399,386	Group Art Unit: 1656
Confirmation No.: 9293	Examiner: STEADMAN, DAVID J
Filed: April 30, 2019	

For: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

DECLARATION UNDER 37 C.F.R. § 1.132

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

Sir:

I, Dr. Hikaru MIZUMURA, hereby declare and state:

I am a citizen of Japan;

I have received the degrees of Bachelor of Science in 1993, Master of Science in 1996, and Doctor of Philosophy (Ph. D.) in 1999 from Saitama University;

I was employed by RIKEN (the Institute of Physical and Chemical Research) from April 1, 1999 to March 31, 2002 as Special Postdoctoral Researcher of Laboratory of Cellular and Molecular Biology, with responsibility for research on endonuclease from yeast mitochondria;

I was employed by Tokai University School of Medicine from April 1, 2002 to March 31, 2005 as Postdoctoral Researcher of Department of Molecular Neuroscience, with responsibility for research on biochemical characterization of ALS2 protein;

I was employed by The University of Tokyo from April 1, 2005 to August 31, 2007 as Postdoctoral Researcher of Laboratory of Microbiology, with responsibility for research on DNA replication in virulence bacteria Staphylococcus aureus;

I have been employed by Seikagaku Corporation since September 1, 2007, where I hold a position as chief researcher of LAL Research Department, Central Research Laboratories, with responsibility for research and development of reagents for measuring endotoxin and/or glucan by using horseshoe crab proteins; and

I am the first-named inventor of the above-identified application; I am familiar with the subject matter of the above-identified application and the Office Action mailed October 8, 2020 in the above-identified application.

This Declaration is submitted to explain and demonstrate that our invention provides unexpected superior results over Ding et al. (US 5,858,706; hereafter "Ding") in view of Current Protocols in Molecular Biology (1993) 16.23.1-16.23.13, John Wiley and Sons, Inc., 2002 (hereafter "Current Protocols") and as evidenced by Mizumura et al. (WO 2012/118226 A1; hereafter "Mizumura"), and thus patentability of our invention over the cited references.

The following additional experiments were conducted by me or under my direct supervision.

Recombinant Factor C was prepared by use of ExpiCHO-S (product of Thermo Fisher Scientific), which is a Chinese hamster ovary cell line, as a host through the following procedure.

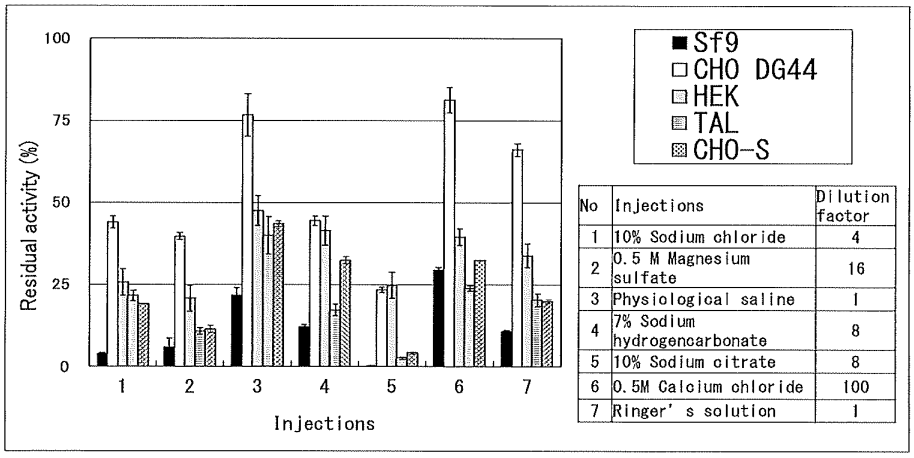
The same Factor C expression plasmid as mentioned in the paragraph [0108] of the specification was used in the following experiments. As described in paragraph [0108] of the

specification, DNA encoding Factor C of Japanese horseshoe crab, *Tachypleus tridentatus*, was inserted between the *Eco*RI recognition site and the *Xba*I recognition site of pCI-neo (product of Promega), which is a vector for expression in mammalian cells, to thereby construct a Factor C expression plasmid. A kozak sequence (GCCACC) was added just before the start codon of Factor C.

The Factor C expression plasmid was introduced into suspension-cultured ExpiCHO-S cells through transfection using ExpiFectamine CHO Reagent (product of Thermo Fisher Scientific). The transfected ExpiCHO-S cells were cultured in ExpiCHO Expression Medium (product of Thermo Fisher Scientific) at 37°C under feeding of 8% CO_2 without replacement of the medium. Eighteen hours after transfection, three-tenth volume of ExpiCHO Feed (product of Thermo Fisher Scientific) was added to the culture broth. Six days after transfection, the culture broth was recovered, and was centrifuged (3,300×g, 30 minutes, 4°C), to thereby recover a culture supernatant. The supernatant was filtered through a 0.22-μm filter, and the thus-recovered fraction (i.e. filtrate) was employed as recombinant Factor C produced in CHO-S cells.

The reactivity of the recombinant Factor C expressed in CHO-S cells to endotoxin in various injections was measured by the same procedure as described in paragraphs [0136] and [0137] and Fig. 7 of the specification, except that sodium iothalamate injection was not used due to discontinuation of its sale. Figure S1 compares the reactivity of recombinant Factor C expressed in CHO DG44 with the reactivity of recombinant Factor C expressed in CHO-S, Sf9 or HEK293, or a native Factor C (TAL).

Figure S1 shows that the enzymatic activity of the recombinant Factor C expressed in CHO DG44 cells is *less* susceptible to inhibition by ions in an injection as compared to the enzymatic activity of a Factor C expressed in CHO-S cells. Furthermore, Figure S1 shows that a Factor C expressed in CHO-S cells is equally susceptible to inhibition by ions in an injection as compared to the native Factor C (TAL) and less susceptible to inhibition by many injections (6 injections except sample No.6) than the recombinant Factor C (Sf9).



No	Injections	Dilution factor
1	10% Sodium chloride	4
2	0.5 M Magnesium sulfate	16
3	Physiological saline	1
4	7% Sodium hydrogencarbonate	8
5	10% Sodium citrate	8
6	0.5M Calcium chloride	100
7	Ringer' s solution	1

Figure S1: Factor C expressed in CHO DG44 cells is *less* susceptible to ions in injections (Nos. 1-6), as compared to Factor C expressed in CHO-S cells, Sf9 cells or HEK cells or native Factor C (TAL).

Furthermore, experiments performed by Applicant show that the Factor C produced in CHO-S cells has smaller molecular weight (suggesting having lower terminal sialic acid amount) than the Factor C produced in CHO DG44 cells used in the Examples of the present

specification. Also, as stated in the specification, differences in molecular weight of Factor C expressed in different host cells such as Sf9, CHO-DG44 or HEK cells is attributable to the differences in post-translation modification (for instance, sugar chain modification) employed in each of the cells. *See* paragraph [0127] and Figure 2 of the specification. Therefore, a Factor C produced in CHO DG44 cells is *structurally different* from a Factor C produced in CHO-S, HEK and Sf9 cells.

I declare further that all statements made herein of my 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 the application or any patent issuing thereon.

Date: January 08, 2021

Hikaru Mizumura

Dr. Hikaru MIZUMURA

Electronic Acknowledgement Receipt

EFS ID:	41827916
Application Number:	16399386
International Application Number:	
Confirmation Number:	9293
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Customer Number:	23373
Filer:	Hui Chen Wauters/Sharon Jones
Filer Authorized By:	Hui Chen Wauters
Attorney Docket Number:	Q246419
Receipt Date:	03-FEB-2021
Filing Date:	30-APR-2019
Time Stamp:	16:09:56
Application Type:	Utility under 35 USC 111(a)

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	Affidavit-traversing rejectns or objectns rule 132	Q246419ExecutedDeclaration132asfiled.pdf	229365 4b6291d7407a9fafd927a7e99e54e9f003db1589	no	5

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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.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16/399,386
Confirmation Number	9293
Filing Date	April 30, 2019
First Named Inventor	Hikaru MIZUMURA
Art Unit	1656
Examiner Name	STEADMAN, DAVID J
Attorney Docket Number	Q246419

U.S. PATENTS

Examiner Initials*	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.	6645724	B1	2003-11-11	Ding et al.	

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FOREIGN PATENT DOCUMENTS

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NON-PATENT LITERATURE DOCUMENTS

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, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	2.	Communication of Notice of Opposition dated November 11, 2020, issued by the European Patent Office in EP Application no. 18190686.8	
	3.	A certified translation of the priority document JP 2012269840 filed December 10, 2012	
	4.	CARLESSO, E. et al., "The rule regulating pH changes during crystalloid infusion", Intensive Care Med., 2011, 37(3): 461-468	
	5.	The Wikipedia site relating to "Blood Plasma" as of Nov. 19, 2012, provided by the WayBack Machine	
	6.	The Wikipedia site relating to "Horseshoe Crab" as of Nov. 23, 2011, provided by the WayBack Machine	
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	9.	NETTLESHIP, JOANNE E., "Structural Biology of Glycoproteins", Glycosylation, 2012, p. 41-62. Available from: https://www.intechopen.com/books/glycosylation/structural- biologyof- glycoproteins	
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	11.	Declaration by Dr. Hikaru Mizumura submitted to the USPTO, signed Oct. 11, 2017 in US14/650767	
	12.	Excerpt from Essential Cell Biology (2nd Edition), Alberts et al., Taylor and Francis Group, New York and London, 2004 (page 209)	
	13.	HOSSLER, P. et al., "Optimal and consistent protein glycosylation in mammalian cell culture", Gycobiol. 19(9): 936-949, 2009	
	14.	Declaration by Dr. Hikaru Mizumura submitted to the USPTO, signed Feb. 22, 2018 in US14/650767	

*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	16/399,386
	Confirmation Number	9293
	Filing Date	April 30, 2019
	First Named Inventor	Hikaru MIZUMURA
	Art Unit	1656
	Examiner Name	STEADMAN, DAVID J
	Attorney Docket Number	Q246419

NON-PATENT LITERATURE DOCUMENTS			
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, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	15.	Inhibition data performed using a Limulus Factor C expressed in HEK 293 cells and HEK 293 GnTI- cells filed with Opposition against EP3441466B dated November 5, 2020	

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)		
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	Filing Date	April 30, 2019
	First Named Inventor	Hikaru MIZUMURA
	Art Unit	1656
	Examiner Name	STEADMAN, DAVID J
	Attorney Docket Number	Q246419

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.

☑ The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

☑ A certification statement is not submitted herewith.

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

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	/Hui C. Wauters/	Date (YYYY-MM-DD)	2021-02-03
Name/Print	Hui C. Wauters	Registration Number	57,426

*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.

Electronic Patent Application Fee Transmittal

Application Number:	16399386
Filing Date:	30-Apr-2019
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Filer:	Hui Chen Wauters/Sharon Jones
Attorney Docket Number:	Q246419

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

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 $0 paid	1251	1	220	220
Miscellaneous:				
RCE- 1ST REQUEST	1801	1	1360	1360
		Total in USD ($)		**1580**

Electronic Acknowledgement Receipt	
EFS ID:	41824361
Application Number:	16399386
International Application Number:	
Confirmation Number:	9293
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Customer Number:	23373
Filer:	Hui Chen Wauters/Sharon Jones
Filer Authorized By:	Hui Chen Wauters
Attorney Docket Number:	Q246419
Receipt Date:	03-FEB-2021
Filing Date:	30-APR-2019
Time Stamp:	13:28:37
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$1580
RAM confirmation Number	E202123D29003394
Deposit Account	194880
Authorized User	Sharon Jones
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.16 (National application filing, search, and examination fees) 37 CFR 1.17 (Patent application and reexamination processing fees)	

37 CFR 1.19 (Document supply fees)

37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Continued Examination (RCE)	Q246419RCEasfiled.pdf	162570 1a7166f64fa2b47883b443ac0ef518bafe5a5999	no	1
Warnings:					
This is not a USPTO supplied RCE SB30 form.					
Information:					
2		Q246419Amendment114casfiled.pdf	186956 ee328e465a117ab0209821a1dcc37653dec8fcf8	yes	13

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Amendment Submitted/Entered with Filing of CPA/RCE	1	1
Claims	2	7
Applicant Arguments/Remarks Made in an Amendment	8	13

Warnings:					
Information:					
3	Extension of Time	Q246419EOTasfiled.pdf	142889 4ea7c8999e932b72988faa7e7f65259db83baf10	no	1
Warnings:					
Information:					
4		Q246419IDSasfiled.pdf	201810 5c965942a3a49fca260ba3bc0b48e29297de55b6	yes	5

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Transmittal Letter	1	2

	Information Disclosure Statement (IDS) Form (SB08)		3	5	
Warnings:					
Information:					
5	Non Patent Literature	Q246419NPLREFNoticeofOpposition.pdf	9715548 7bbb37b3f00581b169d370b19ac09985d48cc1d8	no	53
Warnings:					
Information:					
6	Non Patent Literature	Q246419NPLREFCertifiedTranslationofPriorityDocument.pdf	11121726 7aa0845a6a82a6bc18d721eea4cef658a760fd92	no	56
Warnings:					
Information:					
7	Non Patent Literature	Q246419NPLREFCarlessoetal.pdf	2417901 9bfd0b27718add055252bc17bf30780961a43ba5	no	8
Warnings:					
Information:					
8	Non Patent Literature	Q246419NPLREFWikipediasiterelatingtoBloodPlasma.pdf	1206840 bb2f42cb4a4656aa17a177e0ee43c39b5adaf4e1	no	5
Warnings:					
Information:					
9	Non Patent Literature	Q246419NPLREFWikipediasiterelatingtoHorseshoeCrab.pdf	1065711 a136f946bcc0b9660b2d472a7eb6e0210beb6898	no	4
Warnings:					
Information:					
10	Non Patent Literature	Q246419NPLREFHaradaSuzukietal.pdf	2348576 089cd0dcca710b4bd5bcde01becff6e309300e59	no	8
Warnings:					
Information:					
11	Non Patent Literature	Q246419NPLREFTomiyaetal.pdf	6390905 75b7319a29bd35cbc137a53aed9ffeb592e29908	no	18

Warnings:					
Information:					
12	Non Patent Literature	Q246419NPLREFNettleshipetal.pdf	5634215 75c9cec1b793893fddf62b6ee3098148c976a2e1	no	22
Warnings:					
Information:					
13	Non Patent Literature	Q246419NPLREFThomasetal.pdf	5982627 0533bef9910b4338f9d4ba77f9ed74510777428d	no	14
Warnings:					
Information:					
14	Non Patent Literature	Q246419NPLREFDeclarationbyDrMizumura10112017.pdf	750219 df2a1ae76bbf1c8c582a30ad9483d58d4c45582c	no	5
Warnings:					
Information:					
15	Non Patent Literature	Q246419NPREFExcerptfromEssentialCellBiology.pdf	719272 ba0c4329378ce1d7f8ba5c53fff286d72ead6915	no	3
Warnings:					
Information:					
16	Non Patent Literature	Q246419NPLREFHossleretal.pdf	5464772 1534010fa0a9f236cbb23e7cf5b1bc6c84de8234	no	14
Warnings:					
Information:					
17	Non Patent Literature	Q246419NPLREFDeclarationbyDrMizumura02222018.pdf	885105 3b62cd45bf2a40a5b1a5c9cea542e6e8bbeec383	no	5
Warnings:					
Information:					
18	Non Patent Literature	Q246419NPLREFInhibitiondata.pdf	926585 73fd7627ffe8c4c14d3e80055dbaa8f654cf29df	no	7
Warnings:					
Information:					

19	Fee Worksheet (SB06)	fee-info.pdf	32941 f56c5fc11d1da1907d95f28dcecb61a1ff6c1fb5	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	55357168	

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.

REQUEST FOR CONTINUED EXAMINATION (RCE) TRANSMITTAL

Application Number	16/399,386	Filing Date	April 30, 2019	Confirmation Number	9293	Docket Number	Q246419
First Named Inventor	Hikaru MIZUMURA		Examiner Name	STEADMAN, DAVID J		Art Unit	1656
Title	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN						

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.

SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments attached 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 _____

☐ Do NOT consider the amendment(s)/reply under 37 CFR 1.116 previously filed on _____

☑ Attached

- ☑ Amendment/Reply
- ☑ Information Disclosure Statement (IDS)
- ☑ Affidavit(s)/Declaration(s) **Declaration Under 37 C.F.R. § 1.132 executed by Dr. Hikaru MIZUMURA.**
- ☑ Other Petition for Extension of Time

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.

☑ Payment by credit card.

☑ The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

CORRESPONDENCE ADDRESS

Direct all correspondence to the address for SUGHRUE MION, PLLC filed under the Customer Number listed below:

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

SIGNATURE OF REGISTERED U.S. PATENT PRACTITIONER

Signature /Hui C. Wauters/ Date February 3, 2021

Name Hui C. Wauters Registration No. 57,426

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Docket No: Q246419
Hikaru MIZUMURA, et al.	
Appln. No.: 16/399,386	Group Art Unit: 1656
Confirmation No.: 9293	Examiner: STEADMAN, DAVID J
Filed: April 30, 2019	

For: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

AMENDMENT UNDER 37 C.F.R. § 1.114(c)

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

Sir:

In response to the Office Action dated October 8, 2020, please amend the above-identified application as follows on the accompanying pages. A Request for Continued Examination (RCE) is being filed concurrently. A Petition and payment for a one month extension of time are being filed herewith, making this Amendment due on or before February 8, 2021.

TABLE OF CONTENTS

AMENDMENTS TO THE CLAIMS 2

REMARKS 8

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions and listings of claims in the application:

LISTING OF CLAIMS:

1. (currently amended): A horseshoe crab Factor C protein having activity of Factor C,

wherein the horseshoe crab is selected from the group consisting of *Tachypleus tridentatus, Limulus polyphemus, and Carcinoscorpius rotundicauda*, and wherein the horseshoe crab Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or Human Embryonic Kidney (HEK) cell, and

wherein the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a corresponding native horseshoe crab Factor C and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell.

2. (currently amended): A Factor C protein having activity of Factor C,

wherein the Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or Human Embryonic Kidney (HEK) cell, and

wherein the expressed Factor C protein comprises an amino acid sequence selected from the group consisting of:

(A) the amino acid sequence of SEQ ID NO: 2, and the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor

C of *Tachypleus tridentatus* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell;

(B) an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:2, and having activity of Factor C, and the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Tachypleus tridentatus* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell;

(C) the amino acid sequence of SEQ ID NO: 4, and the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Carcinoscorpius rotundicauda* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell; and

(D) an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:4, and having activity of Factor C, and the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Carcinoscorpius rotundicauda* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell, and

wherein the Factor C protein exhibits a residual activity of at least one selected from the group consisting of:

(1) 10% or higher in the presence of 21 mM sodium citrate; and

(2) ~~25% or higher in the presence of 52 mM sodium hydrogen carbonate;~~

~~(3) 25% or higher in the presence of 214 mM sodium chloride;~~

~~(4)~~ 15% or higher in the presence of 16 mM magnesium sulfate.

3. (original): The Factor C protein according to claim 2, which is water soluble.

4. (original): The Factor C protein according to claim 2, which has a molecular weight of 115 kDa to 140 kDa as measured by SDS-PAGE under non-reducing condition.

5. (original): The Factor C protein according to claim 4, which has a molecular weight of 127 kDa ± 5 kDa.

6. (previously presented): The Factor C protein according to claim 2, which exhibits a residual Factor C activity of 35% or higher in the presence of 52 mM sodium hydrogen carbonate.

7. (previously presented): The Factor C protein according to claim 2, which exhibits a residual Factor C activity of 35% or higher in the presence of 214 mM sodium chloride.

8. (previously presented): The Factor C protein according to claim 2, which exhibits a residual Factor C activity of 25% or higher in the presence of 16 mM magnesium sulfate.

9. (original): The Factor C protein according to claim 2, which exhibits a residual Factor C activity of 35% or higher in the presence of 2.5 mM calcium chloride.

10. (canceled).

11. (canceled).

12. (previously presented): A method for producing the Factor C protein of claim 2, the method comprising culturing a Chinese Hamster Ovary (CHO) DG44 cell or Human Embryonic Kidney (HEK) cell recombinantly expressing said Factor C protein under conditions suitable for the expression of the Factor C protein to produce the Factor C protein.

13. (original): An endotoxin assay kit comprising the Factor C protein according to claim 2 and one or more elements.

14. (withdrawn): A method for measuring endotoxin in a test specimen using an endotoxin assay agent comprising the Factor C protein according to claim 2.

15. (withdrawn): The method according to claim 14, wherein the test specimen comprises at least one of:

(A) an ion;

(B) a cation;

(C) a metal ion;

(D) an alkali metal ion or an alkaline earth metal ion; and

(E) one or more kinds of ions selected from the group consisting of sodium ion, potassium ion, calcium ion, and magnesium ion.

16. (withdrawn): The method according to claim 15, wherein the test specimen comprises a cation and the final concentration of the cation after mixing the test specimen with the assay agent is 1 mM or higher.

17. (withdrawn): The method according to claim 14, wherein the test specimen is an injection.

18. (withdrawn): A product which has been subjected to an endotoxin assay using an endotoxin assay agent comprising the Factor C protein according to claim 2,

wherein the product is selected from the group consisting of water for medical use, a pharmaceutical product, an infusion solution, a blood product, a medical apparatus, a medical instrument, a biological specimen, a native protein, a recombinant protein, a nucleic acid, and a saccharide.

19. (previously presented): The Factor C protein according to claim 2, which exhibits a residual activity of 20% or higher in the presence of 21 mM sodium citrate.

20. (new): The Factor C protein according to claim 2, which exhibits a residual activity of 25% or higher in the presence of 52 mM sodium hydrogen carbonate.

21. (new): The Factor C protein according to claim 2, which exhibits a residual activity of 25% or higher in the presence of 214 mM sodium chloride.

22. (new): The Factor C protein according to claim 1, wherein the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a corresponding native horseshoe crab Factor C and a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell.

23. (new): The Factor C protein according to claim 2, wherein the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a corresponding native horseshoe crab Factor C and a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell.

REMARKS

In the present Amendment, claim 1 has been amended to incorporate the subject matter of claim 10. Claim 10 has been cancelled. Claim 2 has been amended to delete the residual activity in the presence of sodium hydrogen carbonate and sodium chloride and to add the term "and". The deleted recitations are recited in new claims 20 and 21, respectively. New claims 22 and 23 have been added. Support for claims 22 and 23 is found, for example, in paragraphs [0024] and [0025] of the specification. No new matter has been added, and entry of the Amendment is respectfully requested.

Upon entry of the Amendment, claims 1-9 and 12-23 will be pending, of which claims 14-18 are withdrawn from consideration.

Response to Claim Objection

At page 3 of the Office Action, claim 2 is objected to because, per the Examiner, the conjunction "and" should be added immediately after "chloride;" between parts (3) and (4).

In response, claim 2 has been amended to add the term "and". Accordingly, withdrawal of the claim objection is requested.

Response to § 103 Rejection

At page 4 of the Office Actin, claims 1-10, 12, 13 and 19 are rejected under 35 U.S.C. § 103 as being unpatentable over Ding et al. (US 5,858,706; hereafter "Ding") in view of Current Protocols in Molecular Biology (1993) 16.23.1-16.23.13, John Wiley and Sons, Inc., 2002 (hereafter "Current Protocols") and as evidenced by Mizumura et al. (WO 2012/118226 A1; hereafter "Mizumura").

This rejection should be withdrawn because Ding and Current Protocols do not disclose or render obvious the claimed invention, either alone or in combination.

1. In the Amendment Under 37 C.F.R. § 1.111 filed September 17, 2020, Applicant explained that the claimed Factor C protein provides an unexpected result in terms of salt tolerance compared to the native Factor C; that is, neither Ding nor Current Protocols discloses or suggests the effects of the claimed invention of providing improved sensitivity of endotoxin assay in the presence of a salt, by increasing terminal sialic acid of Factor C contained in endotoxin assay reagents.

In response, the Examiner states that even though the combination of Ding and Current Protocols does not expressly teach or suggest providing improved sensitivity of endotoxin assay in the presence of a salt, by increasing terminal sialic acid of Factor C, Ding's recombinant *Tachypleus tridentatus* or *Carcinscorpius rotunicauda* horseshoe crab Factor C produced using the CHO DG44 cells of Current Protocols would have increased (α-2,3) linked terminal sialic and improved sensitivity of endotoxin assay in the presence of a salt.

Applicant respectfully traverses.

Applicants can rely upon unexpectedly superior results provided by the claimed invention to establish the patentability of the claimed invention. Also, under the law, Applicants are only required to compare the closest prior art to effectively rebut a *prima facie* case of obviousness, and Applicants may compare the claimed invention with prior art that is more closely related to the invention than the prior art relied upon by the Examiner. MPEP 716.02(e). Applicants cannot be required to compare the claimed invention with an invention suggested by a combination of references relied upon by the Examiner, *In re Chapman*, 357 F.2d 418, 148 USPQ 711 (CCPA 1966) (Requiring applicant to compare claimed invention with polymer suggested by the combination of references relied upon in the rejection of the claimed invention

under 35 U.S.C. 103 "would be requiring comparison of the results of the invention with the results of the invention." 357 F.2d at 422, 148 USPQ at 714.) and MPEP 716.02(e).

Accordingly, the Examiner's comparison of the claimed invention with the combination of Ding and Current Protocols is not proper.

2. Applicant submits that a person skilled in the art, reviewing the cited references, would not have arrived at the claimed Factor C which is expressed in CHO DG44 cells, because the cited references do not disclose or even remotely suggest using CHO DG44 cells to produce Factor C. Applicant notes that independent claims 1 and 2 of the present application recite CHO DG44 and HEK, but the Office Action denies inventive step only as to CHO DG44.

Applicant submits herewith a Declaration Under 37 C.F.R. § 1.132 executed by Dr. Hikaru MIZUMURA, the first-named inventor of the present application, to demonstrate that the claimed Factor C, which is produced by being expressed in CHO DG44 cells, has an unexpectedly superior property of being *less* susceptible to inhibition by ions in an injection compared to a Factor C, which is produced by being expressed in CHO-S cells (and other cell lines such as Sf9).

Also, as explained in the Declaration, a Factor C produced by CHO-S cells has a smaller molecular weight than a Factor-C produced in CHO DG44 cells. Therefore, the claimed Factor C is also structurally different from the Factor C expressed in CHO-S cells. Applicant notes that Ding does not disclose or even remotely suggest a horseshoe crab Factor C expressed in a particular *type* of CHO cell. Moreover, Ding does not even suggest that a Factor C might potentially have different properties based on the *type* of CHO cell line that is used for the expression of the Factor C. Mizumura does not compensate for these deficiencies.

Accordingly, one of ordinary skill in the art would have found it surprising that a Factor C expressed in CHO DG44 cells has a superior property of being less susceptible to inhibition by ions as compared to a Factor C expressed in CHO-S cells.

Accordingly, the claimed invention is non-obvious over Ding and Current Protocol., even as evidenced by Mizumura.

3. As shown in FIG. 7 of the present specification and in the Declaration, items (1) 10% or higher residual activity in the presence of 21 mM sodium citrate; and (2) 15% or higher residual activity in the presence of 16 mM magnesium sulfate; of amended claim 2 are met by CHO DG44 and HEK but not met by Sf9 and CHO-S.

4. The Examiner states on page 7 of the Office Action that "one would have been motivated to do this because Ding discloses using CHO cells as an expression host and Current Protocol teaches a method for expressing a desired gene using a CHO cell line, noting that CHO DG44 cell line greatly increases the ease of gene amplification."

However, the Examiner's understanding is not correct as explained in the following (i) and (ii).

(i) Current Protocol discloses that three kinds of cells, CHO DXB11, CHO DG44 and CHO GRA, are cells used for gene amplification utilizing DIHYDROFOLATE REDUCTASE (hereinafter referred to as DHFR cell) at Materials on page 1 and that CHO K1 is used for gene amplification utilizing GLUTAMINE SYNTHETASE (hereinafter referred to as GS cell) at Materials on page 6.

Further, Current Protocol discloses on page 1 that "The DHFR system is probably more widely used, and results in very high levels of amplification (up to 1000 copies per cell in some cases) and expression; however, the DHFR amplification process is lengthy and may require

several months to isolate and characterize a stable, amplified line. In contrast, the GS system typically requires only a single round of selection for amplification to achieve maximal expression levels. In this system the length of time necessary to isolate stably amplified clones from the primary transfectants is dramatically reduced to 2 months or less." (emphasis added.)

Accordingly, when selecting kinds of CHO cells in order to "increase the ease of gene amplification" based on Current Protocol as asserted by the Examiner, one skilled in the art would naturally select GS cell such as CHO K1, but would not select DHFR cell such as CHO DG44. This is because Current Protocol discloses that maximal expression levels can be achieved in short period by using GS cell, whereas DHFR cell requires time and labor.

(ii) Further, even if gene amplification becomes easy by using CHO DG44, it could not be anticipated that salt tolerance ability, namely residual activity in the presence of an ion, is improved in expressed Factor C.

As described in paragraph [0136] of the present specification, the experiments as to residual activity shown in FIG. 7 were evaluated by using control group (injection water containing endotoxin) as a comparative subject. Gene amplification or amount of recombinant protein does not relate to such results.

5. The Examiner considers that the unexpected results are not commensurate in scope with the claimed invention.

Specifically, the Examiner states that claim 1 does not require the claimed horseshoe crab Factor C protein to possess the characteristic that correlates with the applicant's alleged unexpected result of improved sensitivity in the presence of the salt, i.e., (α-2,3) linked terminal sialic acid in a greater amount, as compared with a corresponding native horseshoe crab Factor C and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell.

In response, claim 1 has been amended to incorporate the subject matter of claim 10, that is, to recite greater amount of (α-2,3)-linked terminal sialic acid, as required by the Examiner. Accordingly, the unexpected results are commensurate in scope with the claimed invention.

In view of the above, the present claims, including new claims 20-23, are not obvious and are patentable over Ding and Current Protocols, either alone or in combination. Reconsideration and withdrawal of the §103 rejection based on Ding and Current Protocols are respectfully requested.

Allowance is respectfully requested. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,

/Hui C. Wauters/

Hui C. Wauters
Registration No. 57,426

SUGHRUE MION, PLLC
Telephone: 202.293.7060
Facsimile: 202.293.7860

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

Date: February 3, 2021

Attachment: Declaration Under 37 C.F.R. § 1.132 executed by Dr. Hikaru MIZUMURA.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)		Docket Number Q246419 Confirmation Number 9293	
Application Number	16/399,386	Filed	April 30, 2019
For	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN		
Art Unit	1656	Examiner	STEADMAN, DAVID J

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))	$220.00	$110.00	$55.00	$220.00
☐	Two month (37 CFR 1.17(a)(2))	$640.00	$320.00	$160.00	
☐	Three month (37 CFR 1.17(a)(3))	$1480.00	$740.00	$370.00	
☐	Four month (37 CFR 1.17(a)(4))	$2320.00	$1160.00	$580.00	
☐	Five month (37 CFR 1.17(a)(5))	$3160.00	$1580.00	$790.00	

☐ Previous Payment Amount _____ Date Submitted _____

☐ 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.

☐ 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, **except for the Issue Fee and the Publication Fee**, or credit any overpayment, to Deposit Account Number 19-4880.

☑ Payment made via EFS-Web.

I am the
☐ applicant/inventor.
☐ assignee of record of the entire interest. See 37 CFR 3.71.
37 CFR 3.73(b) statement is enclosed (Form PTO/SB/96).
☑ attorney or agent of record. Registration Number 57,426
☐ attorney or agent under 37 CFR 1.34. Registration number _____

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

/Hui C. Wauters/ — Signature
February 3, 2021 — Date
Hui C. Wauters — Typed or printed name
202.293.7060 — 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 1 form is submitted.

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Docket No: Q246419
Hikaru MIZUMURA, et al.	
Appln. No.: 16/399,386	Group Art Unit: 1656
Confirmation No.: 9293	Examiner: STEADMAN, DAVID J
Filed: April 30, 2019	

For: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

INFORMATION DISCLOSURE STATEMENT
UNDER 37 C.F.R. §§ 1.97 and 1.98

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

Sir:

In accordance with the duty of disclosure under 37 C.F.R. § 1.56, Applicant hereby notifies the U.S. Patent and Trademark Office of the documents which are listed on the attached PTO/SB/08 (modified) form and/or listed herewith.

One copy of each of the listed documents is submitted herewith, except for U.S. Patents.

The present Information Disclosure Statement is being filed: (1) No later than three months from the application's filing date; (2) Before the mailing date of the first Office Action on the merits (whichever is later); or (3) Before the mailing date of the first Office Action after filing a request for continued examination (RCE) under §1.114, and therefore, no Statement under 37 C.F.R. § 1.97(e) or fee under 37 C.F.R. § 1.17(p) is required.

The submission of the listed documents is not intended as an admission that any such document constitutes prior art against the claims of the present application. Applicant does not

waive any right to take any action that would be appropriate to antedate or otherwise remove any listed document as a competent reference against the claims of the present application.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,

/Hui C. Wauters/

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Registration No. 57,426

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WASHINGTON OFFICE
23373
CUSTOMER NUMBER

Date: February 3, 2021

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/399,386	04/30/2019	Hikaru MIZUMURA	Q246419	9293

23373 7590 10/08/2020

SUGHRUE MION, PLLC
2000 PENNSYLVANIA AVENUE, N.W.
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WASHINGTON, DC 20006

EXAMINER	
STEADMAN, DAVID J	

ART UNIT	PAPER NUMBER
1656	

NOTIFICATION DATE	DELIVERY MODE
10/08/2020	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):

PPROCESSING@SUGHRUE.COM
USPTO@sughrue.com
sughrue@sughrue.com

Office Action Summary	**Application No.** 16/399,386	**Applicant(s)** MIZUMURA et al.	
	Examiner DAVID J STEADMAN	**Art Unit** 1656	**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 17 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) 1-10 and 12-19 is/are pending in the application.

5a) Of the above claim(s) 14-18 is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-10,12-13 and 19 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 CORRESPONDENCE

Status of the Application

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Claims 1-10 and 12-19 are pending in the application.

Applicant's amendment to the claims, filed on September 17, 2020, is acknowledged. This listing of the claims replaces all prior versions and listings of the claims.

Applicant's amendment to the specification, filed on September 17, 2020, is acknowledged.

Applicant's remarks and declaration under 37 CFR 1.132 filed on September 17, 2020 in response to the non-final rejection mailed on June 30, 2020 have been fully considered.

The text of those sections of Title 35 U.S. Code not included in the instant action can be found in a prior Office action.

Rejections not reiterated from a previous Office action are hereby withdrawn.

Election/Restriction

Claims 14-18 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to nonelected inventions, there being no allowable generic or linking claim. Election was made without traverse in the reply filed on June 17, 2020.

Claims 1-10, 12, 13, and 19 are being examined on the merits.

Information Disclosure Statement

The information disclosure statement (IDS) submitted on September 17, 2020 is in compliance with the provisions of 37 CFR 1.97. Accordingly, the IDS is being considered by the examiner.

Terminal Disclaimer

The terminal disclaimer filed on September 17, 2020 disclaiming the terminal portion of any patent granted on this application which would extend beyond the expiration date of U.S. Patent No. 10,144,923 has been reviewed and is accepted. The terminal disclaimer has been recorded.

The terminal disclaimer filed on September 17, 2020 disclaiming the terminal portion of any patent granted on this application which would extend beyond the expiration date of any patent granted on Application Numbers 15/983,725 and 16/331,810 has been reviewed and is accepted. The terminal disclaimer has been recorded.

Claim Objection

Claim 2 is objected to because there is no conjunction between parts (3) and (4) and in the interest of improving claim form and grammar, it is suggested that the conjunction “and” be added immediately after “chloride;” in part (3) of claim 2.

Claim Rejections - 35 USC § 103

Claims 1-10, 12, 13, and 19 are rejected under 35 U.S.C. 103 as being unpatentable over Ding et al. (US 5,858,706; cited on the IDS filed on April 30, 2019; hereafter "Ding") in view of *Current Protocols in Molecular Biology* (1993) 16.23.1-16.23.13, John Wiley and Sons, Inc., 2002 (cited on the IDS filed on June 17, 2020; hereafter "Current Protocols") and as evidenced by Mizumura et al. (WO 2012/118226 A1; cited on the IDS filed on April 30, 2019; hereafter "Mizumura").

As amended, claims 1 and 10 are drawn to a horseshoe crab Factor C protein having activity of Factor C, wherein the horseshoe crab is selected from the group consisting of *Tachypleus tridentatus, Limulus polyphemus*, and *Carcinoscorpius rotundicauda*, and wherein the horseshoe crab Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or Human Embryonic Kidney (HEK) cell.

As amended, claims 2-9 and 19 are drawn to a Factor C protein having activity of Factor C,

wherein the Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or Human Embryonic Kidney (HEK) cell, and

wherein the expressed Factor C protein comprises an amino acid sequence selected from the group consisting of:

(A) the amino acid sequence of SEQ ID NO: 2, and the Factor C protein contains (α-2,3) linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Tachypleus tridentatus* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell;

(B) an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:2, and having activity of Factor C, and the Factor C protein contains (α-2,3) linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Tachypleus tridentatus* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell;

(C) the amino acid sequence of SEQ ID NO: 4, and the Factor C protein contains (α-2,3) linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Carcinoscorpinus rotundicauda* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell; and

(D) an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:4, and having activity of Factor C, and the Factor C protein contains (α-2,3) linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Carcinoscorpinus rotundicauda* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell, and

wherein the Factor C protein exhibits a residual activity of at least one selected from the group consisting of:

(1) 10% or higher in the presence of 21 mM sodium citrate;

(2) 25% or higher in the presence of 52 mM sodium hydrogen carbonate;

(3) 25% or higher in the presence of 214 mM sodium chloride;

(4) 15% or higher in the presence of 16 mM magnesium sulfate.

As amended, claim 12 is drawn to a method for producing the Factor C protein of claim 2, the method comprising culturing a Chinese Hamster Ovary (CHO) DG44 cell or Human Embryonic Kidney (HEK) cell recombinantly expressing said Factor C protein

under conditions suitable for the expression of the Factor C protein to produce the Factor C protein.

Claim 13 is drawn to an endotoxin assay kit comprising the Factor C protein according to claim 2 and one or more elements.

Regarding claims 1, 2, and 12, the reference of Ding discloses a mammalian host cell, including a Chinese Hamster Ovary (CHO) cell, transformed with a recombinant vector for expressing a cDNA encoding a Factor C (column 9, Example 1). Ding discloses cDNAs for expressing a horseshoe crab Factor C polypeptide, including the representative examples of *T. tridentatus* and *C. rotundicauda* Factor C polypeptides (column 3, lines 14-20). Ding discloses a method for producing the Factor C polypeptide by culturing the host cell and recovering the Factor C protein (column 9, Example 1). Ding discloses activation of the Factor C by endotoxin (column 1, lines 44-47).

Regarding claim 2, Ding does not disclose the amino acid sequence of *T. tridentatus* factor C has the amino acid sequence of SEQ ID NO: 2. However, the evidentiary reference of Mizumura is cited to show that the amino acid sequence of *T. tridentatus* factor C is identical to SEQ ID NO: 2 of this application.

Regarding claim 3, Ding discloses solubilization of the factor C (column 7, lines 456-53) and thus Ding's Factor C is considered to be water-soluble.

Regarding claim 13, Ding discloses the recombinant Factor C for use in an assay for endotoxin detection and teaches elements for conducting such an assay (column 8, lines 50-60), which is considered to be an endotoxin assay kit as encompassed by claim 13.

The differences between Ding and the claimed invention is that Ding does not disclose CHO DG44 or HEK cells as an expression host as recited in claims 1, 2, and 12, does not explicitly disclose the a greater amount of (α-2,3) linked terminal sialic acid as recited in claims 2 and 10, and does not explicitly disclose the residual activity and molecular weight limitations as recited in claims 2, 4-9, and 19.

The reference of Current Protocols generally teaches a method for using CHO cells as an expression host for amplifying and expressing a desired gene (see whole document). Current Protocols teaches CHO DG44 as a cell line that greatly increases the ease of gene amplification (p. 16.23.11).

It would have been obvious to one of ordinary skill in the art before the effective filing date to combine the teachings of Ding and Current Protocols to modify Ding to use CHO DG44 cell line of Current Protocol as an expression host. One would have been motivated to do this because Ding discloses using CHO cells as an expression host and Current Protocol teaches a method for expressing a desired gene using a CHO cell line, noting that CHO DG44 cell line greatly increases the ease of gene amplification. One would have had a reasonable expectation of success to modify Ding to use the CHO DG44 cell line of Current Protocol as an expression host because Ding teaches cDNAs for expressing a horseshoe crab Factor C polypeptide using CHO cells and Current Protocol teaches a method for expressing a desired gene using a CHO DG44 cell line.

Regarding claims 2 and 19, the combination of Ding and Current Protocols does not explicitly teach or suggest the activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells exhibits a residual activity of 10% or 20% or higher in the presence of 21 mM sodium citrate. However,

Figure 7 of this application shows that horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells exhibits a residual activity of 20% or higher in the presence of 10% sodium citrate with a dilution factor of 8, which, according to the specification is about 21 mM sodium citrate (p. 88). As such, since horseshoe crab Factor C recombinantly expressed and recovered from CHO cells exhibits a residual activity of 20% or higher in the presence of 21 mM sodium citrate, it follows that Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells necessarily exhibits a residual activity of 20% or higher in the presence of 21 mM sodium citrate.

Regarding claims 4 and 5, the combination of Ding and Current Protocols does not explicitly teach or suggest the activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells has a molecular weight as recited in the claims. However, the applicant's specification discloses that *T. tridentatus* factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells has a molecular weight of 128 kDa by SDS-PAGE under non-reducing conditions (p. 92, paragraph [0144] and p. 94, Table 1). As such, since Ding's activated *T. tridentatus* factor C polypeptide is recombinantly expressed and recovered from CHO cells, it follows that Ding's activated Factor C polypeptide expressed from CHO DG44 cells necessarily has a molecular weight of 128 kDa by SDS-PAGE under non-reducing conditions.

Regarding claims 2 and 6, the combination of Ding and Current Protocols does not explicitly teach or suggest the activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells exhibits a residual

activity of 25% or 35% or higher in the presence of 52 mM sodium hydrogen carbonate. However, Figure 7 of this application shows that horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells exhibits a residual activity of 35% or higher in the presence of about 7% sodium citrate with a dilution factor of 8, which, according to the specification is about 52 mM sodium hydrogen carbonate (specification at p. 88). As such, since horseshoe crab Factor C recombinantly expressed and recovered from CHO cells recombinantly expressed and recovered from CHO cells exhibits a residual activity of 35% or higher in the presence of 52 mM sodium hydrogen carbonate, it follows that Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells necessarily exhibits a residual activity of 35% or higher in the presence of 52 mM sodium hydrogen carbonate.

Regarding claims 2 and 7, the combination of Ding and Current Protocols does not explicitly teach the activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells exhibits a residual activity of 25% or 35% or higher in the presence of 214 mM sodium chloride. However, Figure 7 of this application shows that horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO cells exhibits a residual activity of 35% or higher in the presence of about 10% sodium citrate with a dilution factor of 4, which, according to the specification is about 214 mM sodium chloride (specification at p. 88). As such, since horseshoe crab Factor C recombinantly expressed and recovered from CHO cells recombinantly expressed and recovered from CHO cells exhibits a residual activity of 35% or higher in the presence of 214 mM sodium chloride, it follows that Ding's

activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells necessarily exhibits a residual activity of 35% or higher in the presence of 214 mM sodium chloride.

Regarding claims 2 and 8, the combination of Ding2 and Current Protocols does not explicitly teach the activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells exhibits a residual activity of 15% or 25% or higher in the presence of 16 mM magnesium sulfate. However, Figure 7 of this application shows that horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO cells exhibits a residual activity of 25% or higher in the presence of about 0.5 M magnesium sulfate with a dilution factor of 16, which, according to the specification is about 16 mM magnesium sulfate (specification at p. 89). As such, since horseshoe crab Factor C recombinantly expressed and recovered from CHO cells recombinantly expressed and recovered from CHO cells exhibits a residual activity of 25% or higher in the presence of 16 mM magnesium sulfate, it follows that Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells necessarily exhibits a residual activity of 25% or higher in the presence of 16 mM magnesium sulfate.

Regarding claim 9, Ding does not explicitly teach the activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells exhibits a residual activity of 35% or higher in the presence of 2.5 mM calcium chloride. However, Figure 7 of this application shows that horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO cells exhibits a residual activity of 35% or higher in the presence of 0.5 M calcium chloride with a dilution factor of 100,

which, according to the specification is about 2.5 mM calcium chloride (specification at p. 89). As such, since horseshoe crab Factor C recombinantly expressed and recovered from CHO cells recombinantly expressed and recovered from CHO cells exhibits a residual activity of 35% or higher in the presence of 2.5 mM calcium chloride, it follows that Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells necessarily exhibits a residual activity of 35% or higher in the presence of 2.5 mM calcium chloride.

Regarding claims 2 and 10, the combination of Ding and Current Protocol does not explicitly teach or suggest the activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells has (α-2,3) linked terminal sialic acid in a greater amount as compared with a horseshoe crab Factor C expressed in Sf9 as a host cell. However, the applicant's specification discloses that horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO cells has (α-2,3) linked terminal sialic acid, while a native horseshoe crab Factor C polypeptide expressed in Sf9 as a host cell does not have any (α-2,3) linked terminal sialic acid (p. 98, paragraph [0154]). As such, since horseshoe crab Factor C recombinantly expressed and recovered from CHO cells has (α-2,3)-linked terminal sialic acid and a native horseshoe crab Factor C polypeptide expressed in Sf9 as a host cell does not have any (α-2,3) linked terminal sialic acid, it follows that Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells necessarily has a greater amount of (α-2,3)-linked terminal sialic acid than horseshoe crab Factor C polypeptide expressed in Sf9 as a host cell. The combination of Ding and Current Protocols also does not explicitly teach or suggest the

activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells has (α-2,3) linked terminal sialic acid in a greater amount as compared with a native horseshoe crab Factor C. However, the applicant's specification discloses that horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO cells has greater (α-2,3) linked terminal sialic acid than a native horseshoe crab Factor C polypeptide (paragraph [0153]). As such, since horseshoe crab Factor C recombinantly expressed and recovered from CHO cells has greater (α-2,3)-linked terminal sialic acid than a native horseshoe crab Factor C polypeptide, it follows that Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells necessarily has a greater amount of (α-2,3)-linked terminal sialic acid than a native horseshoe crab Factor C polypeptide.

Therefore, the invention of claims 1-10, 12, 13, and 19 would have been obvious to one of ordinary skill in the art at the time of the invention.

RESPONSE TO REMARKS: The applicant argues that the claimed Factor C protein provides an unexpected result in terms of salt tolerance compared to the native Factor C, citing to Fig. 7 of the present specification, whereas Fig. 17 of Ding shows that LPS affinity of recombinant Factor C is inferior to that of native Factor C. The applicant argues that neither Ding nor Current Protocols teaches or suggests the effect of improved sensitivity of endotoxin assay in the presence of a salt by increasing terminal sialic acid of Factor C contained in endotoxin assay reagents. The applicant argues that FIG. 17 of Ding shows a test result of binding ability to LPS of recombinant Factor C (rCrFC) produced in yeasts as a host cell compared with that of native Factor C (CAL).

The applicant explains that a band of about 90-100 kDa corresponds to a complex with LPS, namely endotoxin, which band for CrFC sample is paler than that of CAL sample, and one would have understood that Factor C produced in yeasts (rCrFC in FIG. 17) has lower affinity to endotoxin than that of native Factor C (CAL in FIG. 17), and that the sensitivity of endotoxin assay reagent using rCrFC in the presence of the salt becomes lower than that using native Factor C.

The applicant argues that Ding discloses mammalian cells and yeasts in parallel as host cells for expression of rCrFC that do not produce bacterial endotoxin (lines 3 to 12 in column 3), and rCrFC is especially effectively produced in yeasts (lines 42 to 44 in column 7). The applicant argues that Ding discloses the application of rCrFC to remove endotoxin from a liquid samples by binding of endotoxin in the sample to the immobilized rCrFC, thereby forming bound endotoxin (lines 5 to 11 in column 8) and one reading Ding would have understood that yeasts especially effectively produce recombinant Factor C useful for binding to endotoxin, and Factor C produced in mammalian cells does not have at least higher endotoxin binding properties as Factor C produced in yeasts. According to the applicant, one would have understood from Ding that rCrFC produced in mammalian cells has lower affinity to endotoxin than that of native Factor C, and that highly sensitive endotoxin detection is difficult by using rCrFC produced in mammalian cells.

The applicant argues that in contrast to Ding, the claimed recombinant Factor C having (α-2,3)-linked terminal sialic acid in a greater amount for the endotoxin assay reagent exhibits effects of providing improved sensitivity in the presence of the salt

compared with the endotoxin assay reagent using the native Factor C (see FIG. 7 of the present specification), which effects are unexpected from Ding and Current Protocols.

The applicant argues that the Declaration under 37 C.F.R. § 1.132 executed by Hikaru Mizumura (hereafter "Declaration") describes that the Factor C derived from three species of *T. tridentatus* (SEQ ID NO:2 of the present application), *L. polyphemus* and *C. rotundicauda* (SEQ ID NO:4 of the present application) have amino acid sequence identity at 90% or higher of each other and amino acids discordance in species exist also in function domains (for example, LPS binding domain or C-terminal catalytic domain). According to the applicant, in view of the Declaration, one skilled in the art could understand that a protein comprising an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO: 2 or NO: 4 maintains the Factor C activity.

The applicant argues that the Declaration shows experimental results of evaluation of inhibitory activity by injection according to the method of "5. Inhibition of activity by injection" of the instant specification at paragraphs [0134]-[0138], by using *L. polyphemus* Factor C as one example of the protein having about 90% sequence identity to the amino acid sequence of SEQ ID NO: 2 (*T. tridentatus*) and SEQ ID NO: 4 (*C. rotundicauda*). According to the applicant, the Declaration shows that the protein having about 90% sequence identity to the amino acid sequence of SEQ ID NO: 2 (*T. tridentatus*) and SEQ ID NO: 4 (*C. rotundicauda*) (namely, *L. polyphemus* Factor C) actually shows high reactivity with endotoxin under a high salt concentration condition and one would understand that the effects of the claimed invention (shown in Fig. 7 of

the present specification) is also provided by the Factor C protein of *L. polyphemus* or *C. rotundicauda* of claim 1, or the Factor C protein of item (B) or (D) of claim 2.

The applicant's argument, evidence, and Declaration are not found persuasive. The examiner acknowledges that the combination of Ding and Current Protocols does not teach or suggest providing improved sensitivity of endotoxin assay in the presence of a salt, by increasing terminal sialic acid of Factor C contained in endotoxin assay reagents. However, according to MPEP 2112, the inherent disclosures of a prior art reference may be relied upon in the rejection of claims under 35 U.S.C. 103 and according to MPEP 2112.II, there is no requirement that a person of ordinary skill in the art would have recognized the inherent disclosure *at the time of the invention.* As described in detail above, Ding teaches recombinant expression of *Tachypleus tridentatus* or a *Carcinscorpius rotunicauda* horseshoe crab Factor C using CHO cells and according to the specification, recombinant expression of horseshoe crab Factor C using CHO cells increases (α-2,3) linked terminal sialic, and thus conferring improved sensitivity of endotoxin assay in the presence of a salt. As such, even though the combination of Ding and Current Protocols does not *expressly* teach or suggest providing improved sensitivity of endotoxin assay in the presence of a salt, by increasing terminal sialic acid of Factor C, the preponderance of the evidence supports the position that Ding's recombinant *Tachypleus tridentatus* or *Carcinscorpius rotunicauda* horseshoe crab Factor C produced using the CHO DG44 cells of Current Protocols would have increased (α-2,3) linked terminal sialic and improved sensitivity of endotoxin assay in the presence of a salt.

Regarding the applicant's allegation of an unexpected result, according to MPEP 716.02(b), the burden is on the applicant to establish results are unexpected. The applicant relies on a comparison between the applicant's result and Ding's Example 4 and Figure 17, which, as acknowledged by the applicant, uses a recombinant Factor C produced in *yeast*. From Ding's result using a recombinant Factor C produced in *yeast,* the applicant alleges that one would have extrapolated Ding's result with a recombinant Factor C produced in *yeast* to the expected result using a recombinant Factor C produced in *CHO cells*. However, there is no teaching or suggestion in the prior art of record that a recombinant Factor C produced in *yeast cells* would have the same characteristics – including improved sensitivity in the presence of the salt – as a recombinant Factor C produced in *CHO cells*. As such, the applicant's allegation that one would have expected a recombinant Factor C produced in *CHO cells* to have the same characteristics to a recombinant Factor C produced in *yeast* is taken as an unsupported argument of counsel and arguments of counsel cannot take the place of factually supported objective evidence. See MPEP 2145.

In fact, the prior art does not support the applicant's allegation that one would have expected a recombinant Factor C produced in *CHO cells* to have the same characteristics and produce a similar result as a recombinant Factor C produced in *yeast*. According to the reference of Ng et al. (US 2002/0068325 A1; cited on Form PTO-892), O-linked glycosylation is a rare modification in higher eukaryotes, yet it is possible that most heterologous proteins can become O-glycosylated when produced in yeast and that O-glycosylation of a heterologous protein can result in misfolding and compromised activity and stability (p. 6, paragraph [0069]). Ding's result was achieved

using a recombinant Factor C produced using *P. pastoris* as the yeast expression host and the reference of Damasceno et al. (*Appl. Microbiol. Biotechnol.* 93:31-39, November 2011; cited on the IDS filed on September 17, 2020) teaches that there are significant differences between *P. pastoris* and mammalian cells as to the types of sugars added during glycosylation, including that mammalian cells add sialic acid, whereas *P. pastoris* does not (p. 36, column 1, middle). Given the evidence of the prior art, one would have recognized that glycosylation highly affects the activity and stability of proteins and the glycosylation of heterologous proteins between yeast and mammalian cells is very different. Since it was known that there are significant differences between glycosylation by yeasts (including *P. pastoris*) and mammalian cells, which glycosylation highly affects activity and stability of heterologous proteins, one would *not* have extrapolated a result obtained by a recombinant Factor C produced in *yeast* to a result that would be expected by a recombinant Factor C produced in *CHO cells*. As such, the applicant has failed to establish what result would have been expected for a recombinant Factor C produced by CHO cells, and consequently has failed to establish an unexpected result.

Also, according to MPEP 716.02(d), the unexpected result must be commensurate in scope with the claimed invention. Claim 1 does not require the claimed horseshoe crab Factor C protein to possess the characteristic that correlates with the applicant's alleged unexpected result of improved sensitivity in the presence of the salt, *i.e.,* (α-2,3) linked terminal sialic acid in a greater amount, as compared with a corresponding native horseshoe crab Factor C and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell. The recitation of "wherein the

horseshoe crab Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or Human Embryonic Kidney (HEK) cell" is interpreted as a product-by-process and given a broadest reasonable interpretation in light of the specification, the claims encompass additional production steps or post-translational modifications, *e.g.,* treatment of the Factor C polypeptide expressed in a CHO DG44 cell or HEK cell with a glycosidase to remove (α-2,3)-linked sialic acid (which interpretation is supported by the applicant's specification at Example 9 beginning at p. 94). However, according to the results shown in the applicant's Figure 7, the residual activity of Factor C expressed in Sf9 (with no (α-2,3)-linked sialic acid) or native Factor C exhibits almost no residual activity in 21 mM sodium citrate (see No. 5 of Figure 7; 10% sodium citrate corresponds to 21 mM sodium citrate). As such, even if the applicant had established an unexpected result, the result would not be commensurate in scope with the horseshoe crab Factor C protein of claim 1.

For these reasons, it is the examiner's position that the claimed invention would have been *prima facie* obvious to one of ordinary skill in the art before the effective filing date.

Examiner Comment

The applicant is advised that withdrawal of the restriction requirement between the claims of the elected invention of Group I and the claims of the non-elected and patentably distinct invention of Group II may necessitate a nonstatutory double patenting rejection based on the claims of co-pending application 16/399,575.

Conclusion

Status of the claims:

Claims 1-10 and 12-19 are pending.

Claims 14-18 are withdrawn from consideration.

Claims 1-10, 12, 13, and 19 are rejected.

THIS ACTION IS MADE FINAL. 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 mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to DAVID J STEADMAN whose telephone number is (571)272-0942. The examiner can normally be reached on Monday to Friday, 7:30 AM to 4:00 PM.

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, MANJUNATH N. RAO can be reached on 571-272-0939. 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.

/David Steadman/
Primary Examiner, Art Unit 1656

Notice of References Cited	Application/Control No. 16/399,386	Applicant(s)/Patent Under Reexamination MIZUMURA et al.	
	Examiner DAVID J STEADMAN	Art Unit 1656	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-20020068325-A1	06-2002	Ng, Davis T.W.	C12Y302/01096	435/69.1
	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.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/399,386	MIZUMURA et al.
	Examiner	Art Unit
	DAVID J STEADMAN	1656

CPC - Searched*		
Symbol	Date	Examiner
See attached EAST search history printout	10/02/2020	

CPC Combination Sets - Searched*		
Symbol	Date	Examiner
NONE		

US Classification - Searched*			
Class	Subclass	Date	Examiner
NONE			

* 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
PALM inventor name search, all inventors searched	06/24/2020	/DJS/
EAST inventor name and assignee search, all inventors searched	06/24/2020	/DJS/
Reviewed prosecution history for applications 14/650,767, 15/983,725, and 16/399575	06/24/2020	/DJS/
STIC search of SEQ ID NO: 2 and 4 against amino acid and nucleic acid prior and interference databases, see SCORE for search results	06/24/2020	/DJS/
EAST, STN database keyword search, see attached search history printouts for search strategy and databases searched	06/24/2020	
PALM inventor name search and EAST inventor name and assignee search, all inventors searched	10/02/2020	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/399,386	MIZUMURA et al.
	Examiner	Art Unit
	DAVID J STEADMAN	1656

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16/399,386
	Confirmation Number	9293
	Filing Date	April 30, 2019
	First Named Inventor	Hikaru MIZUMURA
	Art Unit	1656
	Examiner Name	STEADMAN, DAVID J
	Attorney Docket Number	Q246419

U.S. PATENTS

Examiner Initials*	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

U.S. PATENT APPLICATION PUBLICATIONS

Examiner Initials*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No	Foreign Document Number[3]	Country Code[2]	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]

NON-PATENT LITERATURE DOCUMENTS

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, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1.	Final Office Action dated August 4, 2020 issued in U.S. Application No. 16/399,575	
	2.	DAMASCENO et al, "Protein secretion in *Pichia pastoris* and advances in protein production", Appl Microbiol Biotechnol (2012) 93: pp. 31-39, Published online: November 2011	
	3.	Final Office Action dated August 28, 2020 issued in U.S. Application No. 15/983,725	
	4.	KNAPPSKOG et al, "The level of synthesis and secretion of *Ganssia princeps* luciferase in transfected CHO cells is heavily dependent on the choice of signal peptide", Journal of Biotechnology, Vol. 128, 2007, pp. 705-715	

EXAMINER SIGNATURE

Examiner Signature	/David Steadman/	Date Considered	09/23/2020

*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. /DJS/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16/399,386
	Confirmation Number	9293
	Filing Date	April 30, 2019
	First Named Inventor	Hikaru MIZUMURA
	Art Unit	1656
	Examiner Name	STEADMAN, DAVID J
	Attorney Docket Number	Q246419

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).

☐ That each item of information contained in the Information Disclosure Statement filed concurrently herewith: (i) was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement; or (ii) is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement. See 37 CFR 1.704(d).

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

☑ The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

☐ A certification statement is not submitted herewith.

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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	/Hui C. Wauters/	Date (YYYY-MM-DD)	2020-09-17
Name/Print	Hui C. Wauters	Registration Number	57,426

*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. /DJS/

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	23,458	(mizumura-h$ OR oda-t$ OR kawabata-s$). in. OR seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
L2	98	L1 AND (tridentatus OR rotundicauda OR polyphemus)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
L3	57	L2 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
L4	11	L3 and (cho OR "chinese hamster")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
L5	9	L3 and (hek OR (human adj embryo$))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
L6	11	L4 or L5	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:30
L8	753	c12n9/6408.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:52
L9	44	8 and (tridentatus OR polyphemus OR rotundicauda)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:53

EAST Search History (Prior Art)

L10	829,430	(cho OR "chinese hamster")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:53
L11	104,016	(hek OR (human adj embryo$))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:53
L12	14	9 and (10 OR 11)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/10/02 11:53
S5	7,687	(horseshoe adj crab) OR limulus	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:46
S6	13,415	factor adj "c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S7	13,415	"factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S8	335	S5 same S6	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S9	212	S8 and recombinant	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47

EAST Search History (Prior Art)

S10	89	S8 same recombinant	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S11	26	S10 and mammalian	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:48
S12	9	S10 and ((chinese adj hamster) OR CHO)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:02
S13	2	S10 and (hek OR (embryonic adj kidney))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:02
S14	6	S10 and (hek$ OR (embryonic adj kidney))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:03
S15	4	S14 not S13	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:03
S16	2	"6,645,724".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:04
S17	12	"2012118226"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:35

EAST Search History (Prior Art)

S18	4	"5716834".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 15:58
S19	2,454	seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:23
S20	18,057	(mizumura-h$ OR oda-t$ OR kawabata-s$). in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:23
S21	20,466	S19 or S20	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:25
S22	91	S21 and (horseshoe adj crab)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:25
S23	66	S21 and tridentatus	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:25
S24	41	S21 and rotundicauda	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:26
S25	108	S22 or S23 or S24	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:26

EAST Search History (Prior Art)

S26	40	S25 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:26
S27	23	("5,858,706" OR "5,712,144" OR "5,795,962" OR "6,077,946" OR "20090208995" OR "20030054432" OR "20040235080" OR "20140024062").pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 11:34
S28	4	"5,716,834".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/05 18:17
S29	4	"5,858,706".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 11:27
S30	2	"6,645,724".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 15:42
S31	2	"20140249082".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 16:45
S32	2,474	seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:07
S33	18,751	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:07

EAST Search History (Prior Art)

S34	21,180	S32 or S33	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:07
S35	19	S33 AND (tridentatus OR polyphemus OR rotundicauda OR gigas)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:08
S36	13	S35 and "factor C"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:08
S37	2	S36 and (sialic OR sialyl$)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:08
S38	14,664	"factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:53
S39	218	S38 and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:53
S40	7	S38 same (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:54
S41	218	S39 and (cho OR "chinese hamster ovary")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:54

EAST Search History (Prior Art)

S42	805	"factor c".clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:54
S43	2	S42 and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:55
S44	5,676	(cho OR "chinese hamster ovary") same (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:55
S45	211	S44 and S38	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:55
S47	7	S45 and horseshoe	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:56
S48	20	horseshoe and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:45
S49	218	"factor c" and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:47
S50	211	S49 not S48	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:47

EAST Search History (Prior Art)

S51	0	S50 and horseshoe	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:48
S52	2	"factor c".clm. and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:48
S53	6	"factor c".ti. and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:49
S54	7	"factor c".ab. and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:49
S55	1	S54 not S53	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:49
S56	2	"9133495".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:36
S57	19,132	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45
S58	6	S57 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45

EAST Search History (Prior Art)

S59	2,485	seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45
S60	3	S59 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45
S61	7	S58 or S60	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45
S62	19,414	(mizumura-h$ OR oda-t$ OR kawabata-s$). in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S63	6	S62 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S64	2,493	seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S65	3	S64 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S66	7	S63 or S65	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58

EAST Search History (Prior Art)

S67	19,635	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/08/14 09:19
S68	6	S67 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/08/14 09:19
S80	3	("20080131929"|"20100086967"|"20130295613").PN.	US-PGPUB; USPAT	OR	OFF	2018/09/07 10:58
S81	19,653	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/09/07 11:43
S82	6	S81 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/09/07 11:43
S94	6	"10144923".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/10/01 09:23
S96	6	(US-20080131929-$ | US-20100086967-$ | US-20130295613-$).DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/10/01 10:53
S97	20,399	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/10/02 15:15
S98	40	S97 AND (tridentatus OR polyphemus OR rotundicauda OR gigas)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/10/02 15:15

EAST Search History (Prior Art)

S99	23,093	(mizumura-h$ OR oda-t$ OR kawabata-s$). in. OR seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/02/07 10:15
S100	95	S99 AND (tridentatus OR polyphemus OR rotundicauda OR gigas)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/02/07 10:16
S101	54	S100 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/02/07 10:16
S102	24	S101 and ("chinese hamster" OR CHO OR human OR HEK)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/02/07 10:16
S103	627	tridentatus OR rotundicauda	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S104	389	S103 and endotoxin	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S105	263	S104 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S106	224	S103 same "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41

EAST Search History (Prior Art)

S107	216	S105 and S106	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S108	140	S107 and "37"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:42
S109	1,050,017	"2 hours"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:42
S110	114	S108 and S109	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:42
S113	349,173	cascade	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:44
S114	169	S107 and S113	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:45
S115	468	limulus adj test	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 15:30
S116	1	S115 same cation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 15:31

EAST Search History (Prior Art)

S117	3	"6,645,724".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 12:19
S118	13	("20030054432"|"20040235080"|"20080131929"|"20090208995"|"20100086967"|"20130295613"|"20140024062"|"20140249082"|"5712144"|"5716834"|"5795962"|"5858706"|"6077946").PN.	US-PGPUB; USPAT	OR	OFF	2020/04/22 14:32
S119	23,218	(mizumura-h$ OR oda-t$ OR kawabata-s$).in. OR seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 14:37
S120	85	S119 AND (tridentatus OR rotundicauda)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 14:37
S121	54	S120 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 14:38
S122	33	S121 and endotoxin.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 14:39
S123	23,351	(mizumura-h$ OR oda-t$ OR kawabata-s$).in. OR seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:10
S124	95	S123 AND (tridentatus OR rotundicauda OR polyphemus)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:10
S125	55	S124 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:11

EAST Search History (Prior Art)

S126	10	S125 and (cho OR "chinese hamster")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:11
S127	8	S125 and (hek OR (human adj embryo$))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:12
S128	10	S126 or S127	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:12

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S69	2,075	(dg44 OR dg-44 OR chodg44 OR cho-dg44)	USPAT	OR	OFF	2018/08/14 09:27
S70	5,851	"factor c"	USPAT	OR	OFF	2018/08/14 09:27
S71	92	S69 and S70	USPAT	OR	OFF	2018/08/14 09:27
S72	17,574	horseshoe OR crab	USPAT	OR	OFF	2018/08/14 09:28
S73	2	S71 and S72	USPAT	OR	OFF	2018/08/14 09:28
S74	40	(dg44 OR dg-44 OR chodg44 OR cho-dg44).clm.	USPAT	OR	OFF	2018/08/14 09:30
S75	380	"factor c".clm.	USPAT	OR	OFF	2018/08/14 09:30
S76	0	S74 and S75	USPAT	OR	OFF	2018/08/14 09:30
S77	2,254	(horseshoe OR crab).clm.	USPAT	OR	OFF	2018/08/14 09:31
S78	0	S74 and S77	USPAT	OR	OFF	2018/08/14 09:31
S79	0	(dg44 OR dg-44 OR chodg44 OR cho-dg44) same "factor c"	USPAT	OR	OFF	2018/08/14 09:31
S83	2,086	(dg44 OR dg-44 OR chodg44 OR cho-dg44)	USPAT	OR	OFF	2018/09/07 11:44
S84	5,873	"factor c"	USPAT	OR	OFF	2018/09/07 11:44
S85	92	S83 and S84	USPAT	OR	OFF	2018/09/07 11:44
S86	17,635	horseshoe OR crab	USPAT	OR	OFF	2018/09/07 11:44
S87	2	S85 and S86	USPAT	OR	OFF	2018/09/07 11:44
S88	41	(dg44 OR dg-44 OR chodg44 OR cho-dg44).clm.	USPAT	OR	OFF	2018/09/07 11:44
S89	383	"factor c".clm.	USPAT	OR	OFF	2018/09/07 11:44

EAST Search History (Interference)

S90	0	S88 and S89	USPAT	OR	OFF	2018/09/07 11:44
S91	2,260	(horseshoe OR crab).clm.	USPAT	OR	OFF	2018/09/07 11:44
S92	0	S88 and S91	USPAT	OR	OFF	2018/09/07 11:44
S93	0	(dg44 OR dg-44 OR chodg44 OR cho-dg44) same "factor c"	USPAT	OR	OFF	2018/09/07 11:44

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 16/399,386	Filing Date 04/30/2019	☐ 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	09/17/2020	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 18	Minus	** 20	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 2	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".	/YOLANDA CHADWICK/
*** 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.

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Docket No: Q246419
Hikaru MIZUMURA, et al.	
Appln. No.: 16/399,386	Group Art Unit: 1656
Confirmation No.: 9293	Examiner: STEADMAN, DAVID J
Filed: April 30, 2019	

For: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

AMENDMENT UNDER 37 C.F.R. § 1.111

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

Sir:

In response to the Office Action dated June 30, 2020, please amend the above-identified application as follows on the accompanying pages.

TABLE OF CONTENTS

AMENDMENTS TO THE SPECIFICATION....2

AMENDMENTS TO THE CLAIMS....3

REMARKS....8

AMENDMENTS TO THE SPECIFICATION

Please replace the paragraph under the title at page 1 with the following amended paragraph:

Cross-Reference To Related Applications

This is a Continuation of U.S. Patent Application 15/983,725 filed May 18, 2018; which is a continuation of U.S. Patent Application 14/650,767 filed June 9, 2015, now U.S. Patent No. 10,144,923, which is National Stage of International Application No. PCT/JP2013/083082 filed December 10, 2013, claiming priority based on Japanese Patent Application No. 2012-269840 filed December 10, 2012, the contents of all of which are incorporated herein by reference in their entirety.

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions and listings of claims in the application:

LISTING OF CLAIMS:

1. (currently amended): A horseshoe crab Factor C protein having activity of Factor C,

wherein the horseshoe crab is selected from the group consisting of *Tachypleus tridentatus, Limulus polyphemus, and Carcinoscorpius rotundicauda*, and wherein the horseshoe crab Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or Human Embryonic Kidney (HEK) cell.

2. (currently amended): A Factor C protein having activity of Factor C,

wherein the Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or Human Embryonic Kidney (HEK) cell, and

wherein the expressed Factor C protein comprises an amino acid sequence selected from the group consisting of:

(A) the amino acid sequence of SEQ ID NO: 2, and the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Tachypleus tridentatus* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell;

(B) an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:2, and having activity of Factor C, and the Factor C protein contains (α-

2,3)-linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Tachypleus tridentatus* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell;

(C) the amino acid sequence of SEQ ID NO: 4, and the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Carcinoscorpius rotundicauda* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell; and

(D) an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:4, and having activity of Factor C, and the Factor C protein contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a native horseshoe crab Factor C of *Carcinoscorpius rotundicauda* and/or a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell, and

~~wherein the Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or HEK cell, and~~

wherein the Factor C protein exhibits a residual activity of at least one selected from the group consisting of:

(1) 10% or higher in the presence of 21 mM sodium citrate;

(2) 25% or higher in the presence of 52 mM sodium hydrogen carbonate;

(3) 25% or higher in the presence of 214 mM sodium chloride;

(4) 15% or higher in the presence of 16 mM magnesium sulfate.

3. (original): The Factor C protein according to claim 2, which is water soluble.

4. (original): The Factor C protein according to claim 2, which has a molecular weight of 115 kDa to 140 kDa as measured by SDS-PAGE under non-reducing condition.

5. (original): The Factor C protein according to claim 4, which has a molecular weight of 127 kDa ± 5 kDa.

6. (currently amended): The Factor C protein according to claim 2, which exhibits a residual Factor C activity of ~~25~~35% or higher in the presence of 52 mM sodium hydrogen carbonate.

7. (currently amended): The Factor C protein according to claim 2, which exhibits a residual Factor C activity of ~~25~~35% or higher in the presence of 214 mM sodium chloride.

8. (currently amended): The Factor C protein according to claim 2, which exhibits a residual Factor C activity of ~~15~~25% or higher in the presence of 16 mM magnesium sulfate.

9. (original): The Factor C protein according to claim 2, which exhibits a residual Factor C activity of 35% or higher in the presence of 2.5 mM calcium chloride.

10. (original): The horseshoe crab Factor C protein according to claim 1, which contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a corresponding native horseshoe crab Factor C protein and/or a corresponding horseshoe crab Factor C protein recombinantly expressed using Sf9 as a host cell.

11. (canceled).

12. (currently amended): A method for producing the Factor C protein of claim 2, the method comprising culturing a Chinese Hamster Ovary (CHO) DG44 cell or Human Embryonic Kidney (HEK) cell recombinantly expressing said Factor C protein under conditions suitable for the expression of the ~~horseshoe crab~~ Factor C protein to produce the Factor C protein.

13. (original): An endotoxin assay kit comprising the Factor C protein according to claim 2 and one or more elements.

14. (withdrawn): A method for measuring endotoxin in a test specimen using an endotoxin assay agent comprising the Factor C protein according to claim 2.

15. (withdrawn): The method according to claim 14, wherein the test specimen comprises at least one of:

(A) an ion;

(B) a cation;

(C) a metal ion;

(D) an alkali metal ion or an alkaline earth metal ion; and

(E) one or more kinds of ions selected from the group consisting of sodium ion, potassium ion, calcium ion, and magnesium ion.

16. (withdrawn): The method according to claim 15, wherein the test specimen comprises a cation and the final concentration of the cation after mixing the test specimen with the assay agent is 1 mM or higher.

17. (withdrawn): The method according to claim 14, wherein the test specimen is an injection.

18. (withdrawn): A product which has been subjected to an endotoxin assay using an endotoxin assay agent comprising the Factor C protein according to claim 2,

wherein the product is selected from the group consisting of water for medical use, a pharmaceutical product, an infusion solution, a blood product, a medical apparatus, a medical instrument, a biological specimen, a native protein, a recombinant protein, a nucleic acid, and a saccharide.

19. (new): The Factor C protein according to claim 2, which exhibits a residual activity of 20% or higher in the presence of 21 mM sodium citrate.

REMARKS

In the present Amendment, claims 1, 2 and 12 have been amended to add the full names for CHO and HEK. Claim 2 has also been amended to incorporate the subject matter of claims 6-8 and 11, further reciting species of the native horseshoe crab. Claims 6-8 have been amended based on paragraph [0017] of the specification. Claim 11 has been cancelled. Claim 12 has also been amended to recite "the expression of the ~~horseshoe crab~~ Factor C protein". New claim 19 has been added. Support for claim 19 is found, for example, in paragraph [0017] of the specification. No new matter has been added, and entry of the Amendment is respectfully requested.

Upon entry of the Amendment, claims 1-10 and 12-19 will be pending, of which claims 14-18 are withdrawn from consideration.

Specification/Informalities

At page 3 of the Office Action, the applicant is requested to update the status of prior-filed application no. 14/650,767 (now issued as U.S. Patent 10,144,923) in the disclosure of the specification at p. 1 under "Cross-Reference to Related Applications".

In response, the specification has been amended as requested.

Response to Claim Objection

At page 3 of the Office Action, claims 1, 2, and 12 are objected to in the recitation of "HEK". In the interest of improving claim form, it is suggested that the entire phrase for which the abbreviation is used, i.e., "human embryonic kidney", be recited at least once in the claims.

Claims 1, 2 and 12 have been amended as suggested by the Examiner. Accordingly, withdrawal of the claim objection is requested.

Response to § 112(b) Rejection

At page 3 of the Office Action, claims 11 and 12 are rejected under 35 U.S.C. § 112(b) as being indefinite.

The Examiner states that claims 11 and 12 are indefinite in the recitation of "a corresponding native horseshoe crab Factor C protein and/or a corresponding horseshoe crab Factor C protein recombinantly expressed using Sf9 as a host cell" and "the horseshoe crab Factor C", respectively.

Per the Examiner, claim 2 (from which claims 11 and 12 depend) encompasses Factor C variants of SEQ ID NO: 2 or 4 that do not have a "corresponding native horseshoe crab Factor C protein" or "a corresponding horseshoe crab Factor C protein" (for claim 11) and encompasses variants that are not a horseshoe crab Factor C (for claim 12).

In the interest of compact prosecution, the Examiner suggests that the noted phrase of claim 11 be amended to recite, e.g., "a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell" and the noted phrase of claim 12 be amended to recite, e.g., "the Factor C protein".

Applicant responds as follows.

The recitation of claim 11 has been amended and introduced into claim 2. As to the recitation "corresponding native horseshoe crab Factor C protein", as described for example in paragraph [0020] of the specification, SEQ ID NO:2 is the amino acid sequence of Factor C protein of *Tachypleus tridentatus*, and SEQ ID NO:4 is the amino acid sequence of Factor C protein of *Carcinoscorpius rotundicauda*.

Accordingly, species of horseshoe crab are defined in each of items (A) to (D) of claim 2, and no "corresponding native horseshoe crab Factor C protein" is recited. Specifically, claim 2

as amended recites "as compared with a native horseshoe crab Factor C of *Tachypleus tridentatus*" or "as compared with a native horseshoe crab Factor C of *Carcinoscorpius rotundicauda*".

As to the recitation "a corresponding horseshoe crab Factor C protein recombinantly expressed using Sf9 as a host cell", it has been amended to "a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell" according to the Examiner's suggestion.

The recitation of claim 12 has been amended according to the Examiner's suggestion.

In view of the above, reconsideration and withdrawal of the § 112(b) rejection are respectfully requested.

Response to § 112(a) Rejection

At page 4 of the Office Action, claims 2-9, 12 and 13 are rejected under 35 U.S.C. § 112(a) as failing to comply with the written description requirement.

Claim 2 has been amended to incorporate the subject matter of claim 11. Claim 11 is not subject to this rejection. Accordingly, reconsideration and withdrawal of the § 112(a) rejection are respectfully requested.

Response to § 103 Rejection

At page 9 of the Office Actin, claims 1-13 are rejected under 35 U.S.C. § 103 as being unpatentable over Ding et al. (US 5,858,706; hereafter "Ding") in view of Current Protocols in Molecular Biology (1993) 16.23.1-16.23.13, John Wiley and Sons, Inc., 2002 (hereafter "Current Protocols") and as evidenced by Mizumura et al. (WO 2012/118226 A1; hereafter "Mizumura").

This rejection should be withdrawn because Ding and Current Protocols do not disclose or render obvious the claimed invention, either alone or in combination.

The Factor C of the claimed invention provides unexpected effects in terms of salt tolerance compared to the native Factor C (Fig. 7 of the present specification) whereas Fig. 17 of Ding shows that LPS affinity of recombinant Factor C is inferior to that of native Factor C.

That is, neither Ding nor Current Protocols discloses or suggests the effects of the claimed invention of providing improved sensitivity of endotoxin assay in the presence of a salt, by increasing terminal sialic acid of Factor C contained in endotoxin assay reagents.

Specifically, FIG. 17 of Ding shows test result of binding ability to LPS (Lipopolysaccharide; component of endotoxin) of recombinant Factor C (rCrFC) produced in yeasts as a host cell compared with that of native Factor C (CAL). A band of about 90-100 kDa corresponds to a complex with LPS, namely endotoxin. FIG. 17 shows that the band of about 90-100 kDa of rCrFC sample is clearly paler than that of CAL sample. Therefore, one skilled in the art would naturally understand that Factor C produced in yeasts (rCrFC in FIG. 17) has lower affinity to endotoxin than that of native Factor C (CAL in FIG. 17), and that the sensitivity of endotoxin assay reagent using rCrFC in the presence of the salt becomes lower than that using native Factor C.

Further, Ding discloses mammalian cells and yeasts in parallel as host cells for expression of rCrFC that do not produce bacterial endotoxin (lines 3 to 12 in column 3), and rCrFC is especially effectively produced in yeasts (lines 42 to 44 in column 7). Ding discloses the application of rCrFC to remove endotoxin from a liquid samples by binding of endotoxin in the sample to the immobilized rCrFC, thereby forming bound endotoxin (lines 5 to 11 in column 8). Accordingly, one skilled in the art reading Ding would naturally understand that yeasts especially effectively produce recombinant Factor C useful for binding to endotoxin, and Factor C produced in mammalian cells does not have at least higher endotoxin binding properties as

Factor C produced in yeasts. Namely, one skilled in the art would understand that rCrFC produced in mammalian cells has lower affinity to endotoxin than that of native Factor C, and that highly sensitive endotoxin detection is difficult by using rCrFC produced in mammalian cells.

In contrast, the claimed invention using the recombinant Factor C having (α-2,3)-linked terminal sialic acid in a greater amount for the endotoxin assay reagent exhibits effects of providing improved sensitivity in the presence of the salt compared with the endotoxin assay reagent using the native Factor C (see FIG. 7 of the present specification), which effects are unexpected from Ding and Current Protocols.

Further, Applicant submits herewith a Declaration under 37 C.F.R. § 1.132 executed by Hikaru Mizumura, the first-named inventor of the present application, which describes that the Factor C derived from three species of *T. tridentatus* (SEQ ID NO:2 of the present application), *Limulus Polyphemus* and *C. rotundicauda* (SEQ ID NO:4 of the present application) have amino acid sequence identity at 90% or higher of each other. Further, amino acids discordance in species exist also in function domains (for example, LPS binding domain or C-terminal catalytic domain).

Accordingly, one skilled in the art could understand that a protein comprising an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:2 or NO: 4 maintains the Factor C activity.

The Declaration shows experimental results of evaluation of inhibitory activity by injection according to the method of "5. Inhibition of activity by injection" of the present specification (paragraphs [0134]-[0138] of the specification), by using *L. Polyphemus* Factor C (as to identity between horseshoe crab species) as one example of the protein having about 90%

sequence identity to the amino acid sequence of SEQ ID NO:2 (*T. tridentatus*) and SEQ ID NO: 4 (*C. rotundicauda*).

As shown in the Declaration, the protein having about 90% sequence identity to the amino acid sequence of SEQ ID NO:2 (*T. tridentatus*) and SEQ ID NO: 4 (*C. rotundicauda*) (namely, *L. Polyphemus* Factor C) actually shows high reactivity with endotoxin under a high salt concentration condition.

Accordingly, one skilled in the art would understand that the effects of the claimed invention (shown in Fig. 7 of the present specification) is also provided by the Factor C protein of *Limulus Polyphemus* or *Carcinoscoryius rotundicauda* of claim 1, or the Factor C protein of item (B) or (D) of claim 2.

In view of the above, the present claims are not obvious and are patentable over Ding and Current Protocols, either alone or in combination. Reconsideration and withdrawal of the §103 rejection based on Ding and Current Protocols are respectfully requested.

Response to Double Patenting Rejections

1. At page 18 of the Office Action, claims 1-13 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-8 and 13-20 of U.S. Patent No. 10,144,923 B2 (hereafter "patent").

2. At page 19 of the Office Action, claims 1-13 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1, 2, 4, 9, and 12-15 of copending Application No. 15/983,725 (reference application).

3. At page 19 of the Office Action, claims 1-13 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1 and 4 of copending Application No. 16/331,810 (reference application) in view of Ding and Current Protocols.

Without admitting that the above double patenting rejections are proper, Applicant submits herewith an E-Terminal Disclaimer to obviate the rejection.

Allowance is respectfully requested. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,

/Hui C. Wauters/

Hui C. Wauters
Registration No. 57,426

SUGHRUE MION, PLLC
Telephone: 202.293.7060
Facsimile: 202.293.7860

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

Date: September 17, 2020

Attachment:

Declaration under 37 C.F.R. § 1.132 executed by Hikaru Mizumura.

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Docket No: Q246419
Hikaru MIZUMURA, et al.	
Appln. No.: 16/399,386	Group Art Unit: 1656
Confirmation No.: 9293	Examiner: STEADMAN, DAVID J
Filed: April 30, 2019	

For: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

INFORMATION DISCLOSURE STATEMENT
UNDER 37 C.F.R. §§ 1.97 and 1.98

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

Sir:

In accordance with the duty of disclosure under 37 C.F.R. § 1.56, Applicant hereby notifies the U.S. Patent and Trademark Office of the documents which are listed on the attached PTO/SB/08 (modified) form and/or listed herein.

One copy of each of the listed documents is submitted herewith.

The present Information Disclosure Statement is being filed after the later of three months from the application's filing date and the mailing date of the first Office Action on the merits, but before a Final Office Action, Notice of Allowance, or an action that otherwise closes prosecution in the application (whichever is earlier), and therefore Applicant is filing concurrently herewith a Statement Under 37 C.F.R. § 1.97(e). No fee under 37 C.F.R. § 1.17(p) is required.

The submission of the listed documents is not intended as an admission that any such document constitutes prior art against the claims of the present application. Applicant does not waive any right to take any action that would be appropriate to antedate or otherwise remove any listed document as a competent reference against the claims of the present application.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,

/Hui C. Wauters/

Hui C. Wauters
Registration No. 57,426

SUGHRUE MION, PLLC
Telephone: 202.293.7060
Facsimile: 202.293.7860

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

Date: September 17, 2020

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16/399,386
Confirmation Number	9293
Filing Date	April 30, 2019
First Named Inventor	Hikaru MIZUMURA
Art Unit	1656
Examiner Name	STEADMAN, DAVID J
Attorney Docket Number	Q246419

U.S. PATENTS

Examiner Initials*	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

U.S. PATENT APPLICATION PUBLICATIONS

Examiner Initials*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No	Foreign Document Number[3]	Country Code[2]	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]

NON-PATENT LITERATURE DOCUMENTS

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, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1.	Final Office Action dated August 4, 2020 issued in U.S. Application No. 16/399,575	
	2.	DAMASCENO et al, "Protein secretion in *Pichia pastoris* and advances in protein production", Appl Microbiol Biotechnol (2012) 93: pp. 31-39, Published online: November 2011	
	3.	Final Office Action dated August 28, 2020 issued in U.S. Application No. 15/983,725	
	4.	KNAPPSKOG et al, "The level of synthesis and secretion of *Ganssia princeps* luciferase in transfected CHO cells is heavily dependent on the choice of signal peptide", Journal of Biotechnology, Vol. 128, 2007, pp. 705-715	

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	16/399,386
	Confirmation Number	9293
	Filing Date	April 30, 2019
	First Named Inventor	Hikaru MIZUMURA
	Art Unit	1656
	Examiner Name	STEADMAN, DAVID J
	Attorney Docket Number	Q246419

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).

☐ That each item of information contained in the Information Disclosure Statement filed concurrently herewith: (i) was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement; or (ii) is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement. See 37 CFR 1.704(d).

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

☑ The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

☐ A certification statement is not submitted herewith.

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

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	/Hui C. Wauters/	Date (YYYY-MM-DD)	2020-09-17
Name/Print	Hui C. Wauters	Registration Number	57,426

*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.

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Docket No: Q246419
Hikaru MIZUMURA, et al.	
Appln. No.: 16/399,386	Group Art Unit: 1656
Confirmation No.: 9293	Examiner: STEADMAN, DAVID J
Filed: April 30, 2019	

For: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

DECLARATION UNDER 37 C.F.R. § 1.132

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

Sir:

I, Dr. Hikaru MIZUMURA, hereby declare and state:

I am a citizen of Japan;

I have received the degrees of Bachelor of Science in 1993, Master of Science in 1996, and Doctor of Philosophy (Ph. D.) in 1999 from Saitama University;

I was employed by RIKEN (the Institute of Physical and Chemical Research) from April 1, 1999 to March 31, 2002 as Special Postdoctoral Researcher of Laboratory of Cellular and Molecular Biology, with responsibility for research on endonuclease from yeast mitochondria;

I was employed by Tokai University School of Medicine from April 1, 2002 to March 31, 2005 as Postdoctoral Researcher of Department of Molecular Neuroscience, with responsibility for research on biochemical characterization of ALS2 protein;

I was employed by The University of Tokyo from April 1, 2005 to August 31, 2007 as Postdoctoral Researcher of Laboratory of Microbiology, with responsibility for research on DNA replication in virulence bacteria Staphylococcus aureus;

I have been employed by Seikagaku Corporation since September 1, 2007, where I hold a position as chief researcher of LAL Research & Development Department, Central Research Laboratories, with responsibility for research and development of reagents for measuring endotoxin and/or glucan by using horseshoe crab proteins; and

I am the first-named inventor of the above-identified application; I am familiar with the subject matter of the above-identified application and the Office Action mailed June 30, 2020 in the above-identified application.

This Declaration is submitted to explain and demonstrate that the specification of the above-identified application has a sufficient written description of our invention and those skilled in the art know how to make and use the full scope of our invention without "undue experimentation". The following additional experiments were conducted by me or under my direct supervision.

Factor C from *Tachypleus tridentatus* (TtFC) is a Lipopolysaccharide (LPS)-induced serine protease zymogen revealing a unique mosaic structure with multiple domains, as illustrated in Figure 7 of reference 1 (references are listed on page 4 and copies are submitted herewith). LPS-binding site and serine protease active site are located in the N-terminal Cys-rich region and the C-terminal catalytic domain, respectively[1, 2] (Fig. 1). Both parts are essential for LPS-induced protease activity of TtFC[3, 4].

We have succeeded in cloning cDNA encoding Factor C from *Limulus polyphemus* (=LpFC) hemocyte[5]. Figure 1 is the protein sequence alignment of three types of Factor Cs (TtFC (SEQ ID No:2), CrFC (SEQ ID No:4, from *Carcinoscorpius rotundicauda*) and LpFC). Different amino acids locate within the entire sequence of Factor Cs including the N-terminal Cys-rich region and the C-terminal catalytic domain. Table 1 presented below shows that each of Factor Cs has more than 90% amino acid sequence identity.

(Table 1) Amino acid sequence identity of three types of Factor Cs

Factor C between	Identity (%)
Tt and Cr	990 / 1019 *100 = 97.2
Tt and Lp	936 / 1020 *100= 91.8
Lp and Cr	926 / 1020 *100= 90.8

In spite of the difference in amino acid sequence, all of the three types of Factor Cs are known to express LPS-induced protease activity in common[4, 6, 7].

Therefore, I believe that those of ordinary skill in the art would expect that substitution(s) up to 90% identity in the amino acid sequence of SEQ ID No: 2 or 4 would result in maintaining Factor C activity.

Further, in reference to "*(2) Expression of Factor C in Mammalian Cells (CHO DG44)*" (paragraph [0107] to [0112] of the present specification) and "*5. Inhibition of Activity by Injection*" (paragraph [0134] to [0138] of the present specification), soluble LpFC was

recombinantly expressed in CHO cell as host cell, and the filtrated cell culture supernatant was used for evaluation of salt ion resistance. In this experiment, a DNA coding LpFC which has 91.8 and 90.8 % amino acid sequence identity respectively to SEQ ID No: 2 and 4 was used instead of the DNA encoding TtFC described in Example 1 of the present specification. Figure 2 shows the results from measuring residual activity in the presence of a salt ion. In Figure 2, the data of TtFC is a reprint of Fig.7 in the present specification.

As shown in Figure 2, a protein comprising an amino acid sequence having about 90% sequence identity to the amino acid sequence of SEQ ID NO:2 can also exhibit salt ion resistance.

Therefore, I believe that a protein comprising an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:2 or 4 expressed in the mammalian cell can have salt ion resistance and/or water solubility, and retain activity of Factor C.

References:

(1) *J Biol Chem.* Apr 5, 1991; 266(10):6554-61.

(2) *J Biol Chem.* Feb 9, 2007; 282(6):3962-7.

(3) *Invertebrate Survival Journal.* 2009; 6:59-77.

(4) *Eur J Biochem.* Feb 3, 1986; 154(3):511-21.

(5) US 2019/0241629 A1.

(6) *Biochem Int.* Aug 1990; 21(5):805-13.

(7) *J Biochem.* Jan 1991; 109(1):150-7.

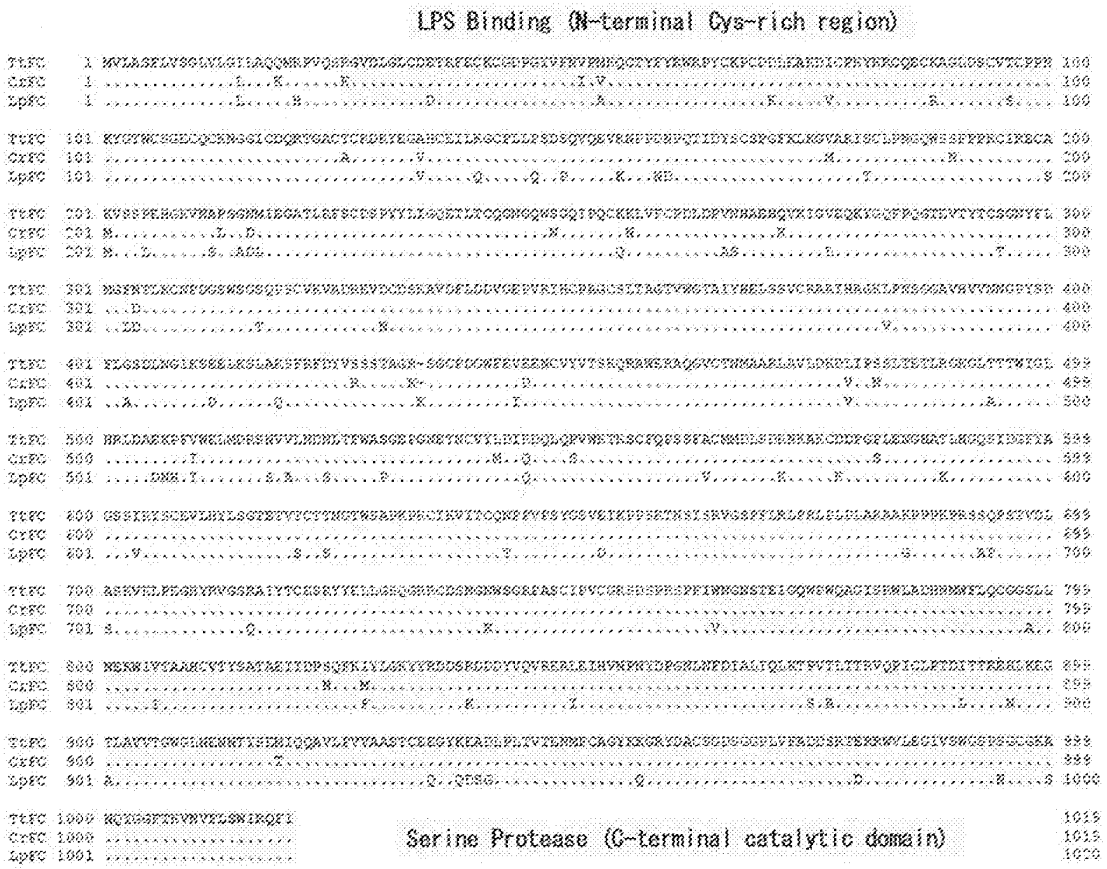


Figure 1 : Protein sequence alignment of three types of Factor Cs

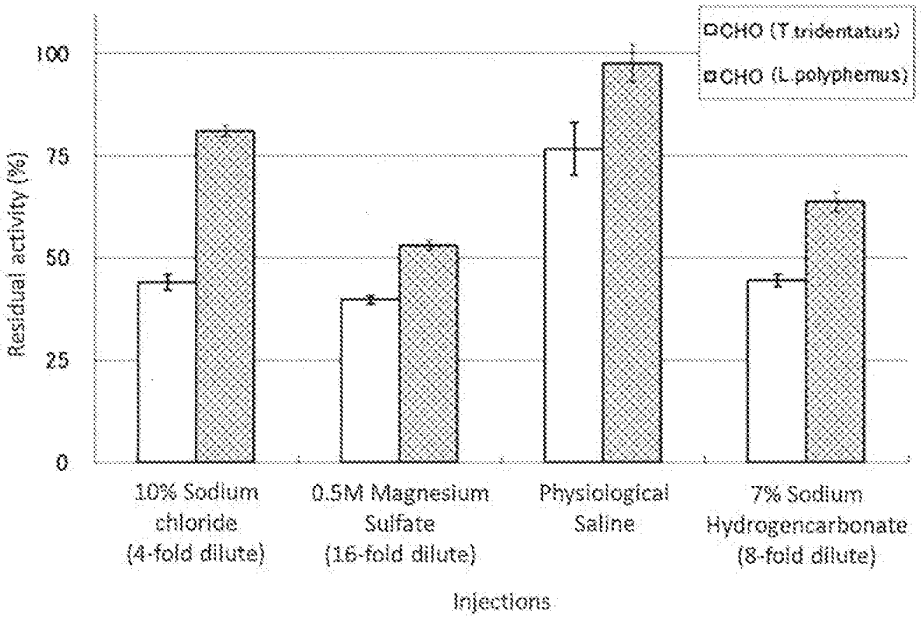


Figure 2: Residual activity of LpFC and TtFC

I declare further that all statements made herein of my 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 the application or any patent issuing thereon.

Date: 09 SEP 2022

Hikaru Mizumura
Dr. Hikaru MIZUMURA

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Printed in U.S.A.
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Limulus Factor C

AN ENDOTOXIN-SENSITIVE SERINE PROTEASE ZYMOGEN WITH A MOSAIC STRUCTURE OF COMPLEMENT-LIKE, EPIDERMAL GROWTH FACTOR-LIKE, AND LECTIN-LIKE DOMAINS*

(Received for publication, October 4, 1990)

Tatsushi Muta‡, Toshiyuki Miyata‡§, Yoshio Misumi¶, Fuminori Tokunaga§, Takanori Nakamura§‖, Yoshihiro Toh§, Yukio Ikehara¶, and Sadaaki Iwanaga‡§**

From the ‡Department of Molecular Biology, Graduate School of Medical Science, and §Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812, and ¶Department of Biochemistry, Fukuoka University School of Medicine, Fukuoka 814-01, Japan

Factor C is an endotoxin-sensitive, intracellular serine protease zymogen which initiates the coagulation cascade system in the limulus hemolymph. We have determined the entire amino acid sequence of factor C using recombinant DNA technique. The zymogen consisted of 994 amino acid residues with a calculated molecular mass of 109,648 Da. Most interestingly, factor C has five repeating units ("Sushi" domain or short consensus repeat) of about 60 amino acid residues each, which have been found in many proteins participating in the mammalian complement system. In addition to a typical serine protease domain in the carboxyl-terminal portion, characteristic segments with an epidermal growth factor-like, a lectin-like, a cysteine-rich, and a proline-rich domain were also found, revealing a unique mosaic protein structure. The serine protease domain was most analogous to human thrombin. Factor C was identified to localize in large granules in the cell, indicating that it is released from the cell by lipopolysaccharide stimulation. Furthermore, we identified a transcript possibly derived by alternative splicing of factor C mRNA, which encodes a protein sharing the amino-terminal portion of factor C. We suggest that factor C, a newly discovered type of serine protease zymogen, is a "coagulation-complement factor" which may play important roles in both hemostasis and host defense mechanisms.

Invertebrates have characteristic host defense systems which differ from mammalian immune systems (1). In Limulus (horseshoe crab), an *Arthropoda* species, this defense system is carried by the hemolymph, which contains a type of cell called an amebocyte. The amebocytes are extremely sensitive to endotoxin (LPS)[1], which is a major component of the cell surface of Gram-negative bacteria. When hemolymph comes into contact with Gram-negative bacteria or LPS, the amebocytes begin to degranulate and hemolymph coagulation is initiated by the granule components (2, 3). This response is thought to be very important for Limulus not only in preventing the leakage of hemolymph, but also in engulfing invading microorganisms.

During the past decade we have studied the molecular mechanism of hemolymph coagulation in Limulus and have established a protease cascade. This cascade is based on three kinds of serine protease zymogens, factor C (4), factor B (5), proclotting enzyme (6), and one clottable protein, coagulogen (7). Endotoxin (LPS) activates the zymogen factor C to the active form, factor $\bar{C}$. Factor $\bar{C}$ then activates factor B to factor $\bar{B}$, which in turn converts the proclotting enzyme to the clotting enzyme. Each activation proceeds by limited proteolysis. The resulting clotting enzyme cleaves two bonds in coagulogen, which is a fibrinogen-like molecule in arthropods, to yield an insoluble coagulin gel. The coagulation cascade is also activated by (1,3)-β-D-glucan. In the presence of (1,3)-β-D-glucan, a protease that is tentatively named factor G is activated, leading to activation of the proclotting enzyme (8).

Factor C that binds LPS exists as a single-chain glycoprotein with an estimated molecular mass of 123 kDa or as a two-chain form, composed of one heavy (H) and one light (L) chain (4, 9). When this protein binds to LPS, it is autocatalytically activated to the active form (factor $\bar{C}$). Upon activation, a Phe-Ile bond in the L chain is cleaved, generating an A and a B chain. The resulting factor $\bar{C}$ is composed of the disulfide-linked H, A, and B chains (mentioned in order of appearance from the amino terminus in the single-chain form of factor C). The activation of factor C by Phe-Ile cleavage was quite unexpected. This is the first known activation mechanism in the coagulation and complement cascade systems involving a chymotrypsin-like specificity.

Our previous results have shown that the B chain of factor $\bar{C}$ has a serine protease domain, while the LPS-binding site is located in the H chain (10, 11). In the A chain we found a complement-like structure composed of about 60 amino acid residues (9). These characteristics of factor C suggest that this protein is a mosaic protein with a structure similar to that of complement component C1r, which is an initiator of the mammalian complement cascade.

To obtain more information about the domain-structure of this molecule, the structure of the LPS-binding site(s) and the mechanism of autocatalytic activation, we have established the cDNA sequence for factor C mRNA. Structural comparison of factor C with other proteins in various systems

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) D90271 and D90272.

This article is dedicated to the memory of Staffan Magnusson.

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[1] The abbreviations used are: LPS, lipopolysaccharide; bp, base pairs; kb, kilobases; EGF, epidermal growth factor.

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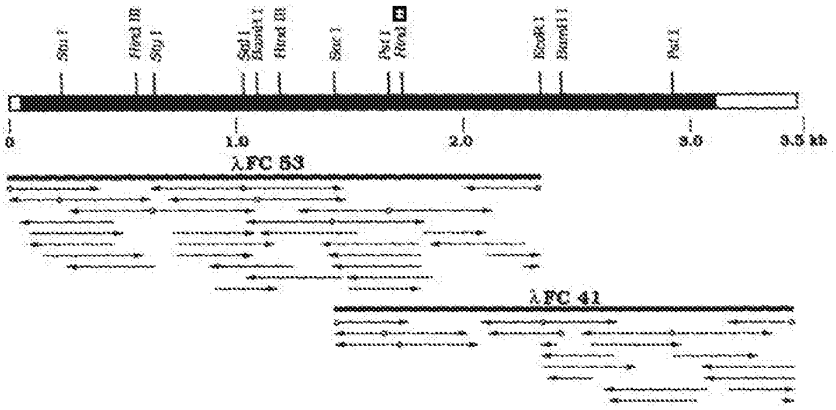


FIG. 1. **Restriction map and sequence strategy.** A *black box* at the top shows the open reading frame. *Arrows* with *circles* show sequencing utilizing restriction sites and *arrows* without *circles* were by deletions.

may offer insights into the evolution of host defense systems in the animal kingdom.

MATERIALS AND METHODS[*]

RESULTS

Cloning of Factor C cDNA—Four-hundred thousand independent clones of the λgt 11 cDNA library constructed from amebocyte poly(A)$^+$ RNA were screened with anti-factor C IgG. Of 16 positive clones purified, the longest positive clone, λFC53, was sequenced. This clone was shown to encode the 5′-noncoding region, the entire H and A chain, and an amino-terminal portion of the B chain (nucleotides 1–2467). To obtain clones encoding the remaining part of the B chain, we prepared a second cDNA library and screened it with the [^{32}P] λFC53 insert as a hybridization probe. The clone λFC41 encoding a cDNA sequence covering the carboxyl-terminal region of factor C starting in the middle part of the H chain (nucleotides 1438–3474), was thus obtained and sequenced (Fig. 1). The composite nucleotide sequence obtained from these two clones spans 3,474 bp (Fig. 2). The first ATG codon at nucleotide 45 begins an open reading frame of 3,057 bp followed by a TGA termination codon and a 365-bp 3′-noncoding sequence before a poly(A) tail. There is one potential polyadenylation or processing signal with the sequence AATAAA in the 3′-noncoding sequence which begins 30 bp before the poly(A) tail. The open reading frame encodes a putative signal sequence of 25 amino acid residues and a mature protein of 994 residues. The molecular mass of the mature protein without carbohydrate chains was calculated to be 109,648 Da. There are six potential *N*-glycosylation sites within the sequence Asn-Xaa-Ser/Thr. The potential glycosylation site (Asn^{715}) has been shown not to be glycosylated.

Partial Amino Acid Sequences of the Peptides Obtained from Factor C—To confirm the sequences of these clones, we determined partial amino acid sequences of factor C. The S-alkylated H and L chains were digested with lysyl endopeptidase or trypsin (Fig. M1). Purified peptides, whose purity was supported by their amino acid compositions, were sequenced. In this way, we obtained the sequences of 347 amino acid residues. Together with the peptide sequences published previously (9), amino acid sequences covering a total of 493 residues (*underlined* in Fig. 2), which comprises 49.6% of the mature protein, have been confirmed by protein sequencing. The amino acid sequences agree completely with the sequence deduced from the cDNA sequence.

Northern Blotting—Northern blot analysis of amebocyte poly(A)$^+$ RNA showed a band at about 3.9-kb hybridizing with λFC53, in addition to a faint 2.0-kb band, which indicates that the composite sequence of 3.5 kb contains almost the full-length factor C cDNA when taking the length of the poly(A) tail into consideration (Fig. 3, *lane 1*).

The faint band at 2.0 kb observed in Northern blotting was thought to be a transcript with similar but not identical sequence to factor C mRNA. To investigate the sequence difference between the two bands, we prepared two kinds of probes from λFC53. One of them was an *Eco*RI-*Sty*I fragment of λFC53, encoding the 5′-terminal part of the cDNA, and the other was the 3′-terminal part of λFC53, a *Pst*I-*Eco*RI fragment, corresponding to the carboxyl-terminal part of the H and A chain. The 2.0-kb band was also observed in the 5′-terminal probe (data not shown). However, it was not detectable in the 3′-terminal probe (Fig. 3, *lane 2*).

Cloning of the Transcript Corresponding to the 2.0-kb Band—By using the difference in reactivity to these two probes, we searched for clones corresponding to the 2.0-kb bands from the positive clones previously obtained. One clone, λFC42, with a length of 1,915 bp, was isolated as a clone that hybridized with the 5′-terminal fragment but not with the 3′-terminal fragment. To deny the possibility that this clone was derived from cloning artifacts, a screening was newly carried out against one million clones. Of 194 clones that hybridized with λFC53, two independent clones, λAS1 and λAS2, did not hybridize with the 3′-terminal fragment. Both of them had the same sequence as λFC42 (Fig. M2).

The sequence analysis revealed that λFC42 contains 55 bp of 5′-noncoding region, 1,494 bp of an open reading frame, 357 bp of 3′-noncoding region with a polyadenylation signal, and a short poly(A) tail. The downstream sequence from nucleotide 1528 was distinct from that of factor C, whereas the portion involving nucleotides 12–1527 was identical to that of nucleotides 1–1516 of factor C (Fig. 4). A protein encoded by these clones has 473 amino acid residues with a molecular mass of 51,652 Da, of which the amino-terminal 466 amino acids are identical to those of factor C. This clone seemed to have arisen by alternative splicing (see "Discussion").

Northern blotting using a probe from the 3′-terminal *Eco*RV-*Eco*RI fragment of λFC42, that is unique to this clone, gave a band at the position corresponding to the 2.0-kb band (Fig. 3, *lane 3*).

[*] Portions of this paper (including "Materials and Methods," Figs. M1 and M2, and Table M1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

FIG. 2. The composite nucleotide sequence (*upper*) and the deduced amino acid sequence (*lower*) of factor C. A polyadenylation signal (AATAAA) is *double-underlined*. The amino acid sequence is *numbered* beginning at the amino terminus of the mature factor C molecule. The amino acid residues that have been confirmed by sequencing of purified peptides are *underlined* and indicate data from Tokunaga *et al.* (9). Amino-terminal amino acid sequencing of each chain identified the three cleavage sites marked by *arrowheads*. The potential carbohydrate attachment sites for (Asn-Xaa-Ser/Thr) are indicated with *closed diamonds* and the putative attachment site confirmed not to carry *carbohydrate* is shown as an *open diamond*. The *small* amino acid residues suspected to form the catalytic triad of the serine protease domain are *circled*.

FIG. 3. Northern blot analysis of factor C mRNA. Five μg of polyA$^+$ RNA from amebocytes were separated by 1.0% agarose gel electrophoresis, transferred to the membrane, and hybridized with ^{32}P-labeled probes. Hybridization probes used were the *Xho*I-*Eco*RI fragment of AFC33 (*lane 2*), or an *Eco*RV-*Eco*RI fragment of AFC32 (*lane 3*), respectively. See text for details.

FIG. 4. The nucleotide and deduced amino acid sequences of AFC42. The sequences of the regions which differ from factor C mRNA are shown. Nucleotides 12–1527 are identical with nucleotides 1–1516 of factor C (Fig. 2). The possible 5′ splice junction consensus sequence, (*T*), is shown by an *underline*. The potential *N*-glycosylation site is shown as a *closed diamond*. A polyadenylation signal (AATAAA) is *double-underlined*.

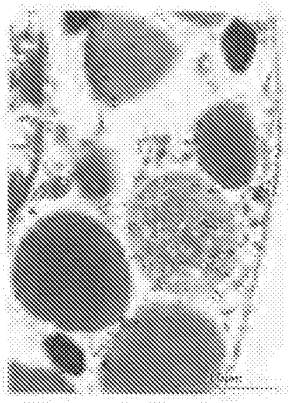


FIG. 5. **Subcellular localization of Factor C.** Hemocytes were fixed, stained with anti-factor C IgG and gold-particle-conjugated second antibody, and then sectioned. Numerous gold particles are present in the large granules. The *bar* is 1 μm.

Subcellular Localization of Factor C—Using anti-factor C polyclonal antibody, the subcellular localization of factor C was examined. Sections of the amebocytes were treated with anti-factor C antibody followed by gold particle-conjugated second antibody. Many gold particle conjugates were observed mainly in large granules in the cell, indicating that factor C antigen is localized in the large granules and exocytosed by stimulation with LPS (Fig. 5).

DISCUSSION

A computer-assisted homology search indicated that several interesting folding structures exist in the sequence of factor C. First, five internally repeated units with about 60 amino acid residues each are present, four in the H chain in addition to one existing in the A chain (Fig. 6*A*). These units are called "Sushi" domains (16) because their disulfide folding structure, which was first determined in β_2-glycoprotein I (17), resembles Sushi, in traditional Japanese cuisine. They are also called short consensus repeat. This kind of domain has been found as tandem repeats in more than 20 proteins, mainly in proteins participating in the mammalian complement system. Most of the complement factors shown to have this domain interact with C3b or C4b (18). Actually, some of the Sushi domains in complement receptor type 1 (CR1) have been shown to be binding sites for C2b or C4b (19). They seem to provide sites for protein-protein interactions in other proteins. Sushi domains in factor C may interact with other proteins such as Limulus factor B or other components. No protein containing the Sushi domain has, until now, been identified in invertebrates. Factor C is the first example of a protein that has a complement-like structure in invertebrates.

Second, an epidermal growth factor (EGF)-like domain is present in the H chain (Fig. 6*B*). This EGF-like domain displays most similarity to that of the laminin B2 chain. Proteins containing this type of domain involve several blood coagulation factors, complement factors, some receptors, including the low density lipoprotein receptor. Cys^{501} and Cys^{511}, Cys^{507} and Cys^{520}, and Cys^{513} and Cys^{531} are thought to form disulfide bonds by analogy with the folding structure determined in bovine factor X (20). Asp^{506}, which is a site for β-hydroxylation in some EGF-like domains, was shown to be unmodified by amino acid and sequence analyses.

Third, a lectin-like domain was unexpectedly found between the third and fourth Sushi domains. This region shows similarity to so called C-type lectins (21) (Fig. 6*C*). No protease has, until now, been known to include a lectin-like domain. This type of domain is also found in proteins whose functions are not limited to binding of carbohydrates, such as the IgE receptor, and pulmonary surfactant-associated protein. Moreover, this domain shows similarity to cartilage link protein and proteoglycan core protein.

There are two potential glycosylation sites in this domain (Asn^{606} and Asn^{714}). Both of these asparagines could not be identified in amino acid sequencing, suggesting that these residues are modified. Cys^{577} and Cys^{587}, Cys^{607} and Cys^{708}, and Cys^{614} and Cys^{630} are thought to be linked to one another based on the disulfide folding structure determined in the lectin from acorn barnacles (22).

Two other characteristic sequences were found in the H chain. One is a Cys-rich region, which is located in the amino-terminal portion of the H chain. Eleven cysteine residues out of 56 are clustered in this region. The other is in the carboxyl-terminal portion of the H chain, in which 15 of 48 residues are proline. This region shows similarity to connecting region of mammalian coagulation factor XII (23) (Fig. 6*D*).

The B chain portion of factor C is composed of a typical serine protease, with a catalytic triad, His^{743}-Asp^{789}-Ser^{885}, judged from similarity with other proteases. Six cysteines out of seven in the B chain are believed to form disulfide bonds between Cys^{760} and Cys^{785}, Cys^{809} and Cys^{836}, and Cys^{877} and Cys^{897} by analogy with those of trypsin (24). The remaining Cys^{860} is considered to pair with Cys^{707} in the A chain. The B chain shows the strongest similarity to human α-thrombin (36.7%). This is consistent with the result that factor C has a similar specificity toward synthetic fluorogenic peptide substrates as α-thrombin (4). The substrate-binding site, Asp^{789}, is conserved, which indicates that factor C is a trypsin type enzyme, although the cleavage site associated with zymogen activation is Phe^{707}-Ile^{708} (9). This proline residue at the P_2 site could be important for cleavage. As a matter of fact, the peptide bond cleaved during conversion to the two-chain form, Arg^{806}-Ser^{807}, is also preceded by a proline residue.

The mosaic structure of factor C is summarized in Fig. 7. Three membrane proteins, endothelial leukocyte adhesion molecule (25), lymph node homing receptor (26), and granule membrane protein 140 (27), have been reported to contain a lectin-like, an EGF-like domain and several Sushi-like domains. They are thought to play roles in cell recognition and the name "Selectin" has been proposed for them. The similarity in domain composition between factor C and these proteins is of interest because these three proteins all seem to be associated with biological defense systems. Factor C may play another role, for example, cell adhesion after degranulation, which seems to be important for the removal of clots and foreign material.

There is a report indicating that all components necessary for hemolymph coagulation exist in the granules of the amebocytes (28). Our immunohistochemical observation with electron microscopy demonstrates that factor C is localized in the large granules in the cell. When LPS comes in contact with amebocytes, these granules are secreted and hemolymph coagulation is initiated by the components of the granules.

We also cloned a minor transcript found on Northern blotting which shares the 5′-terminal sequence with factor C mRNA. Since the boundary in λFC42 contains the sequence GT (nucleotides 1527 and 1528), a conserved sequence at 5′ splice sites, this region appears to be a 5′ splicing junction site and alternative splicing results in the transcription of this clone. The protein encoded by this transcript has the same amino-terminal region as factor C, including the Cys-rich, the EGF-like, and three Sushi domains and the first half of the lectin-like domain (Fig. 7). Only 7 amino acid residues with

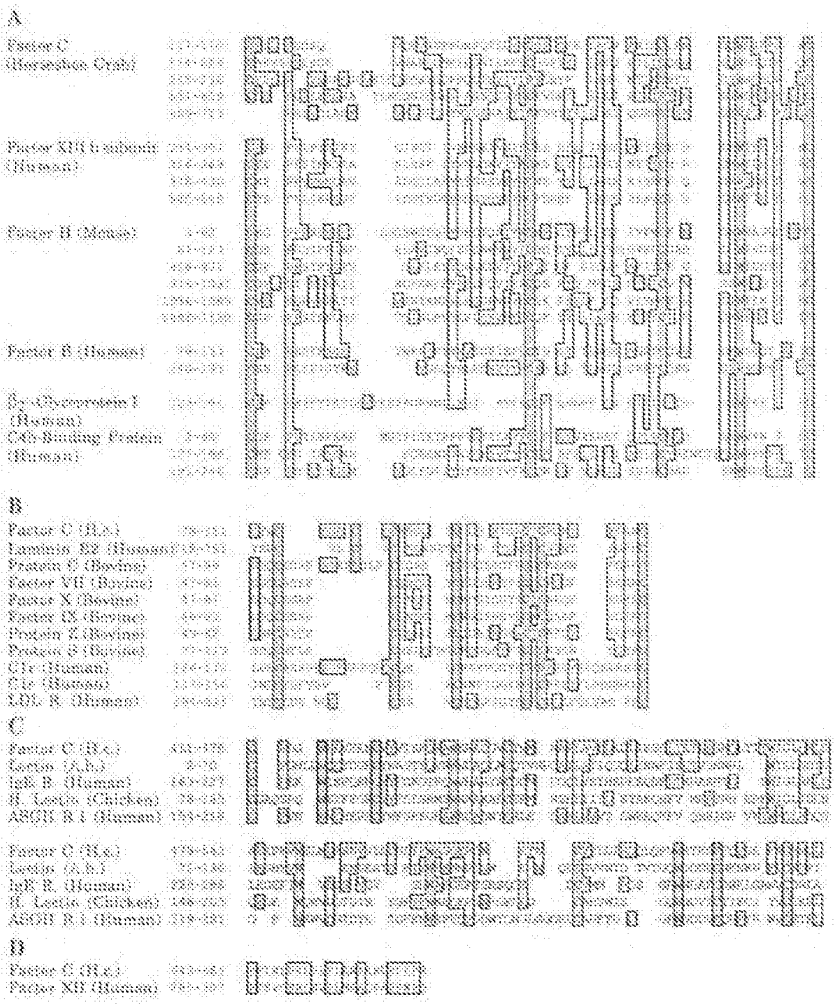


FIG. 6. **Sequence similarities between factor C and other proteins: Sushi domains (*A*); EGF-like domains (*B*); lectin-like domains (*C*); proline-rich regions (*D*).** The sequence references are as follows, *A*, human factor XIII b subunit, mouse factor H, human factor H, human β_2-glycoprotein I, and human C4b-binding protein (29). *B*, human laminin B2 chain, (30), bovine protein C, factor X, factor IX, protein Z, and protein S, and human C1r and low density lipoprotein receptor (31), bovine factor VII (32), and human C1s (33). C, lectin from acorn barnacles (22), human IgE receptor, and chicken hepatic lectin (21) and human asialoglycoprotein receptor 1 (34). *D*, human factor XII (25). Amino acid residues identical with those of factor C are *boxed*. Conserved cysteine and tryptophan residues are *shadowed*.

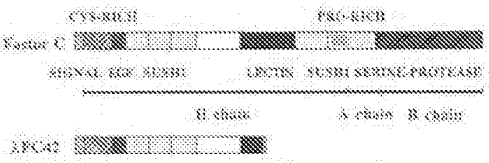


FIG. 7. **The domain structure of factor C.** Factor C has a mosaic structure of several domains including a signal peptide (−25 to −1), one Cys-rich region (1–76), one EGF-like domain (77–111), five Sushi domains (117–176, 174–228, 235–296, 551–609, and 615–728), one lectin-like domain (411–543), one Pro-rich region (618–685, located within the fifth Sushi domain) and one serine protease domain (738–984). The H, A, and B chain correspond to each domain as illustrated. A tentative alternative splicing product encoded by AFC42 contains the signal peptide, the Cys-rich region, the EGF-like domain, three Sushi domains, and the first half of the lectin-like domain.

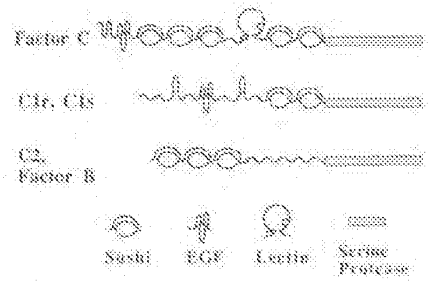


FIG. 8. **Comparison of the structures of serine proteases containing Sushi domains.** The domain structures of serine proteases with Sushi domains including Limulus factor C, C1r, C1s, C2, and complement factor B are illustrated.

one potential *N*-linked glycosylation site (Asn-Val-Thr) at its carboxyl-terminal end and are different from mature factor C.

The mosaic structure of serine proteases containing Sushi domains is shown in Fig. 8. These proteases, except for factor C, have been found only in mammalian complement systems. Proteases shown to have both Sushi and EGF-like domain include only factor C, C1r and C1s, each of which is associated with the initiation of cascade reactions. This structural similarity is suggestive because factor C and C1r can be thought to have similar activation mechanisms. LPS for factor C may correspond to C1q bound to an immune complex for C1r, and both activations occur autocatalytically.

In conclusion, factor C, the putative initiator of the Limulus coagulation cascade, is structurally related to mammalian complement factors. The coagulation cascade functions in preventing not only leakage of hemolymph but also invasion of foreign substances into the hemolymph. The initiator of this cascade, factor C, is a newly discovered type of serine protease zymogen, a "coagulation-complement factor," which

may play an important role both in hemostasis and defense mechanisms. In considering the structural similarity of the initiators of the two systems, the Limulus coagulation and mammalian complement systems may have evolved from a common origin. With regard to the evolution of host defense systems in the animal kingdom, this is consistent with a possible common evolutionary origin of the coagulation and complement cascades.

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SUPPLEMENTAL MATERIAL

to

Limulus Factor C : an endotoxin sensitive serine protease zymogen with a mosaic structure of complement-like, epidermal growth factor-like, and lectin-like domains.

by

Tatsushi Muta, Toshiyuki Miyata, Yoshio Misumi, Fumimaro Tokunaga, Takamori Nakamura, Yoshimitsu Toh, Yukio Ikehara, and Seikaku Iwanaga

MATERIALS AND METHODS

Preparation of amebocyte cDNA libraries ——— A total RNA from the amebocytes (*Tachypleus tridentatus*) was extracted by the method of Chirgwin et al. (12). Poly (A)$^+$ RNA was then fractionated by oligo dT cellulose (Sigma Chemical Co. St. Louis, MO) column chromatography. λgt 11 cDNA libraries were constructed from poly A$^+$ RNA using cDNA Synthesis System (Amersham-Japan) with *EcoRI* linker and cDNA Synthesis kit (Pharmacia LKB Biotechnology, Uppsala) with *EcoRI* adaptor.

cDNA cloning ——— A first screening was carried out by using anti-factor C IgG. Rabbit antiserum raised against factor C was kindly prepared by Dr. H. Igarashi, Tokyo Metropolitan Research Laboratory of Public Health. IgG was purified by Affigel-Protein A (Nippon Bio-Rad laboratories, Tokyo) column chromatography. Goat anti-rabbit IgG horseradish peroxidase conjugate (Nippon Bio-Rad Laboratories) was used as a second antibody and positive clones were developed by 4-chloro-1-naphthol and H_2O_2. The second preparation of libraries constructed with *EcoRI* adaptor was screened with [^{32}P]-DNA insert labelled with multiprime DNA-labelling system (Amersham-Japan). The inserts of positive clones were transferred into pUC118 (Takara Shuzo Co., Kyoto). Subcloning was carried out utilizing restriction sites and directions with Deletion Kit from Takara Shuzo Co. Nucleotide sequence determination was performed on Applied Biosystems 370 A DNA sequencer using fluorescence labelled nucleotide primer (13). Single stranded DNA templates were prepared using MV1184 as host cell and M13KO7 as helper phage (14). All of the sequences were confirmed from both strands with overlapping.

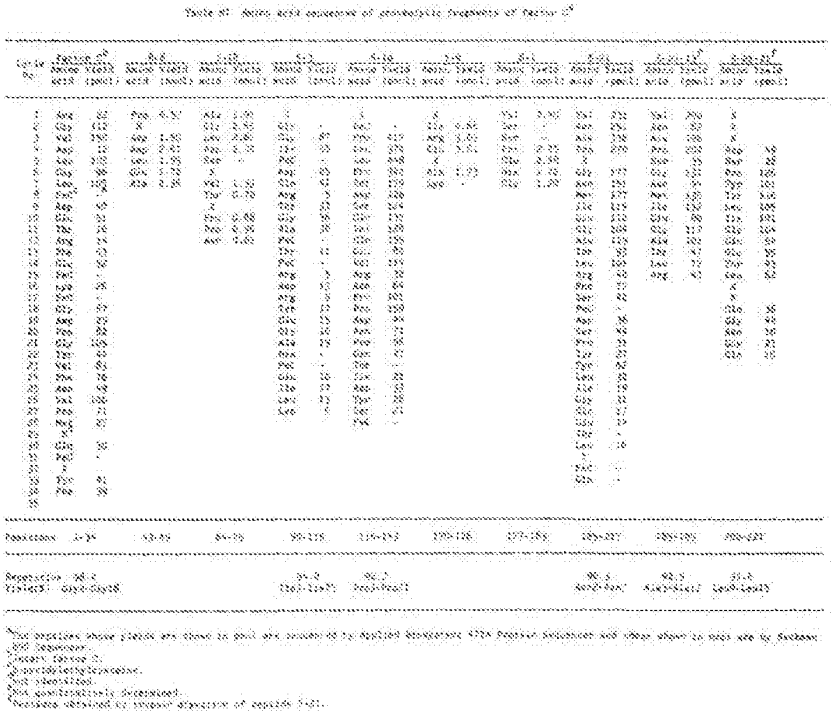
Northern blotting ——— Five μg of poly A$^+$ RNA was electrophoretically separated on 1.0 % agarose gel, transferred to GeneScreen membrane (Nippon Millipore Kogyo K.K., Yonezawa). The filter was hybridized with ^{32}P-labelled probes and washed finally in 0.1 x SSC (0.15 M NaCl, 15 mM sodium citrate), 0.1 % sodium lauroylsulfate at 68°C. The washed filter was applied to autoradiography.

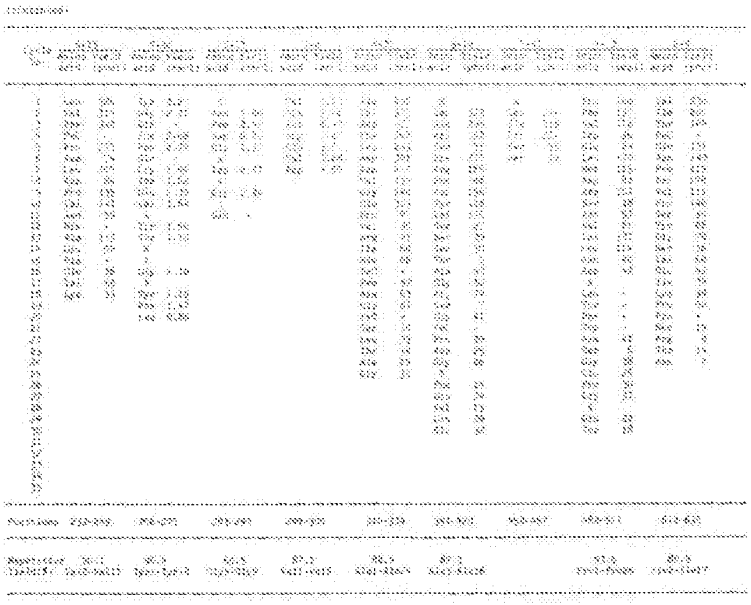
Determination of partial amino acid sequence of factor C ——— Factor C was purified as previously described (15). The purified material was reduced and S-pyridylethylated and the resulting H and L chains were separated as previously described (9). The S-pyridylethylated H chain was digested with lysyl endopeptidase (Wako Pure Chemical Industries, Ltd., Osaka) and the L chain with cyanogen bromide followed by 2-(2-nitrophenyl sulphenyl)-3-methyl-3-bromo indolenine treated trypsin (Worthington Biochemical Corp., Freehold, NJ) (9). The resulting peptides were first separated by gel filtration with Sephadex G-50 fine (1.6 x 141 cm; (Pharmacia Fine Chemicals, Uppsala) and then reversed-phase high performance liquid chromatography using a VYDAC 214TP5415 (4.6 x 150 mm; (The Separation Group, CA) or a TSKgel ODS-120T (4.6 x 150 mm; (TOSOH Co. Ltd., Tokyo) column (Fig. 3B). The purified peptides were subjected to amino acid analysis on PICO-TAG system (Waters, Millipore Corp., Milford, MA) or Hitachi 835 automatic analyzer and to amino-terminal sequence analysis on Applied Biosystems 477 A Protein Sequencer or Beckman System 890 B Sequencer (Table III).

Homology search ——— The Limulus factor C protein sequence was compared with all entries in the NBRF Protein Sequence Database (release 25.0, December, 1989) with GENETYX system (Software Development Co., LTD., Tokyo) on the basis of the algorithm of the computer program FASTP (15).

Subcellular Localization ——— The amebocytes were collected into a sterilized tube and immediately fixed with 4 % paraformaldehyde and 1.5 % glutaraldehyde. The sections mounted on a nickel grid were incubated with factor C IgG after a blocking with 1 % bovine serum albumin. The sections were washed and then incubated with goat anti-rabbit IgG gold colloidal particles (20 nm; (B.V Labs, Inc., San Mateo, CA). After extensive washing, the sections were double stained with 2 % uranyl acetate and lead tartrate and examined under an electron microscope (Hitachi H-500).

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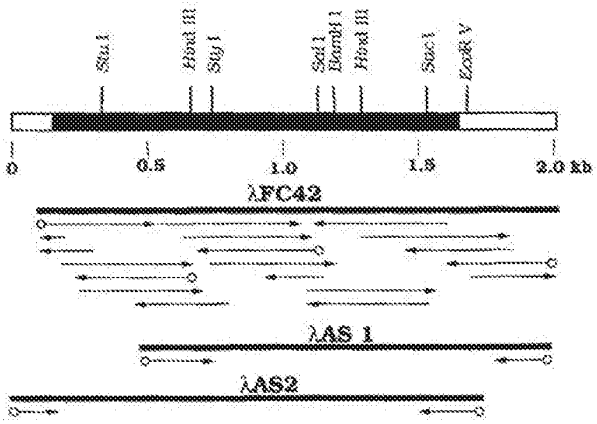


FIG. 3B. Restriction map and sequence strategy of the tentative alternative splicing product of factor C. The black and shadowed box at the top shows the open reading frame. The shadowed box indicates the region encoding the different carboxy acid sequence from factor C. Arrows with circles show sequencing utilizing restriction sites and arrows without circle were by deletions.

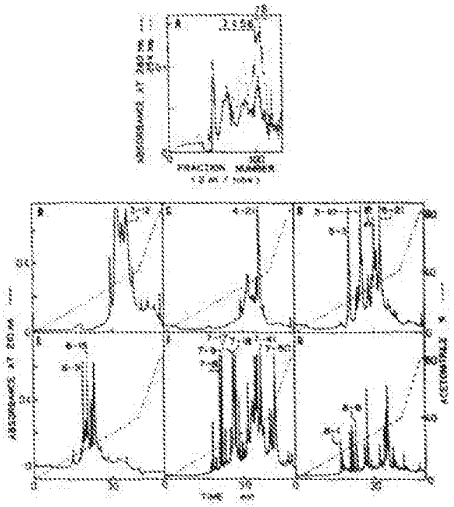


FIG. 3A. Separation of proteolytic fragments from factor C. (A) Gel filtration on a Sephadex G-50 fine column. S-pyridylethylated H chain was digested with lysyl endopeptidase and the digest was separated on the column (1.0 x 141 cm) in 9 % formic acid at the flow rate of 30 ml/h. The solid line shows absorbance at 280 nm and the dotted line shows the absorbance at 254 nm. (B)-(E) Each peak corresponding to a solid bar in Fig. 4A was collected and further purified by reversed phase HPLC employing the indicated gradients of acetonitrile in 0.1% trifluoroacetic acid. The columns used were VYDAC 214TP54 (4.6 x 150 mm) in (B) and TSKgel ODS-120T (4.6 x 150 mm) in (C)-(E). (B) peak 3, (C) peak 4 (D) peak 5, (E) peak 6, (F) peak 7, (G) peak 8. The solid lines show the absorbance at 210 nm, and the dotted lines show the gradients of acetonitrile in 0.1 % trifluoroacetic acid. The flow rate was 1.0 ml/min. The indicated peptides were sequenced.

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A Structural Perspective on the Interaction between Lipopolysaccharide and Factor C, a Receptor Involved in Recognition of Gram-negative Bacteria*[S]

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The recognition of broadly conserved microorganism components known as pathogen-associated molecular patterns is an essential step in initiating the innate immune response. In the horseshoe crab, stimulation of hemocytes with lipopolysaccharide (LPS) causes the activation of its innate immune response, and Factor C, a serine protease zymogen, plays an important role in this event. Here, we report that Factor C associates with LPS on the hemocyte surface and directly recognizes Gram-negative bacteria. Structure-function analyses reveal that the LPS binding site is present in the N-terminal cysteine-rich (Cys-rich) region of the molecule and that it contains a tripeptide sequence consisting of an aromatic residue flanked by two basic residues that is conserved in other mammalian LPS-recognizing proteins. Moreover, we have demonstrated that the Cys-rich region specifically binds to LPS on Gram-negative bacteria and that mutations in the tripeptide motif abrogate its association with both LPS and Gram-negative bacteria, underscoring the importance of the tripeptide in LPS interaction. Although the innate immune response to LPS in the horseshoe crab is distinct from that of mammals, it appears to rely on structural features that are conserved among LPS-recognizing proteins from diverse species.

Innate immunity, which defends the host against infectious microorganisms, is an ancient and ubiquitous system in both vertebrates and invertebrates. Although different organisms employ a variety of environment-specific adaptations to ensure host defense, several generalized features underlie the innate immune response. First, germline-encoded pattern recognition receptors (PRRs)[2] of the host recognize broadly conserved microbial components known as pathogen-associated molecular patterns, including lipopolysaccharide (LPS) of Gram-negative bacteria, β-1,3-glucans of fungi, and peptidoglycans of Gram-positive bacteria (1–3). For example, the *Drosophila* peptidoglycan recognition protein family members and vertebrate Toll-like receptors have been well characterized as PRRs (4, 5). Second, the stimulation of PRRs by pathogen-associated molecular patterns activates intracellular signaling cascades that result in transcriptional activation (6). Finally, phagocytosis and the production of antimicrobial peptides by the host are essential for the clearance and killing of infectious microbes (7, 8).

Hemocytes play a fundamental role in the invertebrate innate immune system both as the initial determinants of non-self-recognition and subsequently as the mediators of phagocytosis, encapsulation, and melanization (9). Granular hemocytes predominate in the hemolymph of the horseshoe crab (accounting for >99% of total hemocytes) and constitute a key component of the host defense against infectious microorganisms, as they contain an array of defense molecules that are deployed to protect against infectious microorganisms (10). Horseshoe crab granular hemocytes are especially sensitive to LPS, a major cell wall component of Gram-negative bacteria. Stimulation of the granular hemocytes with LPS causes the activation of hemolymph coagulation within a few seconds, leading to the immobilization and engulfment invading microorganisms. Hemocytes concurrently release defense molecules by LPS-induced exocytosis, thereby killing infectious microbes (10, 11).

Because of their sensitivity to LPS, it has been thought that granular hemocytes express LPS receptors on their surfaces and that binding of LPS to these receptors could initiate a series of signaling processes important for the innate immunity, as has been observed in vertebrate. A close homolog of vertebrate Toll-like receptors, designated *t*Toll, was identified in the horseshoe crab *Tachypleus tridentatus*. However, the apparent absence of a pathogen-associated molecular pattern binding domain in *t*Toll as well as its relatively nonspecific tissue expression pattern (12) cast doubt upon the idea that *t*Toll itself functions as an LPS receptor in horseshoe crab hemocytes. Another candidate for the putative LPS receptor is Factor C, a serine protease zymogen predominantly stored in hemocyte intracellular granules that initiates the hemolymph coagulation cascade following proteolytic activation. Features of Factor C that are consistent with its potential role as an LPS receptor include its high sensitivity toward LPS (13, 14) and its restricted hemocyte-specific expression pattern (see supplemental Fig. S1). We recently proposed that a portion of Factor C might

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

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[2] The abbreviations used are: PRR, pattern recognition receptor; ALF, anti-LPS factor; EGF, epidermal growth factor; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; HEK, human embryonic kidney; CCP, complement control protein.

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localize to the hemocyte surface and undergo autocatalytic activation upon association with LPS, thereby initiating signal transduction via protease-activated receptors coupled to heterotrimeric GTP-binding proteins (15).

In this study, we have established the essential involvement of the N-terminal Cys-rich region of Factor C in LPS recognition and determined the importance of a tripeptide motif contained within this region of the molecule. Based on these findings, we propose a mode of LPS recognition by Factor C that employs this motif.

EXPERIMENTAL PROCEDURES

Materials—*Salmonella minnesota* Re595 LPS and biotinylated ultrapure *Escherichia coli* O111:B4 LPS were obtained from List Biological Laboratories (Campbell, CA) and InvivoGen (San Diego, CA), respectively. Fluorescein isothiocyanate (FITC)-conjugated LPS (*E. coli* O111:B4) was supplied by Sigma-Aldrich. Biotinylation of *S. minnesota* Re595 LPS was performed with EZ-Link Sulfo-NHS-LC-Biotin reagent (Pierce).

Immunofluorescence Microscopy—One milliliter of *T. tridentatus* hemolymph was collected into 50 ml of pyrogen-free 10 mM HEPES buffer (pH 7.0) containing 0.5 M NaCl, and the diluted hemolymph (200 μl) was plated on coverslips. After a 20-min incubation to allow attachment, hemocytes were fixed with 3.7% formaldehyde for 10 min and washed twice with phosphate-buffered saline (PBS). For LPS treatment, the attached cells were incubated with 50 μg/ml FITC-conjugated LPS for 30 min and then fixed with 3.7% formaldehyde. The cells were blocked with 5% bovine calf serum and incubated for 1 h with anti-Factor C monoclonal antibody 2C12 (16) diluted in blocking buffer. For detection, Cy3-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was used. Cells were imaged with an Olympus BX-FLA fluorescence microscope (Tokyo, Japan).

Cloning and Mutagenesis—To construct Factor C variants with Myc epitope tags, plasmid pMT/FC725, which contains a cDNA encoding the heavy chain of *T. tridentatus* Factor C, or plasmid pMT/FC410–994, which contains a cDNA encoding the light chain of *T. tridentatus* Factor C, was used as a template to amplify regions of cDNAs encoding residues 1–723 (heavy chain), 1–296 (Cys/EGF/CCP123), 1–116 (Cys/EGF), 117–296 (CCP123), 300–543 (LCCL/Lectin), 551–609 (CCP4), 669–723 (CCP5), and 738–994 (Ser protease domain) by PCR by using 5′ primers containing a 5′ AscI site and 3′ primers containing a 3′ KpnI site. The amplified fragments were subcloned into the vector pSecTag2A (Invitrogen). Three tandem copies of the Myc epitope tag derived from p5-Helix/3Myc (17) were then inserted into the 5′ KpnI site (3′ XhoI site) to generate constructs pFC-(1–723)/3Myc, pFC-(1–296)/3Myc, pFC-(1–116)/3Myc, pFC-(117–296)/3Myc, pFC-(300–543)/3Myc, pFC-(551–609)/3Myc, pFC-(669–723)/3Myc, and pFC-(738–994)/3Myc. Mutations (R36E/R38E, K55E/K57E, and W37A) in pFC-(1–116)/3Myc were introduced by standard site-directed mutagenesis (18). The *T. tridentatus ALF* gene was amplified from a hemocyte cDNA library using the oligonucleotides TK260 (5′-aaaaaGGCGCGCCcaaggtggtatatggactcaacttgc-3′) and TK261 (5′-tttttGGTACCttgatattgagagatgaacgtttgtgc-3′) to generate an AscI/KpnI fragment that was used to produce a Myc epitope-tagged construct corresponding to those of Factor C.

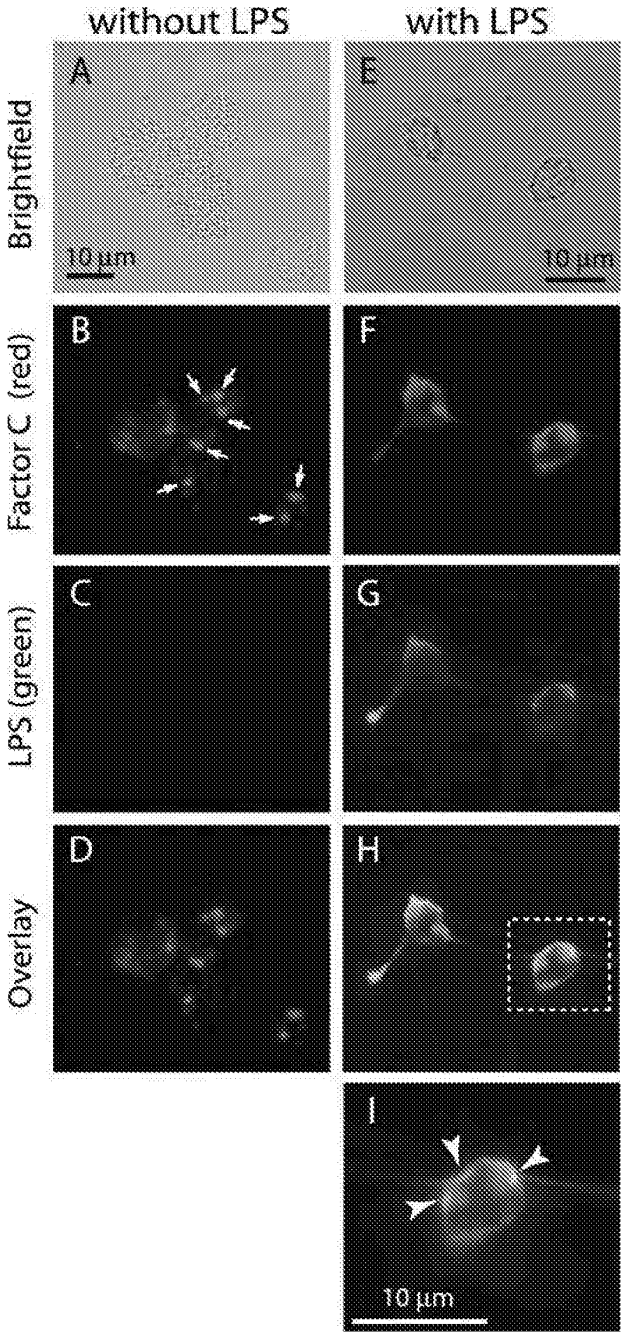


FIGURE 1. **Factor C is a membrane-bound LPS receptor on hemocytes.** The hemocytes from *T. tridentatus* were treated without (*A–D*) or with (*E–H*) FITC-labeled LPS, stained with a monoclonal antibody (2C12) against Factor C (*B* and *F*), and identified by immunofluorescence. *I*, higher magnification image of the *boxed area* in *H*. *Arrowheads* indicate LPS co-localized with membrane-bound Factor C. Note that non-permeabilized hemocytes were used in these experiments. *Scale bar*, 10 μm.

Co-immunoprecipitation—3Myc-tagged Factor C variants and anti-LPS factor (ALF) proteins were transiently expressed in HEK293 cells and secreted into the culture medium. HEK293

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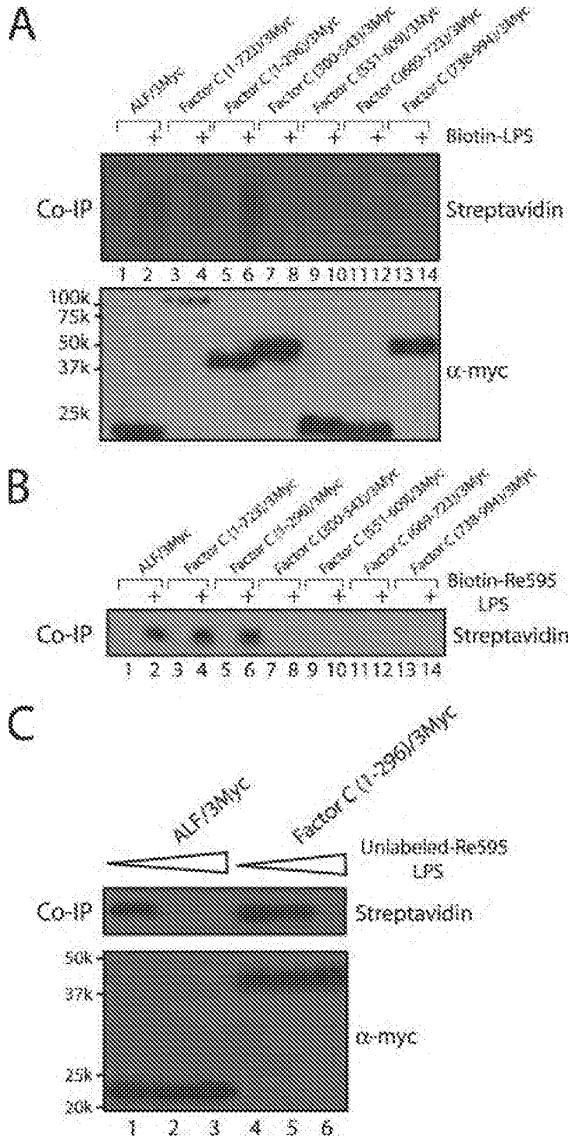


FIGURE 2. **LPS binding assay of Factor C variants.** ALF (positive control) and Factor C variants were incubated with biotinylated *E. coli* O111:B4 LPS (*A*) or *S. minnesota* Re595 LPS (*B*) and subjected to co-immunoprecipitation (*Co-IP*) with an anti-Myc antibody. *C*, binding of Myc-tagged Factor C-(1–296) to biotinylated *S. minnesota* Re595 LPS was specific and was competed by an excess amount of unlabeled Re595 LPS (*lanes 4–6*) as seen in the positive control (*lanes 1–3*). *Lanes 1* and *4*, no competitor; *lanes 2* and *5*, an equal amount of competitor; and *lanes 3* and *6*, 10-fold excess of competitor. Binding to biotinylated LPSs were determined by Western blot analysis with a streptavidin-horseradish peroxidase conjugate (*top*) and anti-Myc monoclonal antibody 9E10 (*bottom*). The positions of protein molecular mass markers are indicated at the *left*.

cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 1% glutamine, 1% penicillin-streptomycin, and 10% bovine calf serum, respectively, at 5% CO_2 and 37° C. Four micrograms of the 3Myc-tagged Factor C variants and ALF expression plasmids were used to transfect HEK293 cells at 50% confluence in a six-well plate using the calcium phosphate method. Conditioned media were collected 2 days after transfection, and the protein expres-

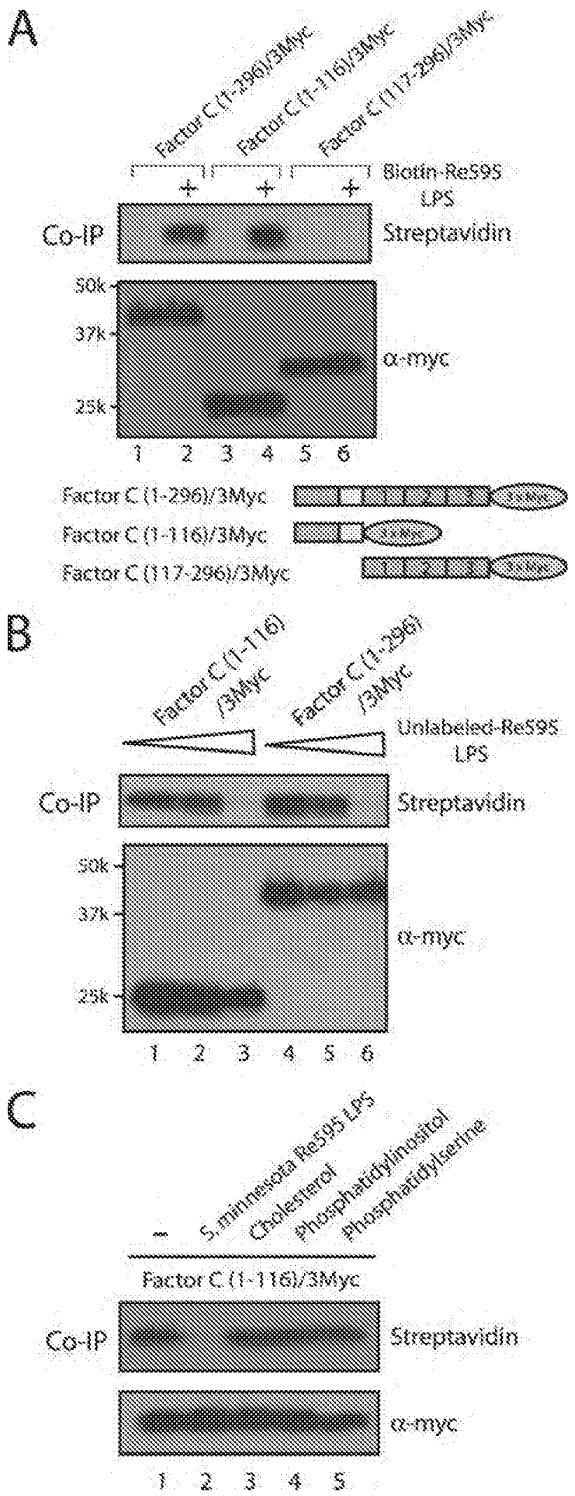


FIGURE 3. **The Cys-rich/EGF-like domain is a potential LPS binding site in Factor C.** *A* and *B*, the LPS binding site was located in the Cys-rich/EGF-like domains of Factor C. The amounts of competitor in *B* were ordered the same as in Fig. 2*C*, and all LPS binding experiments were performed as in Fig. 2. *C*, an excess amount (5-fold excess) of cholesterol (*lane 3*) or acidic phospholipids (*lanes 4* and *5*) had no effect on Factor C-(1–116)/3Myc-LPS complex formation. However, *S. minnesota* Re595 LPS (*lane 2*) effectively disrupted the complex. *Lane 1*, without competitor. *Co-IP*, co-immunoprecipitation.

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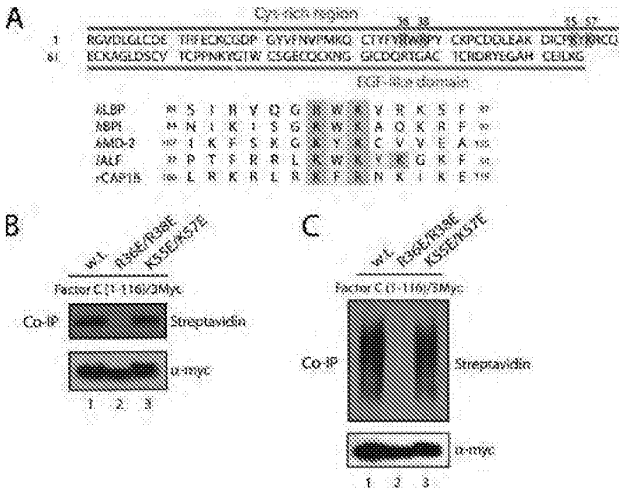


FIGURE 4. **A conserved tripeptide motif in Factor C-(1–116) is critical for LPS interaction.** *A*, the sequence of Factor C-(1–116) with Cys-rich region and EGF-like domain are shown (*top panel*). Two of the tripeptide motif in the Cys-rich region (36–38 and 55–57) are *highlighted* by *cyan* (basic residues) and *yellow* (aromatic residues) and aligned with the LPS-recognizing sites of five different proteins (*bottom panel*). The amino acid sequences are *numbered* according to positions in the mature proteins. *h*LBP, human LPS-binding protein; *h*BPI, human bactericidal permeability-increasing protein; *h*MD-2, human MD-2; *l*ALF, *Limulus* anti-LPS factor; and *r*CAP18, rabbit cationic antimicrobial protein. *B* and *C*, mutational analysis in Factor C-(1–116). Two Factor C-(1–116)/3Myc mutants (R36E/R38E and K55E/K57E) were evaluated for the ability to bind *S. minnesota* Re595 LPS (*B*) and *E. coli* O111:B4 LPS (*C*). Co-immunoprecipitation experiments were performed as in Fig. 2. *w.t.*, wild type.

sion was evaluated by Western blotting with the anti-Myc monoclonal antibody 9E10 (Covance, Berkeley, CA). The clarified supernatants were incubated with 3 μg of biotinylated-Re595 LPS (for the *E. coli* O111:B4 LPS co-immunoprecipitation experiment, 5 μg of biotinylated-LPS was used) at 4 °C for 30 min and then incubated 6 h with 20 μl of agarose beads coupled to the anti-c-Myc polyclonal antibody (Sigma-Aldrich). After four washes with PBS, the immunoprecipitates were separated by SDS-PAGE and immunoblotted with streptavidin-horseradish peroxidase conjugate (Amersham Biosciences) or anti-Myc monoclonal antibody 9E10.

Microbial Organisms and Bacteria Binding Assay—Staphylococcus aureus, Enterococcus hirae, E. coli B, and K12 were cultured in 3% tryptosoy broth at 37 °C overnight. For bacterial immunoprecipitation experiments, 150 μl of the overnight cultures were centrifuged at 12,000 revolutions/min for 2 min and washed twice with PBS. The washed cells were resuspended in conditioned medium from HEK293 cells expressing Factor C variants and incubated at 4 °C for 2 h. After four washes with PBS, the bacteria were directly resuspended in SDS-PAGE loading buffer. Associated proteins were resolved by SDS-PAGE, immunoblotted with the anti-Myc monoclonal antibody 9E10, and subsequently developed with a horseradish peroxidase-conjugated anti-mouse IgG antibody (Bio-Rad). For flow cytometric analysis, Myc-tagged protein bound to bacteria (*E. coli* K12) were incubated with FITC-conjugated anti-Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 1 h and washed twice with PBS, and the FITC-labeled bacteria were analyzed using a FACScan flow cytometer (BD Biosciences).

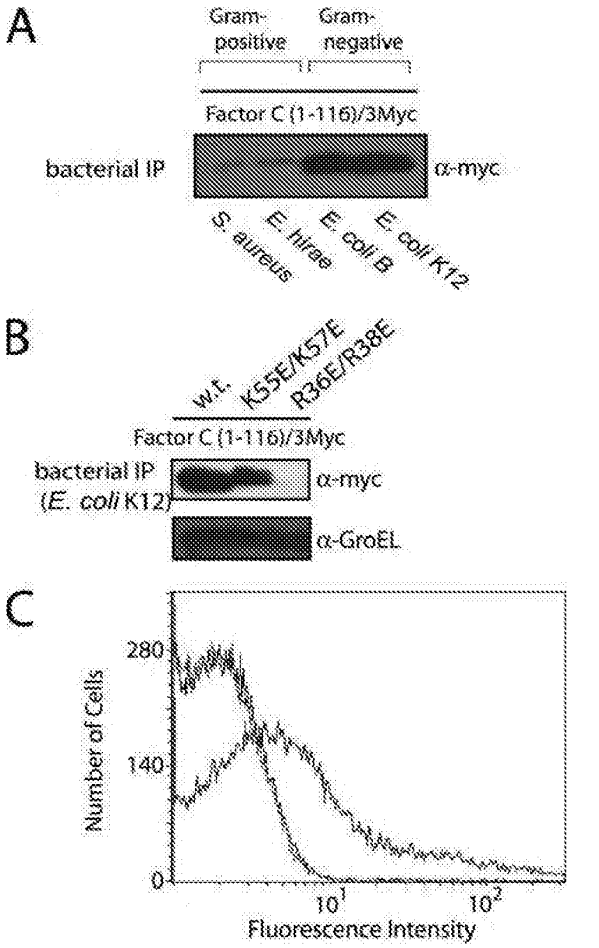


FIGURE 5. **Direct binding of Factor C-(1–116) to Gram-negative bacteria.** *A*, Myc-tagged Factor C-(1–116) specifically bound to Gram-negative bacteria *E. coli* B and K12 but not to Gram-positive bacteria *S. aureus* and *E. hirae*. Factor C-(1–116)/3Myc was subjected to bacterial cell surface immunoprecipitation with the anti-Myc monoclonal antibody 9E10. *B*, bacterial (*E. coli* K12) immunoprecipitation with Factor C-(1–116)/3Myc and its mutants. The R36E/R38E mutant completely lacked the ability to bind bacteria. *C*, flow cytometric analysis of Factor C-(1–116)/3Myc (*blue line*) and its R36E/R38E mutant (*green line*) binding to *E. coli* K12. For detection of surface-bound protein, FITC-labeled anti-myc monoclonal antibody 9E10 was used. As a negative control, untreated bacteria were detected with FITC antibody (*black line*). *w.t.*, wild type.

RESULTS AND DISCUSSION

Factor C Is a Membrane-bound LPS Receptor on the Hemocyte Surface—At least two proteins, ALF and Factor C, have been determined to associate with LPS in the horseshoe crab. We recently reported that Factor C, an LPS-sensitive serine protease zymogen, exists on the surface of hemocytes and initiates signaling through heterotrimeric GTP-binding proteins (15). We reasoned that hemocyte surface-bound Factor C could serve as an LPS receptor, and if so, it would be a likely candidate for the LPS-responsive PRR that induces exocytosis of granular components.

To address this issue, we morphologically investigated whether Factor C and LPS co-localize on the hemocyte surface (Fig. 1). Immunostaining of hemocytes with an antibody to Factor C (red) demonstrated that endogenous Factor C was pres-

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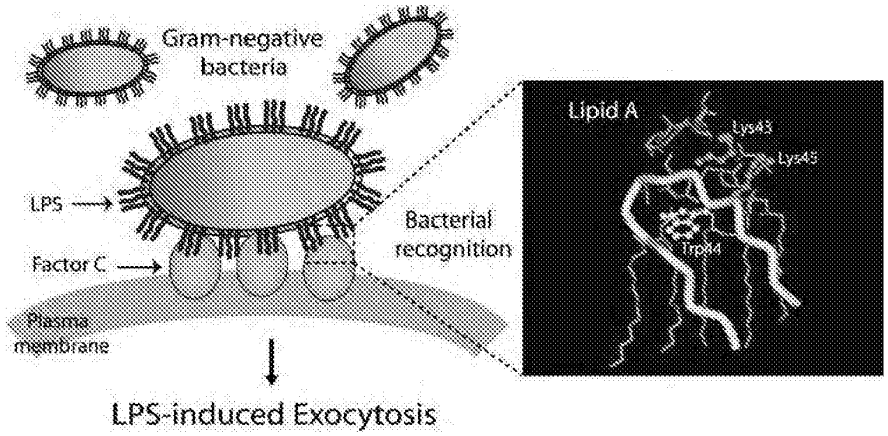


FIGURE 6. **A model of bacterial recognition by Factor C.** The recognition of LPS on Gram-negative bacteria by membrane-bound Factor C initiates the horseshoe crab innate immune response. We propose that the conserved tripeptide motif (Arg^{36}, Trp^{37}, and Arg^{38}) in the Cys-rich region of Factor C interacts with the lipid A portion of LPS. Formation of the Factor C-LPS complex could result in tethering of bacteria to the hemocyte surface and ultimately lead to LPS-induced exocytosis. The modeled structure of the ALF-LPS complex (25) is shown in the *right panel* and may resemble that of the complex between N-terminal elements of Factor C and LPS.

ent in a punctate distribution on the hemocyte surface (Fig. 1*B*, *arrows*). We next tested whether the surface-localized Factor C could bind to LPS. Hemocytes were incubated with FITC-labeled LPS (*E. coli* O111:B4). FITC-LPS accumulated on the cell surface of hemocyte (Fig. 1*G*) and co-localized with Factor C (Fig. 1, *H* and *I*, *arrowheads*). Interestingly, FITC-LPS accumulation was not observed in fixed hemocytes (supplemental Fig. S2), which would be expected to undergo chemical modification and inactivation of surface proteins. These results suggest that Factor C is a membrane-bound LPS receptor in horseshoe crab hemocytes. Factor C can bind to cholesterol and acidic phospholipids with high affinity (14, 15) likely through a different site (19), suggesting that it might associate with plasma membrane lipid rafts enriched with cholesterol and glycosphingolipids (20) or microdomains and subsequently initiate signaling processes through protease-activated receptors following LPS-induced autocatalytic activation.

Identification of the Factor C Domain Essential for LPS Recognition—We therefore attempted to identify the region of Factor C responsible for LPS recognition. Factor C is a multidomain glycoprotein with an estimated molecular mass of ~120 kDa. In addition to the typical serine protease domain at its C terminus, it also contains a Cys-rich region, an epidermal growth factor (EGF)-like domain, five complement control protein (CCP) modules, an LCCL module (derived from a conserved domain of *Limulus* Factor C, Coch-5b2, and Lg11 (21)), and lectin-like domains (supplemental Fig. S3) (22).

We expressed five Factor C variants in HEK293 cells, each containing three copies of the Myc epitope tag (EQKLISEEDL) at its C termini, and tested whether these proteins could form a complex with LPS (Fig. 2*A*). As a positive control, we constructed Myc-tagged ALF, which is known to be an LPS-recognizing protein (23). In co-immunoprecipitation assays, both ALF/3Myc and Factor C-(1–723)/3Myc bound to biotinylated *E. coli* O111:B4 LPS as expected (Fig. 2*A*, *lanes 2* and *4*). The N-terminal fragment, Factor C-(1–296)/3Myc, also bound to LPS (*lane 6*). In contrast, constructs that lacked this N-terminal region, namely Factor C-(300–543)/3Myc, Factor C-(551–609)/3Myc, Factor C-(669–723)/3Myc, and Factor C-(738–994)/3Myc, did not bind to LPS (*lanes 8*, 10, 12, and 14). Consistent with these results, Factor C-(1–296)/3Myc also showed significant binding to biotinylated *S. minnesota* Re595 LPS to an extent that was similar to that of positive controls (Fig. 2*B*, *lanes 2*, *4*, and *6*).

We also investigated the specificity of the Factor C-(1–296)/3Myc-LPS complex (Fig. 2*C*). For both ALF/3Myc and Factor C-(1–296)/3Myc, complex formation with biotinylated LPS was diminished with increasing amounts of unlabeled Re595 LPS, with complex formation completely disrupted by the addition of a 10-fold excess of unlabeled competitor (Fig. 2*C*, *lanes 3* and *6*). Taken together, these results indicate that the N-terminal region of Factor C is sufficient for LPS recognition.

The Cys-rich/EGF-like Domain Encompasses the LPS-recognizing Domain—We performed structure-function analyses to further define the LPS-recognizing sequence within the N-terminal region of Factor C. Two fragments of the previously identified LPS-recognizing domain, Factor C-(1–116)/3Myc and Factor C-(117–296)/3Myc, were generated and tested for their ability to bind to LPS (Fig. 3*A*). Of these two, only the most N-terminal fragment (Factor C-(1–116)/3Myc), which contains the tandem Cys-rich region and EGF-like domain, was capable of forming a complex with biotinylated *S. minnesota* Re595 LPS (Fig. 3*A*, *lane 4*). The interaction between Factor C-(1–116)/3Myc and LPS was determined to be specific, because the complex was disrupted by the addition of excess competing LPS (unlabeled Re595 LPS), as was previously observed for Factor C-(1–296)/3Myc (Fig. 3*B*). The specific interaction between Factor C-(1–116)/3Myc and LPS was further demonstrated by the inability of either cholesterol or acidic phospholipids to disrupt the complex (Fig. 3*C*, *lanes 3–5*). Our results contrast with those presented in a previous study of the Factor C ortholog from the Southeast Asian horseshoe crab *Carcinoscorpius rotundicauda*, which defined a region within the tandem CCP domains as important for LPS binding (24). However, these authors did not specifically investigate the role of the N-terminal region identified in the present study, nor did they include negative controls similar to those described in the following sections.

Mutations within the Cys-rich Region Abolish LPS Binding—From the present study, it is clear that the potential LPS recognition domain in Factor C is located in its N-terminal region, which consists of a Cys-rich region and an EGF-like domain. An interesting feature of the Cys-rich region is the presence of a unique conserved tripeptide motif, namely an aromatic residue immediately flanked by two basic residues, which appears in

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five other known LPS-recognizing proteins (Fig. 4*A*, *bottom*). This sequence motif is present in two copies within the LPS-recognizing domain of Factor C from Arg^{36} to Arg^{38} and from Lys^{55} to Lys^{57} (Fig. 4*A*, *top*).

To test whether these tripeptide motifs are essential for the LPS binding ability, we introduced paired glutamate substitutions into Factor C-(1–116)/3Myc at locations corresponding to basic residue pairs (Arg^{36}/Arg^{38} and Lys^{55}/Lys^{57}). The results of co-immunoprecipitation experiments indicate that the R36E/R38E mutant is incapable of binding to LPS (Fig. 4, *B* and *C*, *lanes 2*), whereas the K55E/K57E mutant retains the ability to bind LPS (Fig. 4, *B* and *C*, *lanes 3*). We also substituted an alanine residue for Trp^{37} to assess the importance of an aromatic residue at this position and found that the resulting W37A mutant also lacked the ability to bind LPS (supplemental Fig. S4). These results establish the essential nature of the Cys-rich region for LPS recognition by Factor C and further highlight the importance of the Arg^{36}-Trp^{37}-Arg^{38} motif.

The Cys-rich/EGF-like Domain of Factor C Binds to Gram-negative Bacteria—The preservation of LPS binding activity in Factor C-(1–116)/3Myc allowed us to explore the direct LPS-dependent recognition of live bacteria. Using bacterial immunoprecipitation, we investigated whether Factor C-(1–116)/3Myc could selectively recognize Gram-negative bacteria, which are rich in LPS on their surface. Four types of live bacteria (two Gram-positive bacteria, *S. aureus* and *E. hirae*, and two Gram-negative bacteria, *E. coli* B and K12) were incubated with Factor C-(1–116)/3Myc, and the association of the protein with the bacteria was assessed by Western blotting with the anti-Myc monoclonal antibody 9E10. Factor C-(1–116)/3Myc was specific for Gram-negative bacteria, showing significant binding to *E. coli* B and K12 but no binding to Gram-positive bacteria (Fig. 5*A*). In addition, the binding of Factor C-(1–116)/3Myc to Gram-negative bacteria (*E. coli*) was confirmed by flow cytometry (Fig. 5*C*). Finally, the R36E/R38E mutant was incapable of binding to *E. coli*, as judged by both bacterial immunoprecipitation (Fig. 5*B*) and flow cytometry (Fig. 5*C*), indicating that the association between Factor C-(1–116)/3Myc and bacteria is LPS-dependent.

Implications for Innate Immunity—Factor C associates with LPS on the hemocyte surface, and the N-terminal Cys-rich region can selectively bind Gram-negative bacteria in an LPS-dependent manner. Taken together, these findings suggest that Factor C may serve as a membrane-bound LPS receptor capable of tethering invading bacteria to the hemocyte surface (Fig. 6), ultimately leading to hemolymph coagulation by Factor C secreted from granules. We propose that the first conserved tripeptide motif (Arg^{36}-Trp^{37}-Arg^{38}) in the Cys-rich region of Factor C plays an important role in the recognition of LPS, an assumption that is consistent with a modeling study of ALF that predicts direct docking of the corresponding tripeptide motif with LPS (25). In this model, two lysine residues that correspond to Arg^{36} and Arg^{38} of Factor C interact with glucosamine (GlcN) I-1-phosphate in lipid A, and a tryptophan residue, which corresponds to Trp^{37} of Factor C, interacts with a hydrophobic portion of lipid A (Fig. 6, *right panel*). Our results are consistent with such a model and underscore the importance of these elements within the Cys-rich region of Factor C for mediating its association with LPS, an essential structural characteristic that appears to be conserved among other LPS-recognizing proteins. Finally, these findings imply that Factor C acts as a PRR for Gram-negative bacteria in the horseshoe crab and lay the groundwork for future studies to elucidate the mechanism whereby hemocyte-bound Factor C potentiates LPS-induced exocytosis degranulation.

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A Structural Perspective on the Interaction between Lipopolysaccharide and Factor C, a Receptor Involved in Recognition of Gram-negative Bacteria

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REVIEW

The lipopolysaccharide-activated innate immune response network of the horseshoe crab

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Abstract

Primary stimulation of the horseshoe crab innate immune system by bacterial lipopolysaccharide (LPS) activates a network of responses to ensure host defense against invading pathogens. Granular hemocytes selectively respond to LPS via a G protein-dependent exocytic pathway that critically depends on the proteolytic activity of the LPS-responsive coagulation factor C. In response to stimulation by LPS, the hemocyte secretes transglutaminase (TGase) and several kinds of defense molecules, such as coagulation factors, lectins, antimicrobial peptides, and protein substrates for TGase. LPS-induced hemocyte exocytosis is enhanced by a feedback mechanism in which the antimicrobial peptide tachyplesin serves as an endogenous mediator. The coagulation cascade triggered by LPS or β-1,3-D-glucans results in the formation of coagulin fibrils that are subsequently stabilized by TGase-dependent cross-linking. A cuticle-derived chitin-binding protein additionally forms a TGase-stabilized mesh at sites of injury. Invading pathogens are agglutinated by both hemocyte- and plasma-derived lectins. In addition, the proclotting enzyme and tachyplesin functionally convert hemocyanin to phenoloxidase. In the plasma, coagulation factor C acts an LPS-sensitive complement C3 convertase on the surface of Gram-negative bacteria. In this manner, LPS-induced hemocyte exocytosis leads not only to coagulation but also activates a sophisticated innate immune response network that coordinately effects pathogen recognition, prophenoloxidase activation, pathogen clearance, and TGase-dependent wound healing.

Key Words: horseshoe crab; lipopolysaccharide; innate immunity; hemolymph coagulation; transglutaminase; complement C3

Introduction

Innate immunity, which defends the host against infectious pathogens, is an ancient and ubiquitous immune system in both vertebrates and invertebrates. Each species employs a variety of environment-specific adaptations to ensure host defense, whereas a generalized recognition strategy against invading pathogens underlies the innate immune reaction. The innate immune system recognizes broadly conserved microbial cell wall components known as pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS) of Gram-negative bacteria, peptidoglycans of Gram-positive bacteria, and β-1,3-D-glucans of fungi via pattern-recognition proteins (Janeway, 1989; Hoffmann, 2003; Akira *et al.*, 2006). Innate immune systems in invertebrates consist of pathways that promote recognition of pathogen-associated macromolecules, hemolymph coagulation, phenoloxidase-mediated melanization, cell agglutination, antimicrobial activity, and phagocytosis (Nappi *et al.*, 2004; Theopold *et al.*, 2004; Iwanaga and Lee, 2005; Kurata *et al.*, 2006; Nakanishi and Shiratsuchi, 2006).

The horseshoe crab belongs to the class Merostomata and is phylogenetically more closely related to Arachnoidea than it is to Crustacea. Fossils of horseshoe crabs, such as *Mesolimulus walchi* and *Limulus coffini*, have been found in deposits from the Paleozoic era to the Cenozoic era in Europe and North America (Størmer, 1952). Extant horseshoe crabs comprise four species, *Limulus polyphemus*, *Tachypleus tridentatus*, *T. gigas*, and *Carcinoscorpius rotundicauda*, each having a distinct geographic distribution; *L. polyphemus* is distributed along the east coast of North America,

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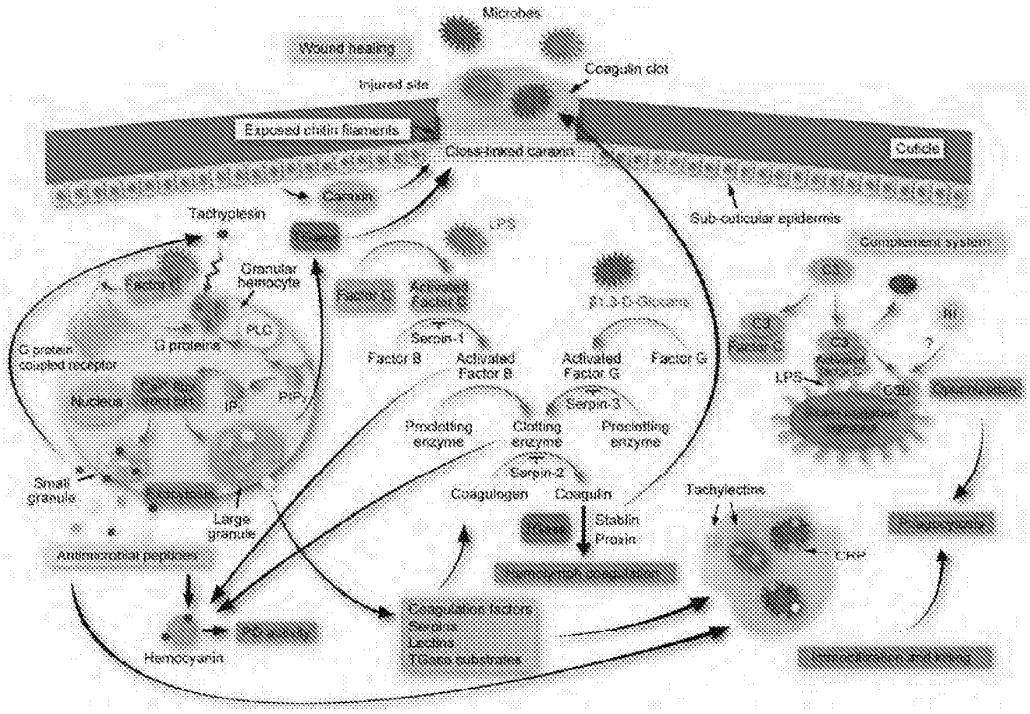


Fig. 1 LPS-activated innate immune response network of the horseshoe crab. Granular hemocytes selectively respond to LPS via a G protein-dependent exocytic pathway that critically depends on the proteolytic activity of the LPS-responsive coagulation factor C. In response to stimulation by LPS, the hemocyte secretes several kinds of defense molecules, such as coagulation factors, lectins, antimicrobial peptides, and protein substrates for TGase involved in protein cross-linking. LPS-induced hemocyte exocytosis is enhanced by a feedback mechanism in which the antimicrobial peptide tachyplesin serves as an endogenous mediator. The coagulation cascade triggered by LPS or β-1,3-D-glucans results in the formation of coagulin fibrils that are subsequently stabilized by TGase-dependent cross-linking with stablin and proxin. A cuticle-derived chitin-binding protein caraxin additionally forms a TGase-stabilized mesh at sites of injury. Invading pathogens are agglutinated by both hemocyte- and plasma-derived tachylectins and CRP. In addition, the proclotting enzyme and tachyplesin functionally convert hemocyanin to phenoloxidase. In the plasma, coagulation factor C also acts an LPS-sensitive complement C3 convertase on the surface of Gram-negative bacteria. An immunocompetent cell with phagocytotic activity against Gram-negative bacteria has not been identified in the horseshoe crab and the complement-dependent clearance system of invading pathogens remains to be examined. PO, phenoloxidase; PLC, phospholipase C; PIP_2, phosphatidylinositol-4,5-biphosphate; IP_3, inositol-1,4,5-triphosphate; ER, endoplasmic reticulum.

and the other three species are mainly distributed throughout Southeast Asia. In Japan, *T. tridentatus* inhabits coastal areas of the northern part of Kyushu Island as well as the Inland Sea. *T. tridentatus* has proven to be a suitable model system for the investigation of arthropod immunity, since, in addition to having a sophisticated innate immune system, it is relatively long-lived; the embryo molts four times within the fertilized egg, and after hatching it molts every year over 15 years to become a mature adult (Sekiguchi *et al.*, 1988). Here we review our current knowledge of horseshoe crab innate immunity at the molecular level with an emphasis on the importance of hemocytes and hemolymph plasma.

LPS-induced hemocyte exocytosis and its endogenous amplification system

In *T. tridentatus*, granular hemocytes, as determined by morphological classification, constitute 99 % of all hemocytes, and play a key role in the innate immune system (Iwanaga *et al.*, 1998; Iwanaga, 2002; Kawabata and Tsuda, 2002). Horseshoe crab hemocytes respond selectively to LPS but not to other PAMPs, such as β-1,3-D-glucans and peptidoglycans (Ariki *et al.*, 2004). A variety of defense molecules are stored in the secretory granules of the hemocyte; large granules contain serine protease zymogens for hemolymph coagulation (factor C, factor G, factor B,

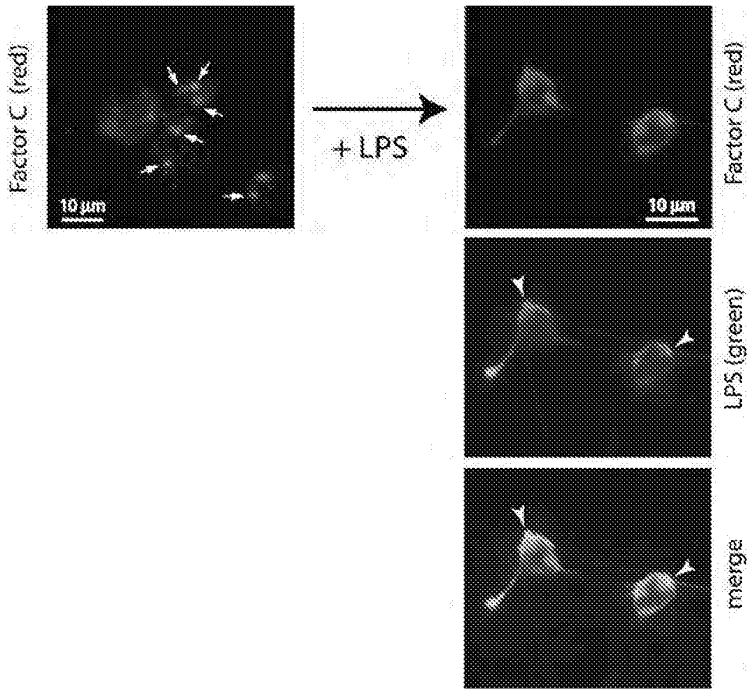


Fig. 2 Factor C is a membrane associated LPS sensor on hemocytes. Hemocytes were treated without (left panel) and with (right panels) FITC-labeled LPS (green), and stained with a monoclonal antibody (2C12) against factor C. For the detection, Cy3-conjugated anti-mouse secondary antibody was used (red). Arrowheads indicate LPS co-localized with membrane bound factor C. Bar = 10 µm.

and the proclotting enzyme), the clottable protein coagulogen, serine protease inhibitors (serpins), lectins, and substrates for transglutaminase (TGase), whereas small granules contains antimicrobial peptides. In response to stimulation by LPS, these defense molecules are rapidly secreted by the hemocyte (Fig. 1).

Factor C is a unique LPS-responsive serine protease zymogen that is stored in the large granules of hemocytes and acts as an LPS sensor to potentiate hemocyte exocytosis. Upon activation by LPS, factor C initiates hemocyte exocytosis via a G-protein-dependent exocytic pathway that is dependent upon the proteolytic activity of factor C. In this respect, the activation of hemocytes by factor C is analogous to the thrombin-thrombin receptor (the protease-activated G protein-coupled receptor, PAR) signaling axis in mammalian platelets (Ariki *et al.*, 2004). Hemocyte exocytosis can be quantitatively assayed by ELISA using an antibody against a granular component such as coagulogen in the presence of 50 mM Mg^{2+} and 10 mM Ca^{2+}, equivalent to the concentrations of these cations in hemolymph plasma. Exclusion of divalent cations from the assay buffer inhibits exocytosis even at high concentrations of LPS. Moreover, in the absence of LPS, hemocyte exocytosis can be induced by synthetic hexapeptides corresponding to the tethered ligands of mammalian PARs, supporting the notion of a PAR-like receptor on the hemocyte surface.

Immunofluorescence microscopy of the hemocyte using an anti-factor C antibody detects factor C, which is localized in a punctate distribution on the hemocyte surface (Kurata *et al.*, 2006; Koshiba *et al.*, 2007) (Fig. 2). When hemocytes are incubated with FITC-labeled LPS, the factor C antigen co-localizes with FITC-LPS accumulated on the cell surface (Koshiba *et al.*, 2007). The accumulation of FITC-LPS is not observed on hemocytes fixed with formaldehyde, as would be expected with chemical modification and inactivation of cell surface proteins. These results also suggest that factor C is a membrane-bound LPS sensor on the hemocyte surface.

Factor C has no obvious transmembrane domain within in its sequence, whereas it strongly interacts with acidic phospholipids (Nakamura *et al.*, 1988a). Surface plasmon resonance analyses indicate that factor C interacts with phosphatidylserine (K_d = 2.5×10^{-9} M) and phosphatidylinositol (K_d = 4.7×10^{-9} M) as well as with cholesterol (K_d = 1.4×10^{-9} M) (Ariki *et al.*, 2004). The interaction of factor C with LPS (K_d = 7.6×10^{-10} M) is

competitively inhibited by the addition of the acidic phospholipids. In contrast, cholesterol does not inhibit the interaction of factor C with LPS, suggesting that factor C interacts with cholesterol through a binding site that is distinct from that for LPS, and raising the possibility that factor C may be localized on cholesterol-rich microdomains or lipid rafts on the hemocyte membrane.

The horseshoe crab hemocyte has an endogenous positive feedback mechanism for LPS-induced hemocyte exocytosis (Ozaki *et al.*, 2005). The hemolymph contains hemocytes at ~10^6 cells/ml. LPS-induced hemocyte exocytosis is highly dependent on the cell density, namely, an increase in cell density from 0.05×10^6 to 0.8×10^6 cells/ml results in a 10^6-fold change in the apparent LPS sensitivity (from 10^{-7} to 10^{-13} g/ml of LPS), suggesting the presence of feedback mechanism for secretion via an unknown secretagogue secreted from hemocytes in response to the stimulation by LPS.

Interestingly, tachyplesin in the exocytosed fluid acts as a secondary secretagogue, thereby dramatically enhancing the sensitivity of the hemocyte to LPS. Tachyplesin (17 residues) is a potent antimicrobial peptide and one of the most abundant components stored in the hemocyte (Nakamura *et al.*, 1988b; Shigenaga *et al.*, 1990). The effective concentration of tachyplesin required for hemocyte exocytosis ranges from 5 to 10 μM, indicating that a high concentration of tachyplesin is required to act as an effective endogenous secretagogue.

Tachyplesin has structural properties in common with mastoparan, a basic tetradecapeptide from wasp venom. Mastoparan interacts directly with G proteins without direct stimulation of the upstream receptor, and induces exocytosis in the mast cell (Higashijima *et al.*, 1988). Consistent with these findings, mastoparan is able to induce hemocyte exocytosis in *T. tridentatus* (Ariki *et al.*, 2004). Moreover, tachyplesin binds to bovine G protein an equilibrium dissociation constant (K_d) of 8.8×10^{-7} M (Ozaki *et al.*, 2005). These data suggest that tachyplesin interacts with G-proteins in the hemocyte in a manner similar to that of mastoparan. In addition, tachyplesin has an ability to bind to hemocyanin (Nagai *et al.*, 2001), the major protein in the hemolymph plasma, suggesting that hemocyanin may serve as a sink for tachyplesin released from hemocytes, thereby spatially restricting its hemocyte-stimulating effect to the site of infection.

On the other hand, insect and mammals conserve a signaling pathway of the innate immune system through cell-surface receptors called Tolls and Toll-like receptors (Hoffmann, 2003; Akira *et al.*, 2006). A Toll-like receptor (tToll) has been identified in *T. tridentatus*, which is most closely related to *Drosophila* Toll in both domain architecture and overall length (Inamori *et al.*, 2000, 2004). The two receptors show a significant sequence identity between their TIR domains (39 %). Interestingly, spätzle, a protein ligand for *Drosophila* Toll, shows significant structurally similarity to horseshoe crab coagulogen (see "Hemolymph coagulation"). Moreover, tToll is nonspecifically expressed in all tissues examined (Inamori *et al.*, 2004), suggesting that tToll does not act as an LPS receptor on granular hemocytes.

In addition, NF-κB and IκB homologues (CrNF-κB and CrIκB) have been identified in the horseshoe crab *C. rotundicauda* (Wang *et al.*, 2006). Gram-negative bacteria infection causes degradation of CrIκB and nuclear translocation of CrNF-κB, leading to up-regulation of immune-related gene expression, including nitric oxide synthase and factor C, indicating that the NF-κB/IκB signaling cascade remains well conserved from horseshoe crabs to mammals, playing a fundamental role in regulating the expression of critical immune defense molecules.

Hemolymph coagulation

The hemocyte releases coagulation factors by LPS-induced exocytosis, leading to the activation of the proteolytic coagulation cascade (Fig. 1). Factor C secreted from hemocytes is autocatalytically activated in the presence of Gram-negative bacteria or LPS. The resulting activated factor C activates coagulation factor B, which in turn converts the proclotting enzyme into the clotting enzyme. The clotting enzyme then promotes the proteolytic conversion of coagulogen to coagulin, which spontaneously forms an insoluble polymer. Alternatively, activated factor G in the presence of β-1,3-D-glucans triggers the activation of the proclotting enzyme to the clotting enzyme. In this manner, factor C and factor G independently serve to couple the recognition of LPS and β-1,3-D-glucans, respectively, to the formation of a physical barrier at the site of microbial invasion.

The vertebrate coagulation system acts locally on the phospholipid surface in cooperation with Ca^{2+} at the site of vascular injury. In an analogous fashion, hemolymph coagulation in the horseshoe is restricted to the surfaces of invading pathogens, such as Gram-negative bacteria and fungi. This mechanistic similarity between the coagulation cascades of vertebrates and horseshoe crabs may lead to the erroneous assumption of a common evolutionary origin (Fig. 3). In fact, fibrinogen homologues of the horseshoe crab, named tachylectins-5A and -5B, act as non-self recognizing proteins rather than as target proteins of the coagulation cascade (Gokudan *et al.*, 1999). Also coagulogen has no structural similarity or evolutionary relatedness to fibrinogen (Bergner *et al.*, 1996).

A protease cascade in *Drosophila* has been well characterized as the morphogenetic cascade for determining embryonic dorsal-ventral polarity, leading to the production of the Toll ligand spätzle (Belvin and Anderson, 1996). The *Drosophila* Toll pathway additionally controls resistance to fungal and Gram-positive bacterial infections (Ferrandon *et al.*, 2007). Spätzle, belongs to nerve growth factor family and possesses a structural similarity to horseshoe crab coagulogen (Smith and DeLotto, 1992; Bergner *et al.*, 1996; Bergner *et al.*, 1997). In addition, a clip-like domain located in the N-terminal region of horseshoe crab coagulation factor B and the proclotting enzyme, originally identified in the proclotting enzyme as a disulfide-knotted domain,

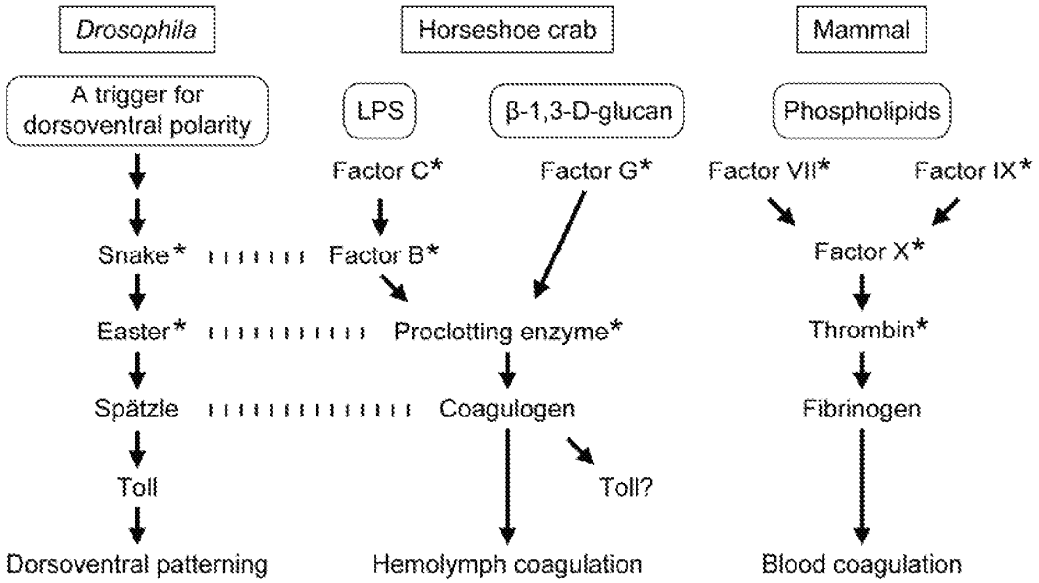


Fig. 3 Comparison of proteolytic cascades between horseshoe crab hemolymph coagulation, mammalian blood coagulation, and *Drosophila* Toll pathway. The serine protease zymogens are indicated by asterisks. Homologous proteins are connected by ladders.

has been identified in the proteins snake and easter of the *Drosophila* Toll pathway (Muta *et al.*, 1990, 1993). The structural similarity between coagulogen to spätzle, as well as that between the serine protease zymogens participating in the two cascades, suggests that the two functionally distinct cascades may have a common evolutionary origin (Fig. 3) (Krem and Di Cera, 2002; Kawabata *et al.*, 2003).

The coagulation cascade in the horseshoe crab is regulated by three types of serpins that form stable 1:1 covalent complexes with target coagulation proteases (Fig. 1); serpins-1, -2, and -3 inhibit activated factor C, the clotting enzyme, and activated factor G, respectively (Miura *et al.*, 1994, 1995; Lal Agarwala *et al.*, 1996). All three serpins are stored in the large granules of hemocytes and are secreted upon hemocyte exocytosis in response to stimulation by LPS. These serpins appear to prevent diffusion of the activated forms of coagulation factors by scavenging activated proteases that escape into the hemolymph from the surface of microbes at the site of injury, and thereby prevent unnecessary clot formation.

Horseshoe crab serpins are more closely related to mammalian serpins than they are to insect serpins. For example, serpin-1 shows higher sequence identities to human plasminogen activator inhibitor (40 %) and human neutrophil elastase inhibitor (39 %) than to an elastase inhibitor from *Manduca sexta* (29 %) and silkworm antichymotrypsin (27 %) (Miura *et al.*, 1994). Mammalian serpin-protease complexes are hypothesized to be rapidly cleared through a cell-surface receptor, which recognizes a hydrophobic consensus sequence that is selectively exposed on the complexed forms of serpins; for instance, Phe-Val-Phe-Leu-Met in the C-terminal region of α_1-antitrypsin (Joslin *et al.*, 1991). This consensus sequence is conserved in horseshoe crab serpins in the corresponding region (ex. Phe-Val-Phe-Phe-Ile for serpin-1), suggesting that a similar clearance mechanism exists in horseshoe crabs.

LPS recognition by factor C

Factor C is a multidomain glycoprotein with an apparent molecular mass of 120 kDa. In addition to a typical serine protease domain at the C-terminus, factor C contains a Cys-rich region, an epidermal factor (EGF)-like domain, five complement control protein (CCP) modules, a C-type lectin domain, and an LCCL module (derived from a conserved domain of *Limulus* factor C, Coch-5b2, and Lg11) (Muta *et al.*, 1991; Trexler *et al.*, 2000) (Fig. 4A).

Structure-function analyses reveal that the LPS-binding site in factor C is present in the N-terminal Cys-rich region of the molecule and contains a tripeptide sequence (-Arg^{36}-Trp^{37}-Arg^{38}-) consisting of an aromatic residue flanked by two basic residues (Koshiba *et al.*, 2007). A recombinant version of the Cys-rich/EGF fragment (positions 1-116) with paired substitutions of Arg^{36}/Arg^{38} to

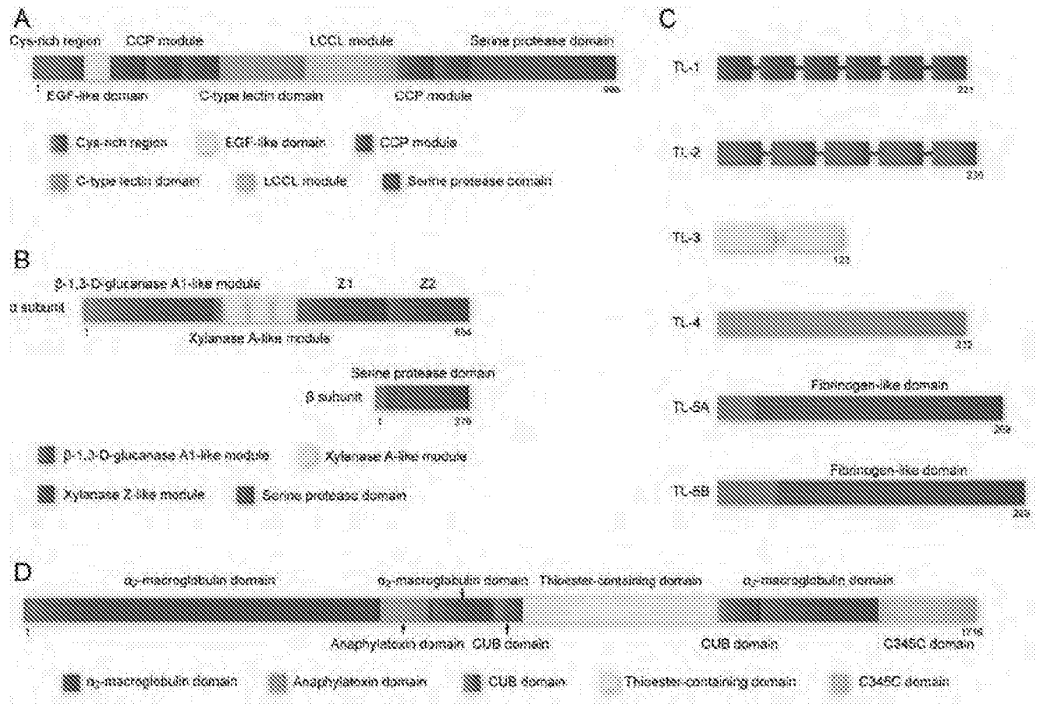


Fig. 4 Schematic domain structure of horseshoe crab innate immune proteins. (A) Domain structure of factor C. EGF, epidermal growth factor; CCP, complement control protein; LCCL, *Limulus* factor C, Coch-5b2, and Lg11. (B) Domain structure of factor G. Z1 and Z2, xylanase Z-like modules. (C) Domain structures of tachylectins. TL, tachylectin. (D) Domain structure of TtC3. CUB, complement-urchin-bone.

Glu^{36}/Glu^{38} ($R^{36}E/R^{38}E$ mutant) is incapable of binding to LPS. Moreover, a mutant protein with substitution of Trp^{37} to Ala^{37} also lacks the ability to bind LPS. These data indicate the essential nature of the tripeptide motif for LPS recognition by factor C. The Cys-rich/EGF fragment interacts with LPS with $K_d = 2.0\times10^{-10}$ M, which is similar to that of the wild-type protein (Shibata *et al.*, unpublished data).

This tripeptide motif is conserved in horseshoe crab anti-LPS factor (Aketagawa *et al.*, 1986) and several mammalian LPS-recognizing proteins such as LPS-binding protein (Schumann *et al.*, 1990) and bactericidal/permeability-increasing protein (Marra *et al.*, 1990) (Fig. 5A). Anti-LPS factor (102 residues) has been identified as an inhibitor for LPS-mediated hemolymph coagulation. Anti-LPS factor is stored in the large granule of hemocytes, and shows sequence similarity to the α-lactoalbumin/lysozyme family. The crystal structure shows that anti-LPS factor has a single domain consisting of three α-helices packed against a four-stranded β-sheet to form a wedge-shaped molecule with a striking charge distribution and amphipathicity (Hoess *et al.*, 1993). The binding site for LPS likely encompasses the extended amphiphilic loop with the tripeptide motif (-Lys^{43}-Trp^{44}-Lys^{45}-) (Pristovsek *et al.*, 2005) (Fig. 5B).

In addition to its physical association with factor C, LPS promotes its autocatalytic activation upon binding to its Cys-rich region. In contrast to wild-type factor C, the $R^{36}E/R^{38}E$ variant is incapable of autoactivation in the presence of LPS (Fig. 6) (Koshiba *et al.*, unpublished data).

β-1,3-D-Glucan recognition by factor G

Factor G is another pattern-recognition protein in the coagulation cascade that acts as a sensitive and specific sensor for β-1,3-D-glucans. In other arthropods, such as crustaceans and insects, the recognition of β-1,3-D-glucans also triggers a serine protease cascade, leading to the activation of prophenoloxidase, a key enzyme in the melanization of pathogens and damaged tissues (Cerenius and Söderhäll, 2004; Kanost *et al.*, 2004; Vetvicka and Sima, 2004). In vertebrates, the recognition of β-1,3-D-glucans by dectin-1, a C-type lectin family member, potentiates the production of cytokines and antifungal reactive oxygen species by dendritic cells and macrophages (Brown, 2006).

Factor G is a heterodimeric serine protease zymogen composed of two non-covalently associated subunits, α and β (Seki *et al.*, 1994) (Fig. 4B). The β subunit contains a serine protease domain,

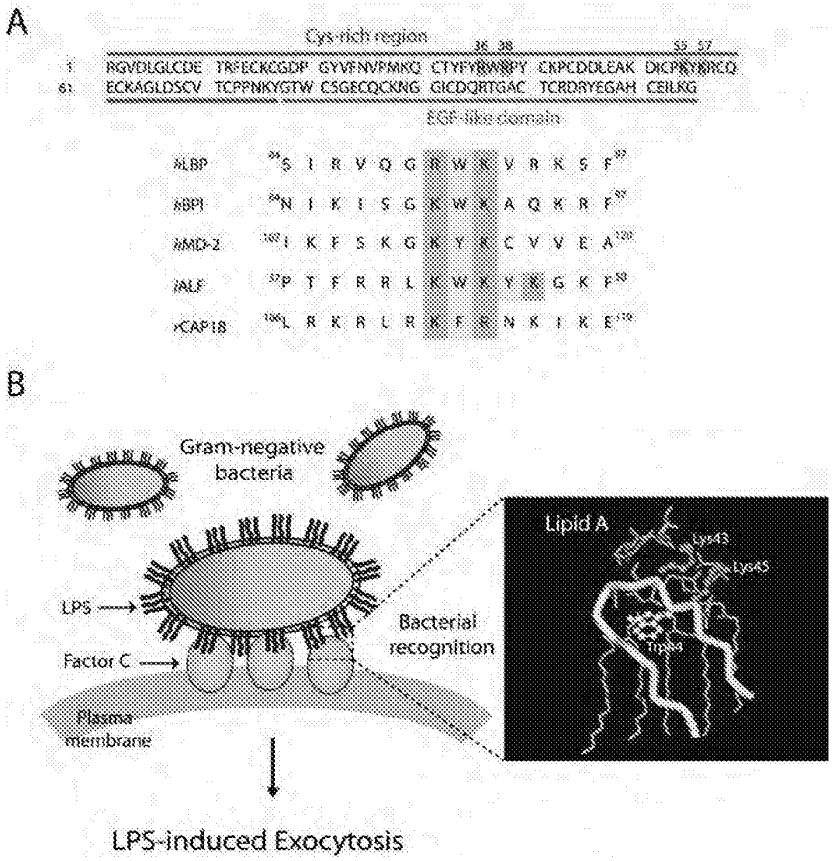


Fig. 5 A model of bacterial recognition by membrane associated factor C via N-terminal Cys-rich region. (**A**) The N-terminal sequence of factor C containing Cys-rich and EGF-like regions (R^{1}-G^{116}) is shown. The tripeptide motifs in the Cys-rich region are highlighted. hLBP, human LPS-binding protein; hBPI, human bacterial permeability-increasing protein; hMD-2, human MD-2; lALF, *Limulus* anti-LPS factor; and rCAP18, rabbit cationic antimicrobial protein. (**B**) The recognition of LPS on Gram-negative bacteria via membrane-associated factor C initiates the horseshoe crab innate immune response. The modeled 3D structure of the lALF-LPS complex is shown in the right panel (Pristovsek *et al.*, 2005) and this model may resemble that of the complex between N-terminal elements of factor C and LPS.

and the α subunit acts as a pattern-recognition subunit. The α subunit comprises three types of non-catalytic glycosidase-like modules: a single β-1,3-D-glucanase A1-like module, three tandem xylanase A-like modules, and two tandem xylanase Z-like modules. Of the three types of glycosidase-like modules, the two xylanase Z-like modules (Z1 and Z2) have been identified as independent binding sites for β-1,3-D-glucans (Takaki *et al.*, 2002). This observation, taken together with the high degree of sequence identity between Z1 and Z2 (91 %), suggests that duplicated binding sites for β-1,3-D-glucans may increase avidity to allow stable and specific recognition of pathogens.

Both Z1 and Z2 show significant sequence similarity to a carbohydrate-binding module of endoglucanase 5A from the aerobic soil bacterium *Cellvibrio mixtus* (45 % sequence identity). Endoglucanase 5A from *C. mixtus* contains an N-terminal catalytic domain and two tandem repeats of non-catalytic family 6 carbohydrate-binding modules, *Cm*CBM6-1 and *Cm*CBM6-2 (Fontes *et al.*, 1998). Our recent structural studies of recombinant Z2 domain by nuclear magnetic resonance spectroscopy clearly indicate that the ligand-binding site in Z2 is located in a cleft on a β-sheet in a predicted β-sandwich structure, which is superimposed onto cleft B in *Cm*CBM6-2 (Ueda *et al.*, unpublished data). Pattern recognition for β-1,3-D-glucans by factor G may be accomplished by a carbohydrate-binding cleft that is evolutionally conserved among invertebrates and bacteria. A crystal structure of the extracellular domain of mouse

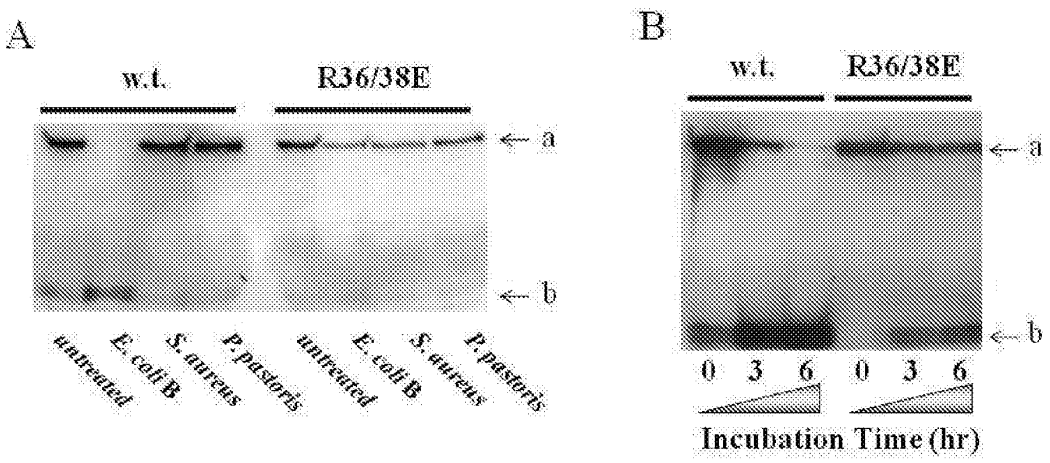


Fig. 6 The R36/38E mutant of factor C abolishes the autocatalytic activation of factor C by Gram-negative bacteria. (A) The wild-type factor C was specifically activated by Gram-negative bacteria (*E. coli* B) but not by Gram-positive bacteria (*S. aureus*) and fungi (*Pichia. pastoris*), whereas the R36/38E mutant was not converted into the active form by *E. coli* B. The wild-type and the mutant factor C contained a Myc-epitope tag at their C-terminal ends and were identified by Western blotting with the anti-Myc monoclonal antibody (9E10). The zymogen form and the active form (the light chain containing the protease domain) of factor C are indicated by the arrows a and b, respectively. (B) The time course for the autocatalytic activation of the wild-type and mutant factor C in the presence of *E. coli* B.

dectin-1 has been determined (Brown *et al.*, 2007), and the putative ligand-binding site of dectin-1 exhibits no structural similarity to that of the predicted structure of Z2.

The "sliding" of factor G molecules on β-1,3-D-glucans seems be essential to increase the frequency of collision between factor G molecules and resultant autocatalytic activation (Takaki *et al.*, 2002). An insoluble β-1,3-D-glucan (curdlan) activates factor G at a minimum concentration of 0.01 ng/ml, whereas a 1000-fold higher concentration of a soluble β-1,3-D-glucan (laminarin) is required for activation (Tanaka *et al.*, 1991). β-1,3-D-glucans have specific molecular structures, and high molecular weight β-1,3-D-glucans have higher ordered structures such as triple helices (Bohn and BeMiller, 1995). Therefore, to induce the autoactivation efficiently, the dual binding of the repeating modules may occur in the case of the interaction of factor G with curdlan but not with laminarin. The importance of dual binding of repeating modules to β-1,3-D-glucans for the autoactivation of factor G remains to be examined.

PAMP recognition by tachylectins

Given the complexity and diversity of pathogen-associated carbohydrate moieties and the essential nature of specific carbohydrate recognition in innate immunity, it has been proposed that a third biochemical alphabet, the sugar code, is indispensable (Gabius *et al.*, 2002). In *T. tridentatus*, four types of lectins have been identified in the large granule of hemocytes, including tachylectin-1 (Saito *et al.*, 1995a), tachylectin-2 (Okino *et al.*, 1995), tachylectin-3 (Saito *et al.*, 1997), tachylectin-4 (Inamori *et al.*, 1999), all of which are secreted upon LPS-induced hemocyte exocytosis (Fig. 1). Although each tachylectin recognizes PAMPs, their carbohydrate ligand specificities differ. Tachylectin-1 interacts with 2-keto-3-deoxyoctonate on the surface of Gram-negative bacteria, whereas tachylectin-2 binds to GlcNAc or GalNAc and recognizes lipoteichoic acids of Gram-positive bacteria. In contrast, tachylectin-3 specifically recognizes a certain sugar moiety on O-antigens of S-type LPS from several specific Gram-negative bacteria, such as *Escherichia coli* O111:B4. Tachylectin-4 also recognizes the O-antigen of *E. coli* O111:B4 and shows the ligand specificity for colitose (3-deoxy-L-fucose), a unique sugar present in the O-antigen. In addition, an isoprotein of tachylectin-1, named tachylectin-P, has also been found in the perivitelline space of the egg (Nagai *et al.*, 1999).

The crystal structure of tachylectin-2 clearly points out the importance of multivalency for achieving high specificity and high affinity (Beisel *et al.*, 1999). Tachylectin-2 (236 residues) belongs to the so-called WD-protein family, characterized by the repetitive sequence of 40-60 residues containing Trp and Asp residues (Neer *et al.*, 1994). Tachylectin-2 contains five tandem WD-repeats of 47 residues and adopts a five-bladed β-propeller structure with five equivalent GlcNAc/GalNAc-binding site (Fig. 7A). Each propeller blade has an independent binding site for the ligand. The specific recognition by tachylectin-2 is reinforced by the short distance between the individual binding sites (25 Å), according

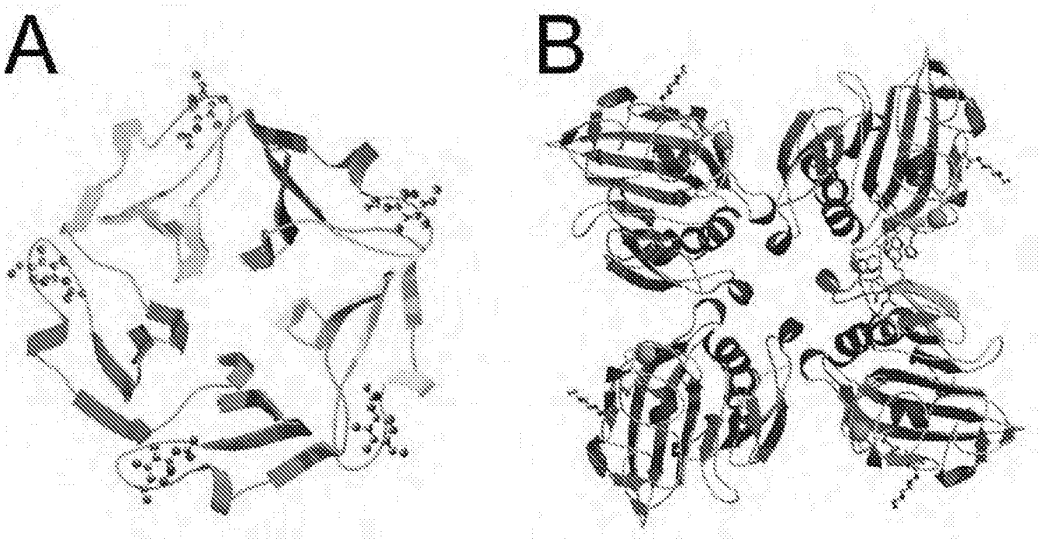


Fig. 7 Crystal structures of tachylectins-2 and -5A. (A) The 5-fold β-propeller structure of tachylectin-2 in complex with GlcNAc. (B) Oligomeric arrangement of tachylectin-5A subunits around the 4-fold crystallographic axis.

to the pentagonal geometry, required to interact with PAMPs. Tachylectin-1 (221 residues) and tachylectin-3 (123 residues), despite lacking significant overall sequence similarity to tachylectin-2, also consist of WD repeats, with six-WD-repeats for tachyectin-1 and two-WD-repeats for tachylectin-3, suggesting β-propeller structures analogous to that of tachylectin-2. Ultracentrifugation analysis shows that tachylectin-3 is present in dimer in solution. In contrast, tachylectin-4 (232 residues) is an oligomeric glycoprotein of 470 kDa and is homologous to the N-terminal domain of *Xenopus* pentraxin-1 (Fig. 4C). The function of the N-terminal domain of pentraxin-1 is unknown.

Hemolymph plasma also contains several lectins, such as isoforms of tachylectin-1 (Chiou *et al.*, 2000; Chen *et al.*, 2001) and three types of C-reactive proteins (CRPs), all of which exhibit functional and structural diversity (Iwaki *et al.*, 1999). Human CRP is an acute-phase reactant, its concentration increasing rapidly in response to stress, injury or infection, and it belongs to a protein family of pentraxin (Osmand *et al.*, 1977; Tennent and Pepys, 1994). Horseshoe crab CRP is a predominant LPS-binding protein and is up-regulated at transcript levels by *Pseudomonas* infection, suggesting the importance of horseshoe crab CRP as a conserved molecule for pathogen recognition (Ng *et al.*, 2004). CRP from the horseshoe crab *L. polyphemus* forms extended fibrilar structures that encapsulate liposomes in the presence of Ca^{2+} (Harrington *et al.*, 2009). Furthermore, the membranes of *Limulus* CRP-treated bacteria exhibit significantly different mechano-elastic properties than those of untreated bacteria, suggesting the protein's role as a primary defense molecule, acting in the entrapment and killing of potential pathogens.

In addition, tachylectins-5A and -5B, with binding specificity for acetyl-group have been identified in hemolymph plasma (Gokudan *et al.*, 1999). The overall sequence identity between tachylectins-5A (269 residues) and -5B (289 residues) is 45 %. About two thirds of the total hemagglutinating activity in the hemolymph plasma can be attributed to tachylectins-5 A and -5B, which strongly agglutinate all types of human erythrocytes at a minimal concentration of 0.004 μg/ml, and also agglutinate both Gram-negative and Gram-positive bacteria. The concentration of tachylectins-5A or -5B in the plasma is at least 10 μg/ml, suggesting that they play an important role in the recognition of invading pathogens at the forefront of the innate immune system.

Tachylectins-5A and -5B show significant sequence similarity to the C-terminal globular domain of the γ-chain of vertebrate fibrinogens and fibrinogen-related proteins, with pairwise identities ranging from 35 to 51 % and highest for the fibrinogen-like domain of human ficolin. Ficolin is composed of an N-terminal Cys-containing oligomerization segment followed by a collagen-like domain and the C-terminal fibrinogen-like domain, forming an overall structure that resembles a bundle-of-tulips (Matsushita and Fujita, 2001; Fujita, 2002). In plasma, ficolin exists as a complex with specific serine proteases, the mannose-binding lectin-associated serine proteases, through the collagen-like domain, leading to complement activation after the recognition of invading pathogens. An analogous collagenous domain is absent in the corresponding regions of tachylecins-5A and 5B.

The crystal structure of tachylecitn-5A is readily superimposed onto that of the C-terminal polymerization domain of the γ-chain of fibrinogen (Kairies *et al.*, 2001) (Fig. 7B). There is a dramatic structural similarity between these proteins, not only with respect to the overall topology, but also within their Ca^{2+}-binding sites. During the final stage of mammalian blood coagulation, thrombin cleaves the N-terminal portion of the fibrinogen α-chain, and the newly created N-terminal sequence containing Gly-Pro-Arg-Pro- is recognized by the polymerization pocket on the γ-chain to form fibrin protofibrils (Pratt *et al*, 1997; Spraggon *et al.*, 1997). The polymerization pocket within the γ-chain structurally corresponds to the acetyl group-binding site of tachylectin-5A, a finding that highlights the evolutionary connection between hemostasis and non-self recognition. Tachylectin-5A is present in oligomer in hemolymph plasma, and its propeller-like arrangement is evident by electron microscopy (Gokudan *et al.*, 1999). Given that tachylectins -2 and -5 exhibit virtually no side- or main-chain conformational changes upon ligand binding, it is likely that the polyvalent nature of these molecules underlies their avidity by allowing them to recognize specific densities or clustering patterns ligands on the surface of pathogens.

Roles of antimicrobial peptides in innate immunity

In arthropods, antimicrobial peptides are widely recognized to be very important for host defense against, and killing of, invasive microbes. However, under isotonic conditions (0.5 M NaCl concentration for horseshoe crabs), the apparent antimicrobial activities of horseshoe crab antimicrobial peptides are dramatically reduced to those under hypotonic conditions. Therefore, the antimicrobial peptides of horseshoe crabs may act as endogenous mediators and regulators in the innate immune system by enhancing such processes as hemocyte exocytosis and conversion of hemocyanin to phenoloxidase (described below).

In *T. tridentatus*, the small granules of hemocytes contain several kinds of cysteine-rich peptides with antimicrobial activities, including tachyplesin (Nakamura *et al.*, 1988b), tachystatins (Osaki *et al.*, 1999), big defensin (Saito *et al.*, 1995b; Kawabata *et al.*, 1997), and tachycitin (Kawabata *et al.*, 1996). The IC_{50} values for these antimicrobial peptides under hypotonic conditions are listed in Table 1 (Osaki *et al.*, 1999). All of the antimicrobial peptides identified in the horseshoe crab have an affinity for chitin, primary target of the innate immune system.

Tachyplesin significantly inhibits the growth of Gram-negative and Gram-positive bacteria and fungi (Table 1). Tachyplesin forms a rigid hairpin loop constrained by two disulfide bridges and adopts the conformation comprising an antiparallel β-sheet connected to a β-turn (Kawano *et al.*, 1990; Laederach *et al.*, 2002; Mizuguchi *et al.*, 2005) (Fig. 8A). When viewed as a planar surface, six basic residues are localized on one face, and six hydrophobic residues are distributed on the opposing face. This amphiphilic structure is presumed

Table 1 Antimicrobial activities of horseshoe crab antimicrobial peptides

	E. coli	*S. aureus*	*C. albicans*
	IC_{50} (μg/ml)		
Tachyplesin	2.5	0.3	0.2
Tachystatin A	25	4.2	3.0
Tachystatin B	No inhibition	7.4	3.0
Tachystatin C	1.2	0.8	0.9
Big defensin	2.5	2.5	20
Tachycitin	33	56	52

to be closely associated with its antimicrobial activity.

Tachystatins A (44 residues), B (42 residues), and C (41 residues) also exhibit a broad spectrum of antimicrobial activity (Table 1). Of the tachystatins, tachystatin C shows the most potent antimicrobial activity, and tachystatin C, but not tachystatins A and B, additionally exhibits hemolytic activity against sheep erythrocytes. Furthermore, tachystatin C has strong cell lysis activity against budding yeast. Tachystatins A and B have 42 % sequence identity. Tachystatin C contains a disulfide motif analogous to that found in tachystatin A, but otherwise shows very low sequence similarity to tachystatin A. Tachystatin A is homologous to ω-agatoxin-IVA of funnel web spider venom, a potent blocker of voltage-dependent Ca^{2+} channels (Mintz *et al.*, 1992). Despite this similarity, tachystatin A exhibits no blocking activity toward P-type Ca^{2+} channel in rat Purkinje cells.

Tachystatin A consists of a cysteine-stabilized triple-stranded β-sheet and shows an amphiphilicity commonly observed in membrane-interactive peptides (Fujitani *et al.*, 2002) (Fig. 8B). Tachystatin A shares structural similarity with ω-agatoxin-IVA. Interestingly, ω-agatoxin-IVA also has antimicrobial and chitin-binding activities. The three dimensional structure of tachystatin A shows no structural homology with well known chitin-binding motifs, suggesting that tachystatin A belongs to a new family of chitin-binding peptides. As expected, tachystatin B shows a significant three dimensional structural similarity to tachystatin A (42 % sequence identity between tachystatins A and B) (Fujitani *et al.*, 2007) (Fig. 8C).

Big defensin has potent antimicrobial activities against both Gram-negative and Gram-positive bacteria, but not against fungi (Table 1). Big defensin (79 residues) is distinct from the mammalian defensins with respect to molecular size (29-34 residues for the latter). It is proteolytically cleaved by trypsin into two domains, the hydrophobic N-terminal domain and a C-terminal domain that is homologous to β-defensins. Interestingly, the N-terminal domain possesses a more potent antimicrobial activity against Gram-positive bacteria than the C-terminal domain. In contrast, the C-terminal domain displays more

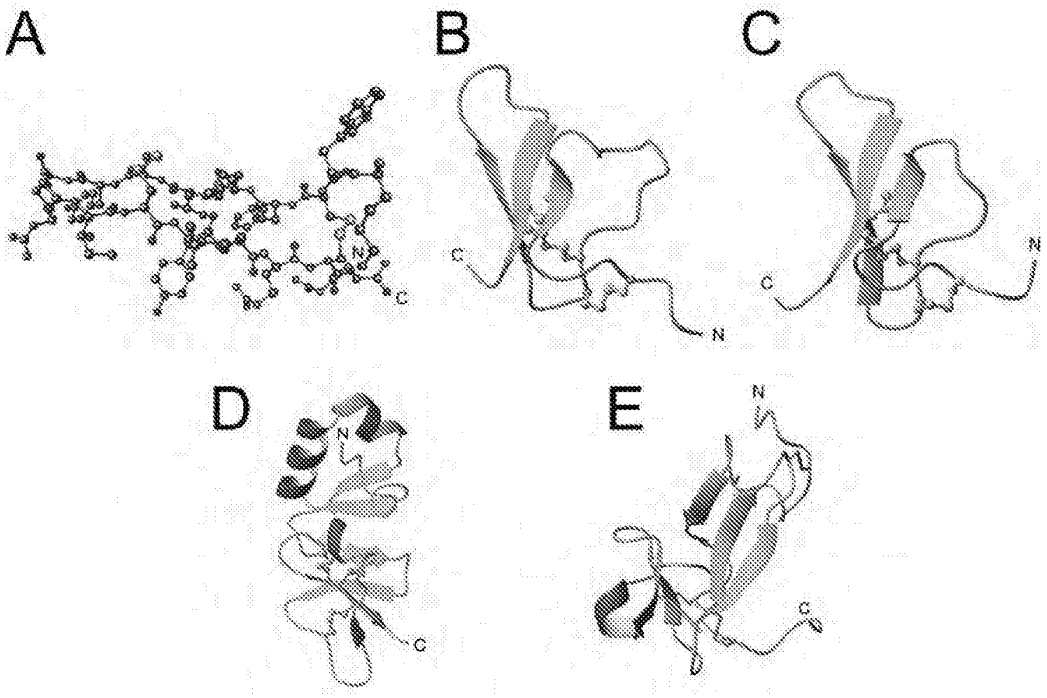


Fig. 8 NMR structures of antimicrobial peptides of the horseshoe crab. (A) Ball-and-stick model of tachyplesin. (B) Ribbon representation of the energy-minimized average structure of tachystatin A. (C) Ribbon representation of the energy-minimized average structure of tachystatin B. (D) Ribbon representation of the energy-minimized average structure of big defensin. (E) Ribbon representation of the energy-minimized average structure of tachycitin.

potent antimicrobial activity than the N-terminal domain against Gram-negative bacteria. These data suggest a new sub-class within the defensin family that possesses two discrete functional domains with different antimicrobial activities. It is noteworthy that tachylectins-5A and -5B enhance the antimicrobial activity of big defensin against Gram-positive bacteria (Gokudan *et al.*, 1999).

Structurally, big defensin represents a new class within the defensin family; the C-terminal domain adopts a β-defensin structure, whereas the N-terminal domain forms a unique globular conformation (Kouno *et al.*, 2008) (Fig. 8D). Interestingly, the hydrophobic N-terminal domain, but not the C-terminal domain, undergoes a conformational change in micelle solution, which may be associated with the antimicrobial activity against Gram-positive bacteria.

Tachycitin (73 residues) contains five disulfide bridges and has no significant sequence similarity to known antimicrobial peptides. The antimicrobial activity of tachycitin is not strong by itself (Table 1). However, tachycitin synergistically enhances the antimicrobial activity of big defensin as demonstrated by a 50-fold reduction in the IC_{50} value of big defensin against Gram-negative bacteria when a small amount of tachycitin is present.

The structure of tachycitin is largely divided into the N-terminal domain and the C-terminal domain (Suetake *et al.*, 2000) (Fig. 8E). In the C-terminal domain, tachycitin forms a hairpin loop connecting a two-stranded β-sheet, a structural motif shared by the chitin-binding site of hevein, an antifungal peptide from the rubber tree *Hevea brasiliensis* (Broekaert *et al.*, 1990). In hevein, the aromatic side chains of the two Trp residues in this loop directly interact with chitin-derived oligosaccharides. The side chain of Tyr and Val residues in the corresponding loop of tachycitin are structurally analogous to these two Trp residues in hevein. Tachyplesin also contains the similar hairpin loop that connects a two-stranded β-sheet. The hydrophobic residues clustered on the one face of their β-hairpin loops possibly participate in chitin-binding sites.

Roles of TGase-dependent protein cross-linking in the innate immune system

In mammals, the blood coagulation cascade culminates in the proteolytic conversion of soluble fibrinogen into insoluble fibrin. The resulting fibrin fibrils are further stabilized by intermolecular ε-(γ-glutamyl) lysine cross-linking by the plasma

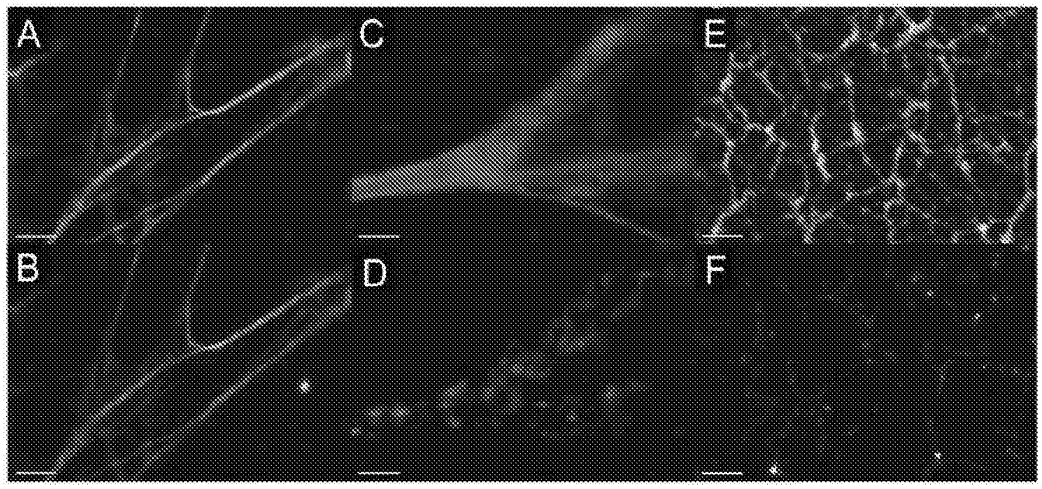


Fig. 9 Stablin as a component of the clotting mesh. (A and B) Co-localization of stablin with coagulin fibrils. Hemocytes were treated with 1 mg/ml LPS (*Salmonella minnesota* R595) for 1 h and stained with a monoclonal antibody against coagulogen (A) and a polyclonal antibody against stablin (B). As secondary antibodies, Alexa Fluor® 488 goat anti-mouse immunoglobulin and rhodamine-conjugated swine anti-rabbit immunoglobulins were used. Bar = 10 μm. (C and D) Bacterial immobilization by the clotting mesh. Hemocytes were treated with *E. coli* expressing enhanced green fluorescence protein for 1 h, stained with the monoclonal antibody against coagulogen (C), and detected by a Cy3-conjugated anti-mouse secondary antibody. *E. coli* was identified by the fluorescence of enhanced green fluorescence protein (D). Bar = 10 μm. (E and F) Involvement of stablin in the formation of the clotting mesh. Hemocytes were preincubated with 0.1 mg/ml of an antibody against stablin (F) or without the antibody (E). Hemocytes were treated with *E. coli* for 1 h, stained with the monoclonal antibody against coagulogen, and detected by the Cy3-conjugated secondary antibody. Bar = 100 μm.

TGase, factor XIIIa (Davie *et al.*, 1991). In crustaceans, hemolymph coagulation directly depends on the TGase-mediated cross-linking of a specific clotting protein (a vitellogenin-related protein) without prior proteolytic cleavage (Doolittle and Riley, 1990; Hall *et al.*, 1999; Theopold *et al.*, 2004).

In horseshoe crabs, hemolymph coagulation cascade triggered by LPS or β-1,3-D-glucans promotes in the conversion of coagulogen to coagulin. In the resulting coagulin homopolymers, coagulin monomers associate non-covalently in a head-to-tail manner (Bergner *et al.*, 1996; Kawasaki *et al.*, 2000). Horseshoe crab TGase is not present in hemolymph plasma. It is ordinarily restricted to cytoplasm of the hemocyte and secreted via an unknown mechanism in response to stimulation by LPS (Tokunaga *et al.*, 1993a, 1993b; Osaki *et al.*, 2002). Although horseshoe crab TGase neither catalyzes monodansylcadaverine incorporation into coagulin nor cross-links coagulin intermolecularly, it cross-links coagulin to other hemocyte-derived proteins, such as the proline-rich protein, proxin (Osaki *et al.*, 2002) and the cysteine-rich protein stablin (Matsuda *et al.*, 2007a), resulting coagulin fibrils with enhanced stability. An anti-stablin antibody strongly inhibits the proper formation of the clotting fibrils.

Proxin and stablin have been detected in the large granules of hemocytes and are secreted by LPS-induced exocytosis. In the absence of TGase, proxin non-covalently interacts with coagulin but not coagulogen. Moreover, proxin exhibits the specific interaction with stablin ($K_d = 4.0\times10^{-9}$ M). In contrast, stablin interacts with LPS and lipoteichoic acids and exhibits bacterial agglutinating activity against both Gram-negative and Gram-positive bacteria. Consequently, stablin co-localizes with coagulin fibrils that are cross-linked with proxin, effectively trapping bacteria (Fig. 9). In addition, stablin binds to chitin, a major component of the arthropod cuticle ($K_d = 1.5\times10^{-8}$ M). These data suggest that proxin and stablin promote not only the formation of the stable clotting fibrils but also the immobilization of invading microbes at injured sites.

Horseshoe crab TGase additionally cross-links carapace-derived chitin-binding proteins, known as caraxins, that are specifically localized to the sub-cuticular epidermis (Matsuda *et al.*, 2007b). One of these homologues, caraxin-1, comprises 135 amino acid residues and consists of N- and C-terminal heptad repeats that flank a central domain consisting of a pentapeptide tandem repeat structure. Recombinant caraxin-1 exists as an oligomer (~20-mer) in solution, and these oligomers are cross-linked by TGase to form an elaborate mesh

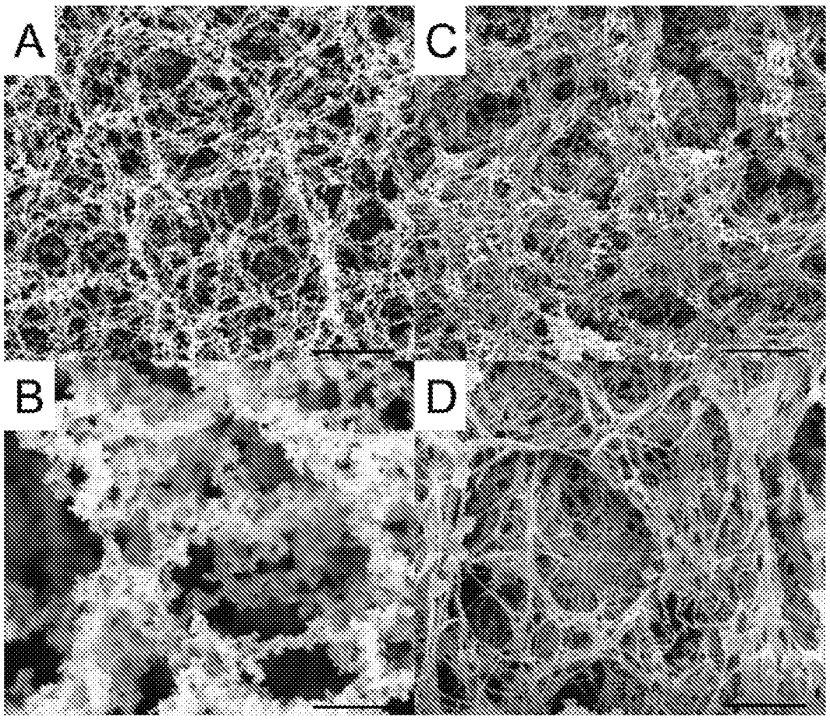


Fig. 10 Scanning electron microscopy of caraxin mesh. (A and C) Caraxin-1 was incubated with TGase at 37 °C for 16 h. Also, hemocytes were stimulated with 1μg/ml LPS for 1 h (B and D). Both samples were fixed in 4 % paraformaldehyde for 1 h. Bars were 20 μm (A and C) and 2 μm (B and D).

of honeycomb structures that is distinguishable, by electron microscopy, from the clotting mesh triggered by LPS (Fig. 10). The α-helical structures of the N- and C-terminal domains of caraxin-1 are essential for proper mesh formation.

Horseshoe crab hemocytes are actively motile, and one of the principal functions of the hemocyte is to seal scars in the cuticle. This function is fulfilled in part by the adherence of hemocytes to injured sites (Armstrong, 1985). The wound repair process of *L. polyphemus* was observed for 180 days by making a cut (1×40×3 mm) on the carapace (Bursey, 1977). According to these observations, a coagulation plug is formed within 10 min, and the coagulum is then infiltrated by hemocytes to form a cellular plug within 24 h. The sub-cuticular epithelial cells begin to migrate into the wound after 15 days, and the epithelial cells span the wound between the cut ends of the exoskeleton by day 30, and then probably secrete cuticular components to complete the wound repair process.

At the initial stage of this wound repair process sufficient quantities of TGase may be secreted from hemocytes recruited to the site of injury, and immediately activated by Ca^{2+} in hemolymph plasma. At the same time caraxin may be secreted from the sub-cuticular epithelial cells. The resulting cross-linked clotting fibrils and caraxin mesh may function to seal the wound to stop bleeding, serve as a barrier to the entry of pathogens into the interior of the animal via the wound, and operate as a transient extracellular matrix for the migration of epithelial cells that facilitate wound healing (Fig. 1).

The complement system

The vertebrate complement system consists of three activation pathways: the classical, lectin, and alternative pathways (Klein and Horejsi, 1997; Fujita, 2002). The classical and the lectin pathways are serine protease cascades initiated by the recognition of microbes by antibodies and lectins, respectively. Although the alternative pathway can be activated spontaneously, it is potently activated upon microbial infection. Activation of each of the three pathways results in generation of a proteolytic complex known as the C3 convertase (C4bC2a in the classical and lectin pathways, or C3bBb in the alternative pathway) that plays a central role in promoting downstream responses. The C3 convertase cleaves C3 to generate C3a, a peptide also known as anaphylatoxin that attracts inflammatory cells to sites of infection. The C3 convertase concomitantly generates C3b, a large polypeptide that covalently

attaches to microbial surfaces and promotes C3 receptor-dependent phagocytosis by leukocytes as well as the formation of the membrane attack complex.

Vertebrate complement factors D (Df) and B (Bf) are involved in the activation of the alternative pathway. Df is a single-chain serine protease circulating in the blood as an active protease that cleaves Bf, resulting in the formation of C3bBb. Although several homologs of vertebrate complement factors are known to be present in ascidians, cyclostomes, and echinoderms, the functional analog of Df that triggers the alternative pathway has not been identified in protostomes (Endo *et al.*, 2006; Dodds and Matsushita, 2007).

The complement-related protein, α_2-macroglobulin, has previously been identified in horseshoe crabs (Iwaki *et al.*, 1996). Recently, a homolog of complement component C3 has been identified in the horseshoe crab *C. rotundicauda* (CrC3), indicating the presence of a complement system capable of promoting the phagocytosis of invading microbes in protostomes (Zhu *et al.*, 2005). In a phylogenetic tree of reactive thioester-containing proteins from vertebrates and invertebrates, CrC3 shows the highest similarity to C3 sequences from lower deuterostomes and forms a clade with amphioxus C3, cnidaria C3-like protein, and sea urchin C3. However, despite the identification of the key complement component in protostomes, the molecular mechanism underlying C3 activation has remained unknown.

In an effort to elucidate the mechanism of C3 activation in horseshoe crabs, we have isolated and characterized an ortholog of C3 (TtC3) from *T. tridentatus* (Ariki *et al.*, 2008). TtC3 consists of 1,716 residues with an overall domain structure that is identical to vertebrate C3, including α_2-macroglobulin domains, complement-urchin-bone (CUB) domains, a thioester-containing domain, an anaphylatoxin domain, and a C345C domain (Fig. 4D). A thioester motif (-Cys-Gly-Glu-Gln-) at residues 1004 to 1007 and a catalytic His at the position 1116 are conserved in the deduced sequence. The sequence identity between TtC3 and CrC3 (98 %) is considerably higher than that between coagulogens from the two species (90 %) (Srimal *et al.*, 1985). TtC3 forms a disulfide-linked three-chain structure (α-, β-, and γ-chains) in hemolymph plasma, unlike vertebrate C3 which is present in a two-chain form.

The horseshoe crab complement system promotes the deposition of TtC3b on the surface of Gram-negative or Gram-positive bacteria in hemolymph plasma. However, evaluation of the ability of PAMPs to promote the proteolytic conversion of TtC3 to TtC3b revealed that LPS, but not zymosan, peptidoglycan, or laminarin, strongly induces this conversion, highlighting the selective response of the complement system to LPS stimulation. Factor C stored in hemocytes has been identified as an LPS sensor and an initiator of hemolymph coagulation in the horseshoe crab innate immune system. As would be expected from these findings, an anti-factor C antibody inhibits both the proteolytic conversion of TtC3 and the deposition of TtC3b on the surface of Gram-negative bacteria in hemolymph plasma. Moreover, activated factor C present on the surface of Gram-negative bacteria directly catalyzes the proteolytic conversion of the purified TtC3, thereby promoting TtC3 deposition. These data indicate that factor C acts as an LPS-sensitive C3 convertase on the surface of invading Gram-negative bacteria in the initial phase of horseshoe crab complement activation (Fig. 1).

In the alternative pathway of the vertebrate complement system, the interaction between C3b and Bb is essential to form C3 convertase (C3bBb). A homolog of complement Bf has been identified in *C. rotundicauda* (Zhu *et al.*, 2005). Although physiological function of this Bf homolog in the horseshoe crab complement system remains unknown, it is likely that it may be responsible for the formation of the second C3 convertase.

TtC3 binds to factor C at $K_d = 4.9\times10^{-8}$M (Ariki *et al.*, 2008). TtC3 is present at a concentration of at least 300 μg/ml in hemolymph plasma, whereas the amount of factor C in hemolymph plasma is very low (~10 μg/ml). The relatively high concentration of TtC3 and its high affinity to factor C suggest that factor C exists in a complex with TtC3 in hemolymph plasma, and that formation of this complex is a prerequisite for the immediate activation of TtC3 by factor C on the surface of Gram-negative bacteria. Factor C antigen has been shown to be present all the tissues examined by Western blotting. In contrast, factor G and the proclotting enzyme are not detectable in hemolymph plasma.

In contrast to these findings, the anti-factor C antibody exhibits no effect on the deposition of TtC3b on *Staphylococcus aureus*, suggesting the presence of a factor C-independent pathway to initiate the opsonization of Gram-positive bacteria (Ariki *et al.*, 2008). PAMPs or complement factors in hemolymph plasma required for the deposition of TtC3b on Gram-positive bacteria remain to be examined.

Factor C and complement Bf from *C. rotundicauda* interact with plasma-derived lectins, including galactose-binding protein, carcinolectin-5, and C-reactive protein (Saux *et al.*, 2008). Also in *T. tridentatus*, hemocytes and hemolymph plasma contain several lectins responsible for microbial agglutination (Kawabata and Tsuda, 2002). Therefore, the protease-lectin complexes on the surface of Gram-positive bacteria may enhance the deposition of TtC3b. Although the complement-dependent clearance system of invading pathogens in horseshoe crabs remains to be examined, phagocytosis of Gram-positive bacteria by hemocytes both *in vivo* and *in vitro* is inhibited by protease inhibitors, raising the possibility that the proteolytic dependence of opsonization by C3b may underlie phagocytosis by hemocytes (Zhu *et al.*, 2005).

Roles of hemocyanin in the innate immune system

In crustaceans and insects, the prophenoloxidase activation system is an important part of the innate immunity, where it acts to detect and kill invading pathogens as well as to synthesize melanin for wound healing and encapsulation of

pathogens (Cerenius and Söderhäll, 2004; Nappi *et al.*, 2004). Arthropod prophenoloxidases are known to be non-enzymatically activated by treatment with detergents, lipids, or organic solvents (Ashida and Yamasaki, 1990). In the horseshoe crab as well, the induction of phenoloxidase activity in hemolymph plasma is evident upon similar treatment (Nellaiappan and Sugumaran, 1996), yet prophenoloxidase has not been identified in horseshoe crabs. Arthropod prophenoloxidases have been sequenced, and show significant homology to hemocyanins (Aspan *et al.*, 1995; Fujimoto *et al.*, 1995; Kawabata *et al.*, 1995).

Both prophenoloxidase and hemocyanin contain two functional copper-binding sites capable of reversibly binding an oxygen molecule (Solomon *et al.*, 1996). Moreover, the origin of arthropod hemocyanins appears to be an ancient prophenoloxidase-like protein (Burmester and Scheller, 1996). Under physiological conditions, arthropod prophenoloxidases require a proteolytic cleavage for activation by a specific protease (Aspan *et al.*, 1995). Interestingly, tarantula hemocyanin is found to express phenoloxidase activity after limited proteolysis with trypsin or chymotrypsin (Decker and Rimke, 1998).

In horseshoe crabs, the clotting enzyme or activated coagulation factor B efficiently produces phenoloxidase activity in hemolymph plasma, whereas activated factor C, activated factor G, or trypsin do not (Nagai *et al.*, 2000). The phenoloxidase activity in hemolymph plasma disappears upon removal of hemocyanin by ultracentrifugation, suggesting that hemocyanin is involved in the prophenoloxidase activation system in horseshoe crabs. Horseshoe crab hemocyanin is composed of six subunits (α, β, γ, δ, ε, and ζ), each of which have a molecular mass of 70 kDa and, in their purified forms, independently express phenoloxidase activity. The clotting enzyme converts the α-subunit of hemocyanin to phenoloxidase in a dose-dependent manner, and the resulting phenoloxidase activity reaches a plateau at a 1:1 molar ratio. The proteolytic cleavage of the hemocyanin subunit is not required for the functional conversion, and the zymogen forms of the coagulation proteases, the proclotting enzyme and coagulation factor B, are effective activators.

A common structural feature of these two coagulation factors is the presence of an N-terminal clip domain (Muta *et al.*, 1990, 1993). Homologous clip domains are present in the N-terminal regions of insect prophenoloxidase-activating enzymes (Jiang *et al.*, 1998; Lee *et al.*, 1998; Satoh *et al.*, 1999). Therefore, the clip domain of the proclotting enzyme or coagulation factor B may promote the interaction of these factors with hemocyanin effect its functional conversion to an active phenoloxidase.

Horseshoe crab hemocyanin is also converted to phenoloxidase by treatment with amphiphilic substances such as SDS and phosphatidylethanolamine (Nagai and Kawabata, 2000). Consistent with the amphiphilic nature of the antimicrobial peptides secreted from the horseshoe crab hemocyte, tachyplesin interacts with the α-subunit of hemocyanin (K_d = 3.4×10^{-6} M) and induces its intrinsic phenoloxidase activity. The phenoloxidase activity induced by tachyplesin is inhibited by phenylthiourea, a typical inhibitor of phenoloxidase.

Although tachyplesin is the most effective activator of hemocyanin, other hemocyte-derived antimicrobial peptides such as tachystatins and tachycitin significantly induce its phenoloxidase activity. Mutation of tachyplesin at Trp^{2} or Tyr^{8} and Try^{13} on its hydrophobic face, but not mutation of basic residues on its cationic face, significantly impair its interaction with the α-subunit, implicating the hydrophobic face of tachyplesin in the functional conversion of hemocyanin to a phenoloxidase.

Horseshoe crab antimicrobial peptides are all capable of binding to chitin, a cell wall component of fungi and also the major structural component of the arthropod cuticle. The antimicrobial peptides likely recognize chitin exposed at sites of injury as well as on the surface of invading microbes. In addition, hemocyanin binds to tachyplesin-coated chitin. Although horseshoe crab hemocyanin present at high concentration in hemolymph plasma (~70 mg/ml) and acts as an oxygen carrier under normal physiological conditions, it may be selectively converted to phenoloxidase by the antimicrobial peptides. We hypothesize that the chitin coated with antimicrobial peptides may serve as a scaffold for the binding of hemocyanin and that the resulting phenoloxidase activity acts as trigger for wound healing in the cuticle (Fig. 1).

In the crayfish *Pacifastacus leniusculus*, an antibacterial peptide astacidin 1 has been identified (Lee *et al.*, 2003). Interestingly, astacidin 1 is released from the C-terminal part of hemocyanin by a cysteine-like protease and is up-regulated by injection of LPS or β-1,3-D-glucans, indicating that hemocyanin acts as a multifunctional protein in the innate immune system. Recently, horseshoe crab hemocyanin and human hemoglobin have been found to be activated by microbial proteases and to be enhanced by LPS, resulting in the production of reactive oxygen species as an antimicrobial strategy (Jiang *et al.*, 2007).

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Lipopolysaccharide-sensitive serine-protease zymogen (factor C) found in Limulus hemocytes

Isolation and characterization

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Bacterial endotoxin (lipopolysaccharide, LPS) induces coagulation of horseshoe crab hemolymph. Our previous studies had demonstrated that a hemolymph factor, designated factor B, was associated with the LPS-mediated activation of the Limulus clotting system [Ohki et al. (1980) *FEBS Lett. 120*, 318–321]. On further purification of factor B we found that an additional component, designated factor C, was required to generate factor B activity in the presence of LPS in order to activate the proclotting enzyme. To elucidate the role of factor C in the LPS-mediated reaction, factor C was isolated and characterized from the hemocyte lysate under sterile conditions. The preparation exhibited a single band on sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) in the absence of 2-mercaptoethanol, while two protein bands on SDS-PAGE were observed after reduction. Thus, factor C had a M_r of 123000 consisting of a heavy chain of M_r = 80000 and a light chain of M_r = 43000. Factor C was converted to an activated form in the presence of LPS with a M_r = 123000, designated factor $\overline{C}$. Upon activation, cleavage of the light chain occurred resulting in the accumulation of two new fragments of M_r = 34000 and 8500 on reduced SDS-PAGE. A diisopropylfluorophosphate-sensitive active site was localized in the light chain (M_r = 34000) of factor $\overline{C}$. The reconstitution experiments, using factor C, factor B, proclotting enzyme and LPS, demonstrated that all of these proteins are essential for the endotoxin-mediated coagulation system. On the basis of these results we propose that a cascade pathway of LPS-induced activation of the Limulus clotting system consists of three sequential activations of hemolymph serine protease zymogens.

The bacterial endotoxin-induced clotting phenomenon of the hemocyte lysate from horseshoe crab is thought to be a defense mechanism that serves to immobilize invading Gram-negative bacteria [1]. Because of this specific property and its extreme sensitivity to endotoxins, Limulus hemolymph has been applied in the last decade to estimate or quantify nanogram quantities of endotoxins in human body fluids and pharmaceutical products [2–4]. We have studied the Limulus clotting system to define the overall molecular events in the hemolymph clotting and to compare these reactions with those of mammalian blood coagulation [5, 6]. Our recent studies [5, 6, 10, 11] have shown that there are sequential activations of intracellular serine protease zymogens similar to the mammalian coagulation system [7] and that a new factor, factor B, is involved in this system [5] in addition to coagulogen and proclotting enzyme [8–10]. Here we describe the existence of another protein, designated factor C, which is sensitive to lipopolysaccharide (LPS), and delineate its involvement in the LPS-mediated initiation reaction of the Limulus clotting system. Furthermore, the isolation, characterization and the mechanism of its activation by LPS are reported herein. We propose a Limulus clotting cascade system on the basis of the reconstitution experiments and our biochemical studies on proclotting enzyme and factor B[1]. A preliminary account has been presented in abstract form [11]. The Experimental Procedure and some of the Results are presented in the miniprint supplement.

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Abbreviations. SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Dip-F, diisopropylfluorophosphate; Nan, *p*-nitroanilide; Boc, *N*-*tert*-butoxycarbonyl; Z, benzyloxycarbonyl; Bz, benzoyl; Bzl, benzyl; Cm, carboxymethyl; LPS, lipopolysaccharide; Mca, 4-methylcoumaryl-7-amide; AMC, 7-amino-4-methylcoumarin; $ClHgBzOH$, *p*-chloromercuribenzoate; HPLC, high-performance liquid chromatography.

Enzymes. Thrombin (EC 3.4.21.5); coagulation factor Xa (EC 3.4.21.6); plasmin (EC 3.4.21.7); plasma kallikrein (EC 3.4.21.34).

RESULTS

In the previous paper [5] we reported that the Limulus intracellular clotting system, mediated with LPS, consists of at least three protein components, factor B, proclotting enzyme and coagulogen. Among them, the latter two components have been purified and characterized [9, 10]. On further purification of factor B we found that an additional component, factor C, is required to generate the factor B activity in the presence of LPS. In order to clarify further the LPS-mediated clotting system, we isolated and characterized factor C from the hemocyte lysate.

Purification of factor C

The hemocyte lysate was first fractionated on a dextran-sulfate–Sepharose CL-6B column, as shown in Fig. 1.

[1] Factor $\overline{C}$ activates factor $\overline{B}$ of M_r = 64000 to its activated form, designated factor $\overline{B}$ of M_r = 36000 (T. Nakamura, T. Horiuchi, T. Morita and S. Iwanaga (1986) *J. Biochem. (Tokyo)*, March issue, in the press).

Factors B and C were separately eluted with the buffer containing 0.5 M NaCl. Fractions containing factor C and devoid of factor B were pooled as indicated in Fig. 1 and applied to a Sepharose CL-6B column. Fig. 2 shows the elution profile of factor C. While the factor C preparation in the first peak produced a single band in the unreduced sample on sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE), several bands were observed in the reduced sample (data not shown). Thus, further purification was performed by using a CM-Sepharose CL-6B column. Fractions containing factor C (Fig. 2) were applied to a CM-Sepharose CL-6B column, and eluted with linear salt gradient of the buffer containing NaCl. Fig. 3 shows that factor C was eluted as a single major peak with the activity of factor C. A summary of the procedures for purification of factor C is shown in Table 1. The yield of purified factor C was 15.5 mg. The purification procedures for factor C were repeated three times using 1.2 l lysate and reproducible results were obtained. The mean yield was 20.7 mg from 173 g hemocytes.

The purity of the final preparation was examined by PAGE in the presence and absence of SDS. On SDS-PAGE the purified zymogen gave a single band before reduction and two protein bands after reduction by 2-mercaptoethanol (Fig. 4A). This suggests that factor C consists of two polypeptide chains connected by disulfide linkage(s). As shown in Fig. 4B the purified factor C gave a single protein band on disc-PAGE at pH 4.3, and the factor C activity was observed at the same position as the protein band stained with Coomassie brilliant blue. Factor C preparation did not exhibit any amidase activity towards either Boc-Leu-Gly-Arg-Nan or Boc-Val-Pro-Arg-Nan. Furthermore, no apparent molecular change in factor C was observed on SDS-PAGE following incubation at 37 °C for 3 h under LPS-free conditions. These results indicate that the factor C preparation did not contain the activated form of factor C and was not contaminated with LPS.

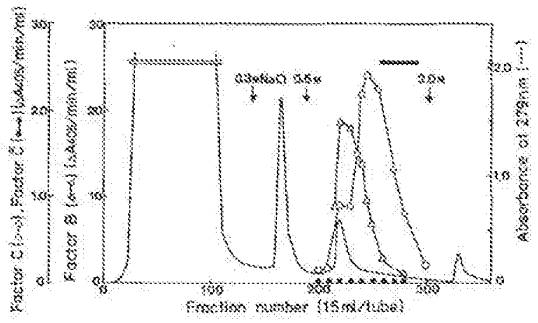


Fig. 1. *Dextran-sulfate—Sepharose CL-6B chromatography of* T. tridentatus *hemocyte lysate.* Details of the procedure are described in the supplement under Experimental Procedure. The fractions indicated by a solid bar were pooled and concentrated. Factor C (○), factor C̄ (●), and factor B (△) activities were assayed by using Boc-Leu-Gly-Arg-Nan as a substrate by the method described. Absorbance at 279 nm (——) was measured

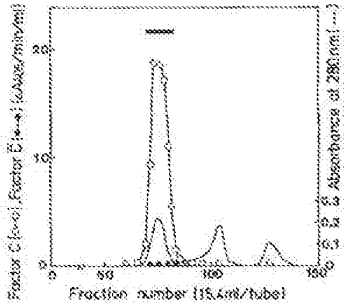


Fig. 2. *Sepharose CL-6B gel filtration of the factor C fraction obtained from a dextran-sulfate—Sepharose CL-6B column.* Details of the procedure are described in the supplement under Experimental Procedure. The fractions indicated by a soid bar were pooled. The activities of factor C (○) and factor C̄ (●) were assayed, using Boc-Val-Pro-Arg-Nan as a substrate by the method described. Absorbance at 280 nm (——)

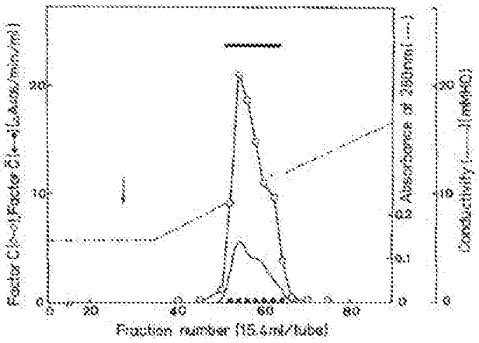


Fig. 3. *CM-Sepharose CL-6B chromatography of the factor C fraction obtained from a Sepharose CL-6B column.* The fractions indicated by a solid bar were collected. The activities of factor C (○) and factor C̄ (●) were assayed, using Boc-Val-Pro-Arg-Nan as a substrate by the method described in the supplement under Experimental Procedure. Absorbance at 280 nm (——) and the conductivity (- - - -) were measured. The gradient elution of NaCl was started at the position indicated by an arrow

Table 1. *Summary of the purification procedure for the* Tachypleus *factor C*

Step	Volume	Total protein	Total activity	Specific activity	Purification	Yield
	ml	mg	units	units/mg	-fold	%
Hemocyte lysate	1150	6095	—	—	—	—
Dextran-sulfate—Sepharose CL-6B	525	98.4	767	7.8	1	100
Sepharose CL-6B	215	25.4	572	22.5	2.9	74.6
CM-Sepharose CL-6B	215	15.5	443	28.6	3.7	57.8

Molecular mass

The molecular mass of the purified factor C was estimated using Ferguson plots [12] from the relative mobilities of the samples on SDS-PAGE. The factor C preparation and marker proteins gave a straight line (data not shown). Using this calibration line, the relative molecular mass of factor C was calculated to be 123000 in the absence of 2-mercaptoethanol. Upon reduction, the relative molecular masses of the two polypeptide chains were calculated to be 80000 (designated heavy chain) and 43000 (designated light chain).

Separation of heavy and light chains of factor C

To ascertain the polypeptide chain structure of factor C, the *S*-alkylated sample was applied to a TMS-250 column. The result is shown in Fig. 5. Two major peaks were obtained. The first peak gave a single band on SDS-PAGE while the second peak gave one major band and one minor band on SDS-PAGE (Fig. 5, inset). According to the mobilities of these bands on SDS-PAGE the first and second peaks contained the heavy chain of $M_r \approx 80000$ and the light chain of $M_r = 43000$. The minor protein band in the second peak is most probably an aggregated form of the light chain, since the apparent M_r of the minor protein band was 86000, which is consistent with the molecular mass of a light-chain dimer.

Amino acid and carbohydrate compositions

Table 2 shows the amino acid and carbohydrate compositions of the intact molecule of factor C and its heavy and light chains. A total of 1027 amino acid residues and 24 and 31 residues of hexose and hexosamine per molecule of factor C were calculated respectively. No neuraminic acid was detected (less than 0.1 residue/mol). The sum of the total amino acid

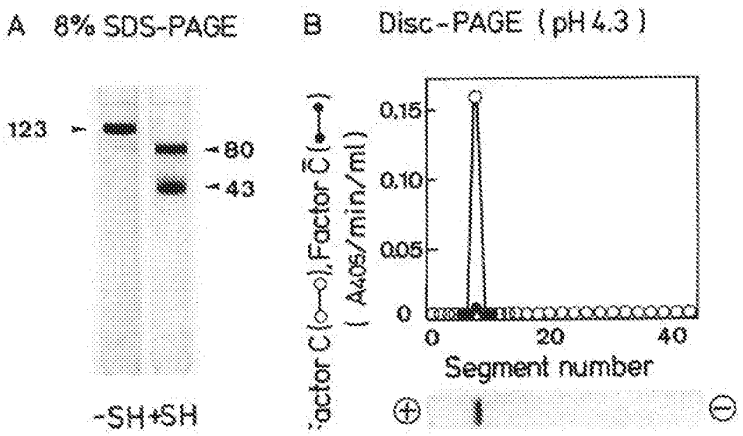


Fig. 4. *PAGE of the purified factor C.* (A) SDS-PAGE (13-μg sample) was performed in the presence (right) or absence (left) of 2-mercaptoethanol. (B) PAGE (7.5% gel) was performed at pH 4.3. One of the two gels was sliced into 1.5-mm segments and the segments were soaked in 0.4 ml 0.5 M Tris/HCl buffer, pH 8.0, containing 0.05% bovine serum albumin, and kept at 4°C for 24 h. Aliquots of the extracts (50 μl) were assayed for factor C (○) and factor C̄ (●) by using Boc-Val-Pro-Arg-Nan as a substrate. The other gel was stained with Coomassie brilliant blue R-250. Arrows show $M_r \times 10^{-3}$

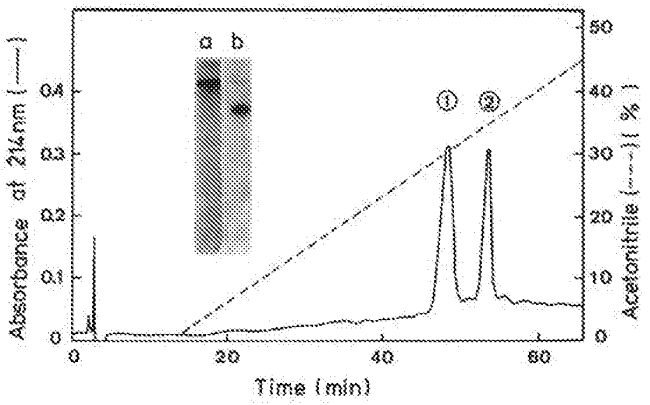


Fig. 5. *Reverse-phase HPLC pattern of S-alkylated factor C on a TMS-250 column.* (A) The *S*-alkylated factor C (64 μg) was injected onto the column and eluted with a linear gradient of acetonitrile/0.1% trifluoroacetic acid. SDS-PAGE of two peaks is shown in the insert. Lanes a and b were proteins from peaks 1 and 2 respectively

Table 2. *Amino acid compositions of S-alkylated factor C and heavy and light chains of factor C*
Amino acid and hexosamine compositions were calculated from extrapolated or average values estimated on samples of 24-h, 48-h and 72-h hydrolysates. Hexosamine, hexose and neuraminic acid were calculated as *N*-acetylhexosamine, mannose and *N*-acetylneuraminic acid respectively. Residue values are based on M_r = 112000 for the factor C polypeptide, 73000 for the heavy chain polypeptide and 39000 for the light chain polypeptide.

Amino acid or carbohydrate	Factor C	Sum of L and H chains	Heavy chain (H)	Light chain (L)
	Residues per molecule			
Cm-cysteine	65.3	65.2	48.9	16.3
Aspartic acid	109.0	108.8	69.4	39.4
Threonine	64.4	59.8	37.9	21.9
Serine	90.7	88.7	57.8	30.9
Glutamic acid	93.3	93.0	59.8	33.2
Proline	65.1	65.6	43.2	22.4
Glycine	92.4	93.7	59.9	33.8
Alanine	53.7	57.4	34.2	23.2
Valine	59.4	58.7	38.6	20.1
Methionine	10.5	9.8	6.7	3.1
Isoleucine	38.1	37.1	21.8	15.3
Leucine	67.3	69.2	43.6	25.6
Tyrosine	37.0	35.9	21.2	14.7
Phenylalanine	29.4	29.0	19.6	9.4
Lysine	57.1	57.8	40.5	17.3
Histidine	21.0	21.1	14.7	6.4
Tryptophan	23.0	24.8	13.3	11.5
Arginine	50.4	51.1	30.0	20.8
Total	1027.1	1026.8	661.4	365.4
Glucosamine	20.2	20.6	13.3	7.3
Galactosamine	11.3	11.0	7.4	3.6
Hexose	24.4	—	—	—
Neuraminic acid	< 0.1	—	—	—

and hexosamine residues of the two chains essentially corresponded to the total residues of intact factor C.

LPS-mediated activation of factor C

The activation of factor C in the presence of LPS (2 μg/ml) was followed by measuring the generation of amidase activity due to newly generated activated factor C (factor $\bar{C}$). In parallel with this experiment the structural change of the zymogen during the activation was examined by SDS-PAGE. As shown in Fig. 6A the activation reaction proceeded rapidly and was complete in 90 min. No generation of amidase activity was observed in the absence of LPS. During the activation the molecular mass of factor C remained unchanged in unreduced SDS-PAGE (Fig. 6B). In reduced SDS gels, however, the factor C light chain of M_r = 43000 was converted to 34000, while the heavy chain of M_r = 80000 did not change (Fig. 6C). In this experiment we could not detect small fragment(s) derived from the light chain by SDS-PAGE. However, the appearance of the new fragment (M_r = 34000) correlated with the generation of factor $\bar{C}$ activity. These results indicate that the activation of factor C to factor $\bar{C}$ in the presence of LPS involves limited proteolysis of the light chain of factor C.

Isolation and characterization of activated form of factor C, factor $\bar{C}$

Factor C was partially purified essentially as described in Purification of factor C, using dextran-sulfate–Sepharose CL-6B and Sepharose CL-6B. The factor-C-containing sample from Sepharose CL-6B was applied to a benzamidine–CH-Sepharose CL-6B column. Factor C was first passed through the column with the equilibration buffer, as shown in Fig. 7A. Then, the factor-C-containing sample was incubated with LPS (2 μg/ml) to activate factor C to factor $\bar{C}$ at 37°C for 4 h. The reaction mixture containing factor $\bar{C}$ was applied again to the same benzamidine–CH-Sepharose CL-6B column (Fig. 7B). In this column, contaminating proteins were unadsorbed, whereas factor $\bar{C}$ was tightly bound to the column. The column was washed with buffer containing 1 M NaCl to elute trace quantities of contaminating proteins, and factor $\bar{C}$ was subsequently eluted from the benzamidine-Sepharose column with buffer containing 1.0 M guanidine hydrochloride. The yield of factor $\bar{C}$ was 8.8 mg.

The purity of the final preparation was examined by PAGE in the presence and absence of SDS. In SDS-PAGE the preparation gave a single protein band in the absence of 2-mercaptoethanol. In the presence of 2-mercaptoethanol the preparation produced two major protein bands and one faint protein band as shown in Fig. 8A. This faint protein band was also detected by measuring the absorbance at 590 nm on the gel (Fig. 9B). As shown in Fig. 8B, the purified factor $\bar{C}$ gave a single protein band on disc-PAGE at pH 4.3. Moreover, a single peak with factor $\bar{C}$ activity was observed at the same position as the protein band stained with Coomassie brilliant blue. These data suggest that factor $\bar{C}$ consists of three polypeptide chains connected by disulfide bridges.

Molecular mass

The molecular masses of factor $\bar{C}$, the heavy chain, the light chain and a small fragment of factor $\bar{C}$ were estimated using Ferguson plots from the relative mobilities of the

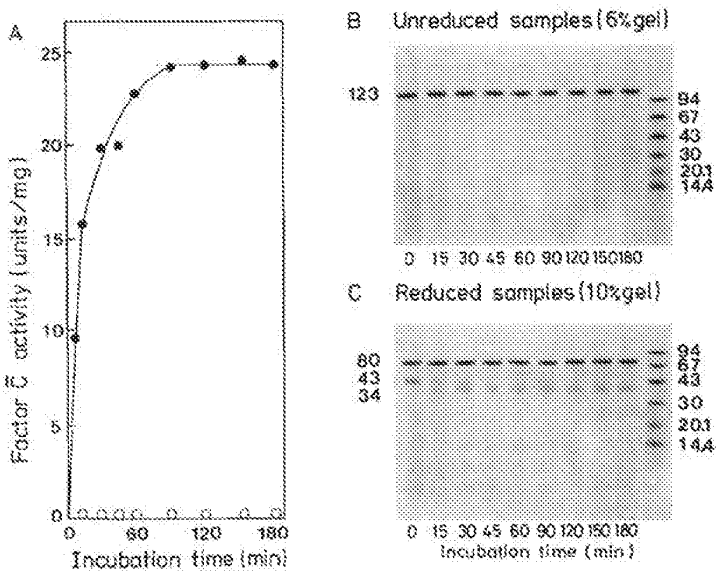


Fig. 6. Time course for activation of factor C to factor $\bar{C}$ in the presence of LPS. Factor C (248 μg/ml) was incubated at 37°C in the presence (●——●) or absence (○——○) of LPS (2 μg/ml) in a total volume of 0.05 M Tris/HCl/0.1 M NaCl (pH 8.0). At the indicated time 5-μl aliquots were withdrawn and assayed for factor $\bar{C}$ activity, and the structural change of factor C was analyzed on SDS-PAGE (6% gels for non-reduced samples and 10% gels for reduced samples). Bands are labeled as $M_r \times 10^{-3}$.

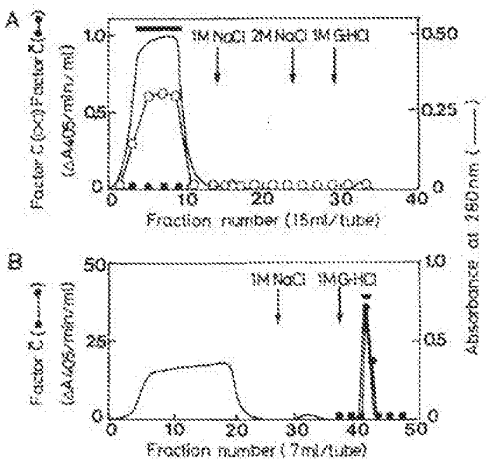


Fig. 7. First benzamidine—CH-Sepharose CL-6B chromatography of the factor-C-containing fraction obtained from a Sepharose CL-6B column (A) and second benzamidine—CH-Sepharose CL-6B chromatography of factor-$\bar{C}$-containing reaction mixture (B). (A) Details of the procedure are described in the supplement under Experimental Procedure. The fractions indicated by a solid bar were pooled. The activities of factor C (○) and factor $\bar{C}$ (●) were assayed by using Boc-Leu-Gly-Arg-Nan as a substrate by the method described. Absorbance at 280 nm (———) was measured. (B) Details of the procedure as described. The factor-$\bar{C}$-containing mixture was applied to the same benzamidine—CH-Sepharose column as described in Fig. 8A. The elution of factor $\bar{C}$ was performed with 0.02 M Tris/HCl buffer, pH 8.0, containing 1.0 M NaCl and 1.0 M guanidine/HCl. The activity of factor C (●) was assayed by the method described. Absorbance at 280 nm (———) was measured

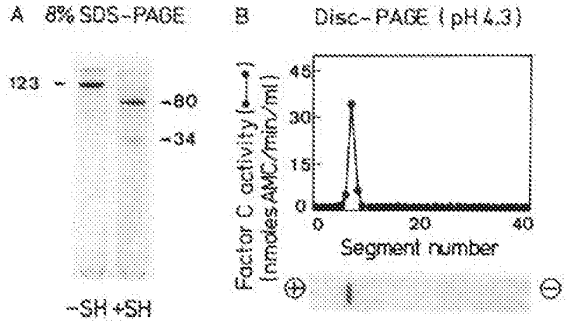


Fig. 8. PAGE of the purified factor $\bar{C}$. (A) SDS-PAGE (11-μg sample) was performed in the presence (right) or absence (left) of 2-mercaptoethanol. Bands are labeled as $M_r \times 10^{-3}$. (B) PAGE (6% gel) was performed at pH 4.3. One of the two gels was sliced into 1.5-mm segments and the segments were soaked in 0.4 ml 0.2 M Tris/HCl buffer, pH 8.0, containing 0.1% bovine serum albumin, and kept at 4°C for 45 h. Aliquots of the extracts (50 μl) were assayed for the factor $\bar{C}$ by using Boc-Val-Pro-Arg-Mca as a substrate. The other gel was stained with Coomassie brilliant blue R-250

samples on SDS-PAGE. In the absence of 2-mercaptoethanol the M_r of factor $\bar{C}$ was the same as that of factor C (M_r = 123000). Upon reduction, the M_r of the heavy chain of factor $\bar{C}$ was identical to the heavy chain of factor C (M_r = 80000), whereas the M_r of the light chain of factor $\bar{C}$ (M_r = 34000) was smaller than the light chain of factor C (M_r = 43000). In addition, a new fragment of factor $\bar{C}$ (M_r = 8500) was detected. Furthermore, the amino acid composition of factor $\bar{C}$ was essentially identical to that of factor C (data not shown).

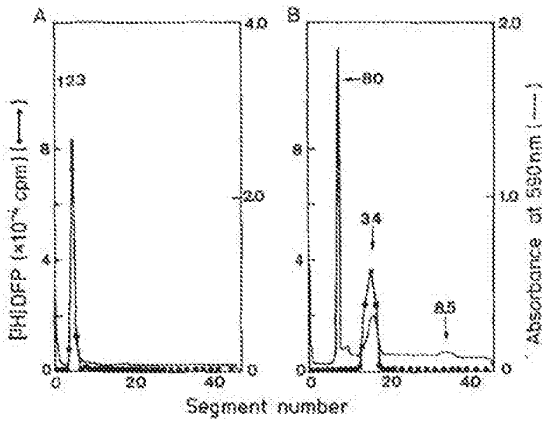


Fig. 9. *Distribution of radioactivity of [^{3}H]Dip-F-labeled factor $\overline{C}$ on SDS-PAGE.* (A) Nonreduced factor $\overline{C}$ (11 μg); (B) reduced factor $\overline{C}$ (11 μg). Experimental details are described in the supplement under Experimental Procedure. Peaks are labeled as $M_r \times 10^{-3}$

Table 3. *Effect of various inhibitors on the activity of factor $\overline{C}$*
The activity of factor $\overline{C}$ (0.05 μM) was measured using Boc-Val-Pro-Arg-Nan as a substrate as described in the supplement in Experimental Procedure. The inhibitory activity of antithrombin III was measured in the presence of heparin (12.5 units/ml)

Inhibitors	Concentration	Remaining activity
		%
None		100
Antithrombin III	0.14 μM	71
	0.27 μM	41
α_2-antiplasmin	0.42 μM	100
Hirudin	15 ATU/ml	100
Benzamidine	5 mM	49
	10 mM	31
	20 mM	17
Dip-F	0.3 mM	1
	1.0 mM	0
ClHgBzOH	1.0 mM	100
	4.0 mM	95
Leupeptin	0.06 mM	88
	0.11 mM	80
	0.23 mM	66

Incorporation of [^{3}H]diisopropylfluorophosphate ([^{3}H]Dip-F) into the light chain

Factor $\overline{C}$ was treated with [^{3}H]Dip-F under the conditions described in Experimental Procedure (see supplement) and the resulting product was subjected to SDS-PAGE. As shown in Fig. 9A a single peak of radioactivity before reduction was found at the position corresponding to factor $\overline{C}$. Upon reduction the radioactive peak migrated to the position which corresponded to the mobility of the light chain, indicating that the Dip-F-reactive site is located in the light chain of $M_r = 34000$ (Fig. 9B). In parallel with this experiment, factor C was also treated with [^{3}H]Dip-F, but it did not bind Dip-F (data not shown).

The effect of inhibitors on factor $\overline{C}$

Table 3 shows the effect of naturally occurring and synthetic proteinase inhibitors on the amidase activity of factor $\overline{C}$. The amidase activity was strongly inhibited by antithrombin III, but not by α_2-antiplasmin. Soybean trypsin inhibitor and hirudin had no inhibitory effect on factor $\overline{C}$ activity. Dip-F had a potent inhibitory effect among the three synthetic inhibitors tested. Benzamidine and leupeptin also had inhibitory activity at high concentrations (5 mM and 0.23 mM respectively), while ClHgBzOH had no apparent inhibitory effect. These results clearly indicate that factor $\overline{C}$ is a serine protease.

Substrate specificity of factor $\overline{C}$

The purified factor $\overline{C}$ activated factor B to factor $\overline{B}$ as described in the next section, but it did not activate Limulus proclotting enzyme [10] and various mammalian plasma coagulation factors, such as factor IX, factor X, prothrombin, plasminogen, protein C and prekallikrein. The mobility of these zymogens remained unchanged in SDS-PAGE after incubation with factor $\overline{C}$ (1/50, w/w, 37°C, 60 min). Moreover, no specific amidase activity appeared after the incubation of factor $\overline{C}$ with each zymogen, and factor $\overline{C}$ did not form any fibrin gel from fibrinogen after incubation (1/50, w/w, 37°C, 60 min). In addition, factor $\overline{C}$ had no effect on coagulogen. These data indicate an extremely high specificity of factor $\overline{C}$ towards Limulus clotting factor B. Table 4 shows the amidase activity of factor $\overline{C}$ toward synthetic fluorogenic peptide substrates for factor Xa, α-thrombin, plasmin, activated protein C and kallikrein [13—16]. Of the peptide substrates examined at 0.2 mM substrate concentration, factor $\overline{C}$ hydrolyzed Bz-Thr-Ser-Arg-Mca most efficiently. Factor $\overline{C}$ possessed an apparent affinity for hydroxyamino acids at the P_2 (Ser) and P_3 site (Ser or Thr) (nomenclature of Schechter and Berger [17]). We also examined four peptidyl-Naa chromogenic substrates for comparison with Mca substrates. Factor $\overline{C}$ most strongly hydrolyzed Boc-Val-Pro-Arg-Nan (Table 4).

Evidence of factor C for involvement in the LPS-mediated initiation step

To elucidate the relationship among the Limulus coagulation factors, i.e. factor C, factor B [18] and proclotting enzyme [10], and to obtain evidence for factor C as the initiation factor, reconstitution experiments were performed using LPS as a trigger for the clotting reaction and Boc-Leu-Gly-Arg-Nan as a substrate for clotting enzyme generated in this reaction. The results are shown in Table 5. No amidase activity toward Boc-Leu-Gly-Arg-Nan was observed in the combination of proclotting enzyme and factor B (experiment 4) or factor C (experiment 5) and factors B and C (experiment 6). However, strong amidase activity was observed in the reaction mixture containing the above three components in the presence of LPS (experiment 7). These results indicate that three components are required for activation of proclotting enzyme. To further confirm this conclusion and to examine factor $\overline{C}$ as an initiation enzyme, the recombination experiments were performed using factor $\overline{C}$, factor B and proclotting enzyme in the absence of LPS (Table 6). A mixture of factor $\overline{C}$, factor B and proclotting enzyme produced strong amidase activity. However, a mixture of factor $\overline{C}$ and proclotting enzyme

Table 4. *Amidase activity of factor $\bar{C}$ toward peptidyl-Mca and peptidyl-Nan substrates*
The activity of factor $\bar{C}$ was measured under the conditions described in the supplement in Experimental Procedure against the peptidyl-Mca or peptidyl-Nan substrates at 0.2 mM or 0.4 mM, respectively. Amidase activity is given as the rate of AMC or *p*-nitroaniline release

Substrates	Amidase activity
	μmol min^{-1} mg^{-1}
Boc-Val-Pro-Arg-Mca	5.8
Boc-Asp-Pro-Arg-Mca	1.3
Boc-Leu-Gly-Arg-Mca	1.0
Z-Leu-Gly-Arg-Mca	1.0
Boc-Glu(OBzl)-Gly-Arg-Mca	3.4
Boc-Gln-Gly-Arg-Mca	1.4
Boc-Met-Gly-Arg-Mca	1.4
Boc-Gly-Ser-Arg-Mca	1.0
Bz-Ser-Ser-Arg-Mca	6.6
Bz-Thr-Ser-Arg-Mca	7.2
Bz-Ser-Thr-Arg-Mca	1.7
Bz-Thr-Thr-Arg-Mca	2.5
Boc-Val-Pro-Arg-Nan	19.7
Boc-Leu-Gly-Arg-Nan	11.8
Boc-Thr-Ser-Arg-Nan	2.7
Boc-Leu-Thr-Arg-Nan	1.1

Table 5. *Recombination experiments with factor C, factor B and proclotting enzyme*
The amidase activity of the clotting enzyme generated in these experiments was measured by the method described in the supplement in Experimental Procedure. The substrate for clotting enzyme was Boc-Leu-Gly-Arg-Nan (0.4 mM). Amidase activity is given as the rate of *p*-nitroaniline release

Experiments	Recombination system	Amidase activity	
		with LPS	without LPS
		nmol/min	
1	proclotting enzyme	0	0
2	factor B	0	0
3	factor C	0.1	0
4	proclotting enzyme + factor B	0	0
5	proclotting enzyme + factor C	0.5	0
6	factor B + factor C	0	0
7	proclotting enzyme + factor B + factor C	14.9	0

Table 6. *Recombination experiments with factor $\bar{C}$, factor B and proclotting enzyme*
The amidase activity of the clotting enzyme generated in these experiments was measured by the method described in the supplement in Experimental Procedure. The substrate for clotting enzyme was Boc-Leu-Gly-Arg-Nan (0.4 mM). Amidase activity is given as the rate of *p*-nitroaniline release

Experiments	Amidase activity
	nmol/min
1. Factor $\bar{C}$ + factor B + proclotting enzyme	5.53
2. Factor $\bar{C}$ + proclotting enzyme	0.58

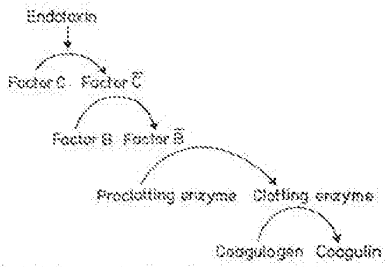


Fig. 10. *A tentative mechanism for the coagulation cascade of the Limulus hemocyte lysate*

showed weak amidase activity. These results indicate that all three components, factor C, factor B and proclotting enzyme, were required to activate proclotting enzyme, and that factor C is an initiation factor of the Limulus clotting system.

DISCUSSION

LPS has been reported to directly mediate the activation of proclotting enzyme resulting in the transformation of coagulogen to coagulin gel [19–21]. However, our recent studies have indicated that both hemocyte lysates from *Limulus polyphemus* and *Tachypleus tridentatus* contain another component, designated factor B, beside coagulogen and proclotting enzyme. All of these proteins are closely associated with the clotting system [5] and proclotting enzyme is insensitive to endotoxin [10]. In the present study we identified and separated an LPS-sensitive component, factor C, from a factor-B-containing fraction of dextran-sulfate–Sepharose CL-6B. The reconstitution experiments demonstrate that factor C is an initiation factor for the activation of proclotting enzyme in the presence of factor B and LPS. The biochemical characterization of factor C shows that it is a glycoprotein with an apparent M_r = 123000, and that it consists of two polypeptide chains; a heavy chain of M_r = 80000 and a light chain of M_r = 43000. Factor C is converted to its activated form (factor $\bar{C}$) in the presence of LPS alone, while no activation occurs without addition of LPS. These data indicate that the activation of factor C is LPS-dependent and possible contaminants, such as a contaminating protease in the factor C preparation, is excluded. Furthermore, factor C binds to LPS to form a high-molecular-mass complex (T. Nakamura, unpublished observations). The activation process is accompanied by a change in the molecular mass of factor C, and the light chain of M_r = 43000 is converted to a species with M_r = 34000. Thus, limited proteolysis of the light chain of factor C occurs during the activation process. Factor $\bar{C}$ activates factor B to factor $\bar{B}$ with proclotting enzyme activator activity. On the basis of these results and our previous results [5, 10, 18] we propose a plausible mechanism for the coagulation cascade found in the hemocyte lysate of horseshoe crab (Fig. 10). In this mechanism, bacterial endotoxins induce the activation of factor C and its active form converts factor B to factor $\bar{B}$, which subsequently activates proclotting enzyme to clotting enzyme. This sequential activation of serine protease zymogens results in the transformation of coagulogen to coagulin gel. Therefore, this cascade system found in the hemocytes may impart an extremely high sensitivity of the lysate to nanogram quantities of endotoxins.

In Limulus the LPS-mediated clotting does take place in cell adhesion, aggregation, degranulation and gel formation, and locally occurs in response to damage. In addition the Limulus clotting system found in the hemocytes appears to be a complex amplification process comparable to the mammalian blood coagulation cascade. These properties of the Limulus hemocytes are very similar to those of mammalian monocytes and macrophages, which may play an important role in extravascular coagulation. It is known that monocytes produce tissue-factor-like activity when the cells are exposed with endotoxins [22]. Macrophages are also known to synthesize factor X and prothrombin [23, 24]. Therefore, a similar coagulation cascade, induced with bacterial endotoxins, may be contained within both Limulus hemocytes and mammalian monocytes. Because of the unique properties and the sensitivity of factor C to LPS with the concomitant activation to a serine protease, it is of great interest to investigate the zymogen activation mechanism of factor C by LPS. A study on the interaction of LPS and factor C may provide a new approach to increase our understanding of the activation mechanism of other serine protease zymogens.

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Supplementary Material to
Lipopolysaccharide-sensitive serine-protease zymogen
(factor C) found in Limulus hemocyte : Isolation and
characterization
by
Takanori NAKAMURA, Takashi MORITA and Sadaaki IWANAGA

Experimental Procedure

Materials

Horseshoe crabs (Tachypleus tridentatus) were collected in Fukuoka prefecture and bled by needle puncture in the presence of cacodylic (10 mM). The hemocytes were sedimented at 1,500 rpm for 10 min at 4°C, and the lysate was prepared according to the method previously described (16). Sephacryl CL-6B, CM-Sepharose CL-6B, and carbon pentyans for the molecular weight determinations were from Pharmacia Fine Chemicals, Uppsala. LPS prepared from Escherichia coli (E. coli) 0111:B4 was a product of Difco Biological Laboratories, Inc. Fluorogenic and chromogenic substrates were from the Protein Research Foundation, Minoh, Osaka. Soybean trypsin inhibitor and bicuculin (3,800 anti-thrombin units/mg) were from Sigma Chemical Co., Ltd., St. Louis. Leupeptin sulfate was purchased from Nippon Kayaku Co., Ltd., Tokyo. Antithrombin III was prepared by a published method (25). Human α_2-antiplasmin was a kind gift from Mr. T. Sasaki, Green Pharmaceutical Co., Ltd., Tokyo. Benzamidine-CH-Sepharose CL-6B and benzamidine-CH-Sepharose 4B were prepared by the method of Bethell et al. (26). Dextran sulfate-Sepharose CL-6B was prepared as described previously (23). [^{3}H]Dip-F, BCS (trans cinnamoyl and BCS II were products of Amersham Co., USA. All other chemicals were of highest quality commercially available. The zymogen-containing fraction used for assay of factor B was partially purified by a dextran sulfate-Sepharose CL-6B column chromatography (18). This preparation enzyme fraction did not possess any detectable levels of the activated form, factor B and factor C. The factor B-containing fraction used for reconstitution experiments (in Tables 5 and 6) was partially purified by a combination of dextran sulfate-Sepharose CL-6B, Sephacryl CL-6B, and benzamidine-CH-Sepharose 4B column chromatography. This factor B-containing fraction had no detectable activated factor B and factor C.

Methods

Factor C assay

The amidase activity of factor C after activation in the presence of LPS was measured using Boc-Val-Pro-Arg-Nan as the substrate. A reaction mixture containing 10-20 µl of sample, 20 µl of 1 M Tris-HCl (pH 8.0) containing 20 mM $MgCl_2$, and 10 µl of factor C (10 µg/ml), in a total volume of 200 µl, was preincubated at 37°C for 10 min to activate factor C. To the mixture, 20 µl of 5 mM Boc-Leu-Gly-Arg-Nan and 30 µl of partially purified proclotting enzyme (8.66 µg/ml) were added and incubated further for 3 min. Finally, 0.8 ml of 0.4 M acetic acid was added and the appearance of amidase activity was measured.

Determination of protein concentration

Protein concentration of factor C was determined from the absorbance at 280 nm. The extinction coefficient $E^{1\%}_{280}$ = 21.3 for factor C was calculated from the amino acid and carbohydrate mass/$S_{20,w}$. An internal standard, ovalbumin, was included in the protein hydrolysates to allow corrective for losses. The same value was assumed for factor B. The protein concentrations of other protein samples were determined according to Bradford (27), using bovine serum albumin as a standard protein.

Electrophoresis

PAGE was performed at pH 4.3 by the method of Reisfeld et al. (28). SDS-PAGE was performed according to Weber and Osborn (29). The gels were stained with Coomassie brilliant blue R-250.

Determination of molecular weight

The molecular weights of factor C, factor B, and the heavy and light chains of factor C (or factor B) were estimated using Ferguson plots from the relative mobilities of the samples on 4 %, 5 %, 6 % and 10 % gels, using heavy meromyosin (Mw= 485,000), phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and α-lactalbumin (14,400) as reference proteins.

Amino acid and carbohydrate analyses

Samples were hydrolyzed for 24, 48 and 72 h at 110°C in evacuated, sealed tubes with 200 µl of 4 N methanesulfonic acid containing 0.2 % tryptamine. Excess 4 N methanesulfonic acid was neutralized by adding 200 µl of 3.5 M NaOH followed by the addition of 200 µl of 0.02 M HCl. Analyses were performed with a Hitachi model 835 amino acid analyzer according to Spackman et al. (30). Neutral sugar was determined by the phenol-sulfuric acid method of Dubois et al. (31), and glucosamine acid was determined by the thiobarbituric acid method (32) as modified by Ozawa and Muteki (33).

The effect of factor C on several coagulation factors

The possible activation of factor IX, factor X, prothrombin, fibrinogen, plasminogen, protein C, and plasma prekallikrein by purified factor C was examined. The activation reactions were performed with a weight ratio of factor C to plasma zymogen of 1 to 50. After incubation at 37°C for 60 min, aliquots of each

reaction mixture, except for plasma prekallikrein, were subjected to SDS-PAGE, in the presence and absence of 2-mercaptoethanol, in order to detect any possible structural change in the plasma zymogens. Other ligands (except for factor IX and fibrinogen) were used to determine the enzyme activities generated using specific fluorogenic substrates for factor Xa, α-thrombin, plasmin, activated protein C, and kallikrein. The substrates used here were Boc-Leu-Gly-Arg-Mca for factor Xa, Boc-Val-Pro-Arg-Mca for α-thrombin, Boc-Val-Leu-Lys-Mca for plasmin, Boc-Leu-Ser-Thr-Arg-Mca for activated protein C, and D-Phe-Arg-Mca for kallikrein.

The effect of inhibitors on the activity of factor D

The effect of various inhibitors on the amidase activity of factor D was measured as follows. A reaction mixture containing 20 μl of factor D (30 μg/ml), 20 μl of 1 M Tris-HCl (pH 8.0), 10 μl of human serum albumin (10 mg/ml; and 150 μl of various inhibitors, to a total volume of 200 μl, was incubated at 37°C for 5 min. Then, 50 μl of 3 mM Boc-Val-Pro-Arg-Mca was added and the amidase activity was measured.

Hydrolysis of peptidyl-Mca and -Nna substrates

A reaction mixture containing 50 μl of 1 M Tris-HCl, pH 8.0, and 20 μl of various Mca substrate (4 mM), in a total volume of 475 μl, was incubated at 37°C in a cuvette. The reaction was started by adding 25 μl (50 μg/ml) of factor D, and the change of the fluorescence at 460 nm was measured following excitation at 380 nm in a Hitachi 650-10 S fluorescence spectrophotometer. For peptidyl-Nna substrates, a reaction mixture containing 20 μl of 1 M Tris-HCl, pH 8.0, 10 μl of human serum albumin (10 mg/ml), and 20 μl of various Nna substrates (5 mM), in a total volume of 190 μl, was preincubated at 37°C for 5 min. The reaction was started by the addition of 30 μl of factor D (65 μg/ml) to the reaction mixture, and then the amidase activity was measured.

Effects of pH on the stability of factor C and factor D, the amidase activity of factor D, and the activation of factor C

To examine the pH stability, factor C (30 μg/ml) or factor D (17 μg/ml) was incubated at 37°C for 5 h at pH 1.5-11.0. After incubation, the remaining amidase activity of factor C or factor D was measured at pH 8.0 as described above. The optimum pH of the amidase activity of factor D between pH 5.5-11.0 and the optimum pH for the activation of factor C by 6% between pH 6.0-11.0 were also examined. The activation of factor C by 1% (10 μg/ml) was performed at 37°C for 60 min, and the amidase activity of factor D generated was measured at pH 8.0 using Boc-Val-Pro-Arg-Mca.

Purification of factor C from hemocyte lysate

All the following procedures were made under sterile conditions. The lysate (1,150 ml) was applied to a Sepharose 6B-CL column (5.0 x 23.5 cm), and the eluting factors were eluted as shown in Fig. 1. The factor C-containing fractions indicated by a solid bar were collected and concentrated by hollow fiber ultrafiltration. The concentrated fraction was applied to a Sephacryl S-300 column (4.0 x 137 cm), equilibrated with 0.02 M sodium acetate (pH 5.6) containing 0.1 M NaCl. Elution was carried out at a flow rate of 60 ml/h. Factor C activity appeared in the first protein peak, as shown in Fig. 2. Fractions 80 to 82 were collected for further purification. The pooled factor C fraction was applied to a CM-Sepharose CL-6B column (2.0 x 25.0 cm), equilibrated with 0.02 M sodium acetate-0.1 M NaCl (pH 5.6). Factor C was eluted with a linear NaCl gradient (0.1 to 0.35 M) in 0.02 M sodium acetate (pH 5.6) (200 ml/chamber) at a flow rate of 72 ml/h. As shown in Fig. 3, one major protein peak appeared and factor C activity coincided with this peak.

Preparation and purification of factor D from hemocyte lysate

The lysate (834 ml) prepared from 68 g (wet weight) of hemocytes was applied to a dextran sulfate-Sepharose CL-6B column (5.0 x 21.5 cm). The elution of factor C was performed by the procedure described in Fig. 1. The pooled and concentrated factor C sample was applied to a Sepharose CL-6B column (3.5 x 122 cm), equilibrated with 0.05 M Tris-HCl (pH 8.0) containing 0.2 M NaCl. Elution was carried out with the same buffer at a flow rate of 35 ml/h. The fractions containing factor C activity were collected and NaCl was added to give a final concentration of 0.5 M. This sample was applied to a benzamidine-CH-Sepharose CL-6B column (1.5 x 14 cm), equilibrated with 0.02 M Tris-HCl-0.5 M NaCl (pH 8.0). The column was then washed with 100 ml of the equilibrating buffer. As shown in Fig. 5A, factor C was eluted in the unadsorbed fraction of the column under these conditions. The proteins adsorbed on the column were eluted in stepwise fashion sequentially with 0.02 M Tris-HCl-0.5 M NaCl (pH 8.0) (150 ml), 0.02 M Tris-HCl-2.0 M NaCl (pH 8.0) (150 ml), and finally 0.02 M Tris-HCl-2.0 M NaCl (pH 8.0) containing 1.0 M guanidine hydrochloride (100 ml) to elute any factor D present in the applied sample. To the factor C pool, $MgCl_2$ and 50% of *E. coli* O111:B4 were added to give a final concentration of 5 mM and 1 mg/ml, and the mixture was incubated at 37°C for 4 h to activate factor C. This reaction mixture was applied to the same benzamidine-CH-Sepharose CL-6B column (1.5 x 14 cm), equilibrated with 0.02 M Tris-HCl-0.5 M NaCl (pH 8.0). The column was initially washed with the equilibrating buffer (100 ml) and subsequently with 0.02 M Tris-HCl-2.0 M NaCl (pH 8.0) (100 ml). Factor D was then eluted from the column with 0.02 M Tris-HCl-1.0 M NaCl (pH 8.0) containing 1.0 M guanidine hydrochloride, as shown in Fig. 5B. Factor D-containing fractions were collected and dialyzed against 0.01 M Tris-HCl-0.1 M NaCl (pH 8.0).

Separation of heavy and light chains of factor C

Factor C (4.6 mg) was reduced with dithiothreitol and alkylated with monoiodoacetamide in 0.6 ml of 0.5 M Tris-HCl (pH 8.0) containing 6 M guanidine hydrochloride and 2 mM EDTA (1G). The resulting S-alkylated protein was chromatographed on a Sephadex G-25 column (2.6 x 38.5 cm), previously equilibrated with 0.1 M ammonium bicarbonate. Then, 240 ml of S-alkylated factor C (56 mg) was applied to a DEAE-650 column equilibrated with 0.1 M trifluoroacetic acid. Elution was performed with a linear acetonitrile gradient (0- 50 %) in 0.1 % trifluoroacetic acid at flow rate of 0.5 ml/min by Beckman HPLC system.

Activation of factor C in the presence of LPS

A reaction mixture containing 200 µl of factor C (821 ng/ml), 200 µl of 0.05 M Tris-HCl (pH 8.0) / 0.25 % Poem-20, and 100 µl of LPS (10 µg/ml), were incubated at 37°C, and samples (5 µl) of the mixture were removed at selected intervals and assayed for factor C activity. At the same time, a 50 µl aliquot was subjected to SDS-PAGE in the presence or absence of 2-mercaptoethanol.

Treatment of factor C with β^3-Sepharose

The purified factor C (20 µg) in 300 µl of 0.02 M Tris-HCl (pH 8.0) containing 0.1 M NaCl, was incubated with 5 µl of 0.034 mM $\{^3S\}$ Dig-F at 37°C for 30min. After incubation, SDS-PAGE analysis of the oligomers (50 µl) were performed before and after reduction with 2-mercaptoethanol. The gels were stained with Coomassie brilliant blue R-250, destained, sliced into 1.5 mm segments, and the radioactivity of the gel slices was counted as described (18).

Reconstitution experiments of factor C, factor B and proclotting enzyme

A series of reaction mixtures containing either 5 µl of purified factor C (18 µg/ml), 20 µl of partially purified factor B (200 µg/ml), 20 µl of partially purified proclotting enzyme (900 µg/ml) or combinations of the above proteins, and 30 µl of 1 M Tris-HCl (pH 8.0) with or without 4 µl of LPS of E. coli 0111:B4 (10 µg/ml), in a total volume of 200 µl, was incubated at 37°C for 15 min. Then, 50 µl of 2 mM Boc-Leu-Gly-Arg-Nan was added to the reaction mixture and incubated further at 37°C for 1 min. Finally, 0.8 ml of 0.6 N acetic acid was added to terminate the reaction, and the absorbance at 405 nm was measured.

Reconstitution experiments of factor C, factor B and proclotting enzyme

A series of reaction mixtures containing either 10 µl of purified factor C (22 µg/ml), 50 µl of partially purified factor B (900 µg/ml) and 20 µl of partially purified proclotting enzyme (900 µg/ml) or combined mixture, 30 µl of 1 M Tris-HCl (pH 8.0) containing 50 mM $MgCl_2$, and 50 µl of 2 mM Boc-Leu-Gly-Arg-Nan, in a total volume of 250 µl, was incubated at 37°C for 6 min. Finally, 0.8 ml of 0.6 N acetic acid was added, and the absorbance at 405 nm was measured.

Results

Effect of pH on the stability and the activation of factor C

The pH stability of factor C was estimated with the various buffer systems at 37°C. Factor C was stable at pH 6.0-10.0 (data not shown). The effect of pH on activation of factor C was also estimated using Boc-Val-Pro-Arg-Nan. The optimum pH for the activation of factor C in the presence of LPS was 7.8 (data not shown).

Effect of pH on the stability and the amidase activity of factor C

The pH stability of factor C was estimated with the various buffer systems at 37°C. Factor C was found to be stable between pH 1.0 and 10.0 (data not shown). The effect of pH on the amidase activity of factor C was also examined using Boc-Val-Pro-Arg-Nan. The optimum pH of the amidase activity of factor C was 8.0 (data not shown).

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(54) **RECOMBINANT PROTEINS DERIVED FROM GENUS LIMULUS, AND DNA MOLECULES ENCODING SAME**

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CPC ***C07K 14/43509*** (2013.01); ***G01N 33/68*** (2013.01); ***G01N 33/579*** (2013.01); ***C12N 9/2477*** (2013.01)

(57) **ABSTRACT**

Provided are all full-length recombinant proteins involved in the clotting mechanism of *Limulus polyphemus*, cDNAs encoding the same, and applications thereof. A recombinant protein containing the amino acid sequence represented by SEQ ID NO: 2 or 4, a recombinant protein containing the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12, a recombinant protein containing the amino acid sequence represented by SEQ ID NO: 14, 16, 18, 20, or 22, variants thereof, cDNAs encoding the same, and utilization thereof.

Specification includes a Sequence Listing.

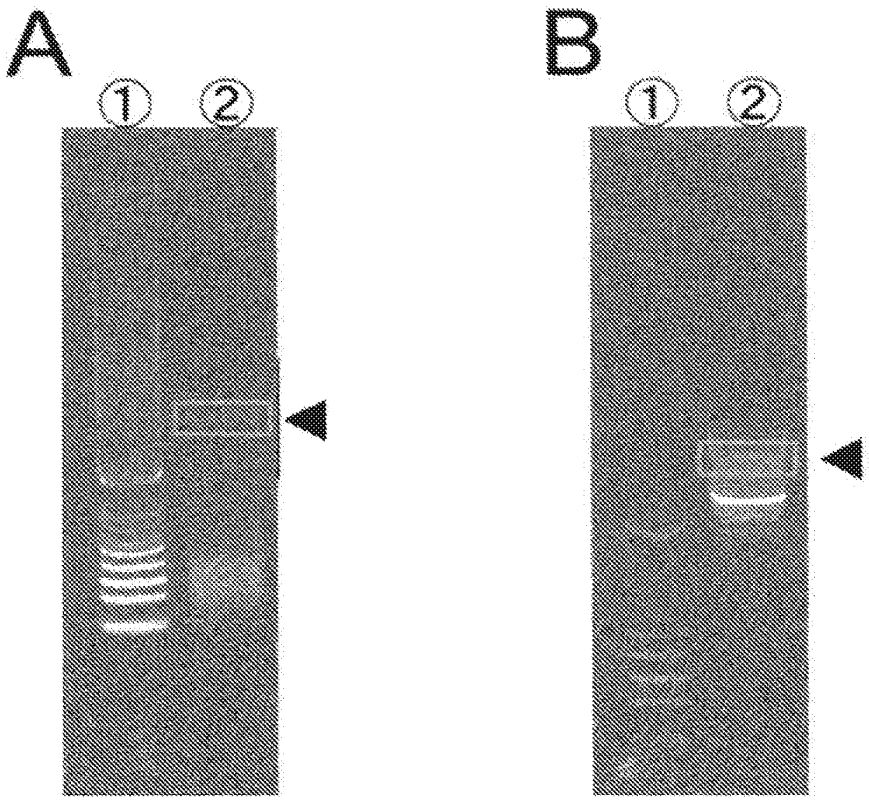


FIG. 1

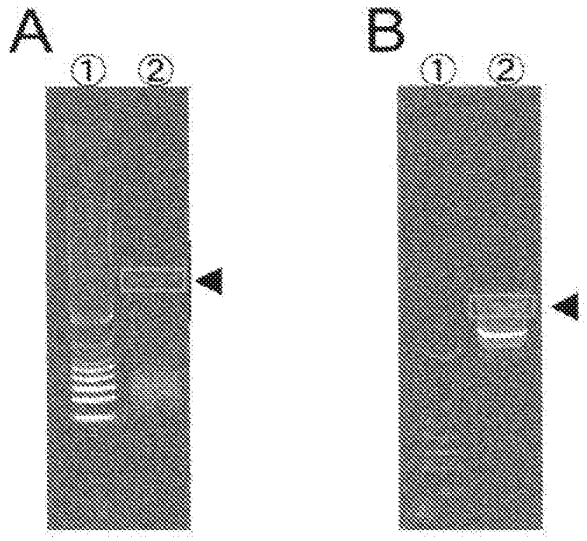


FIG. 2

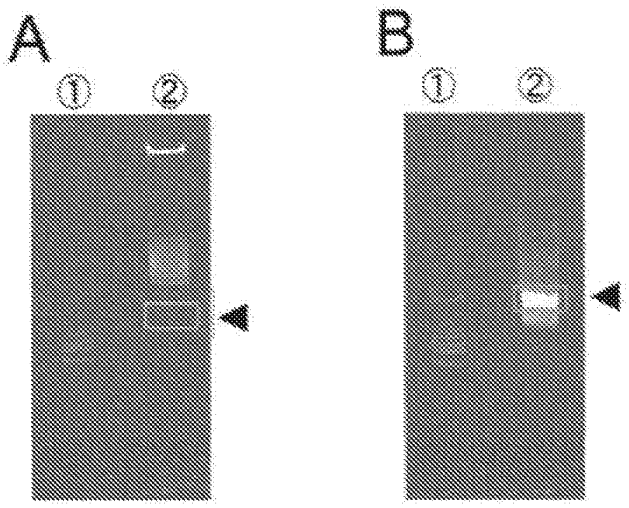


FIG. 3

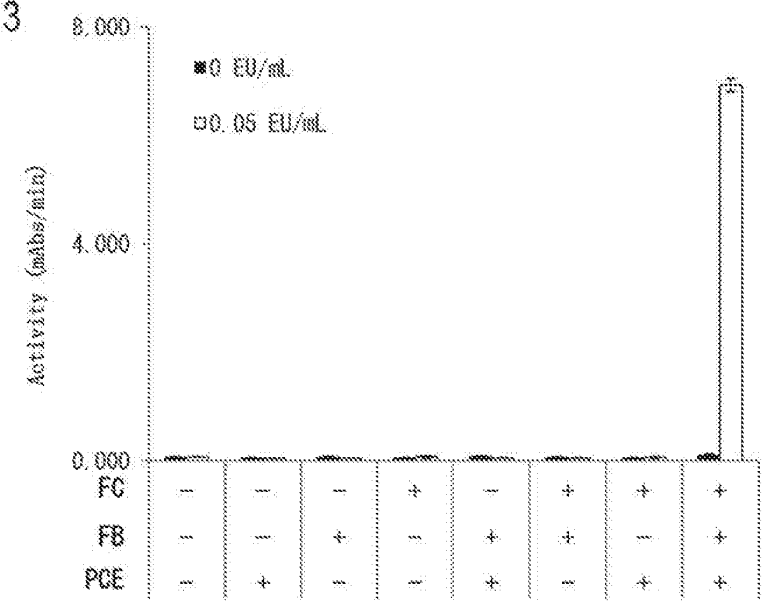
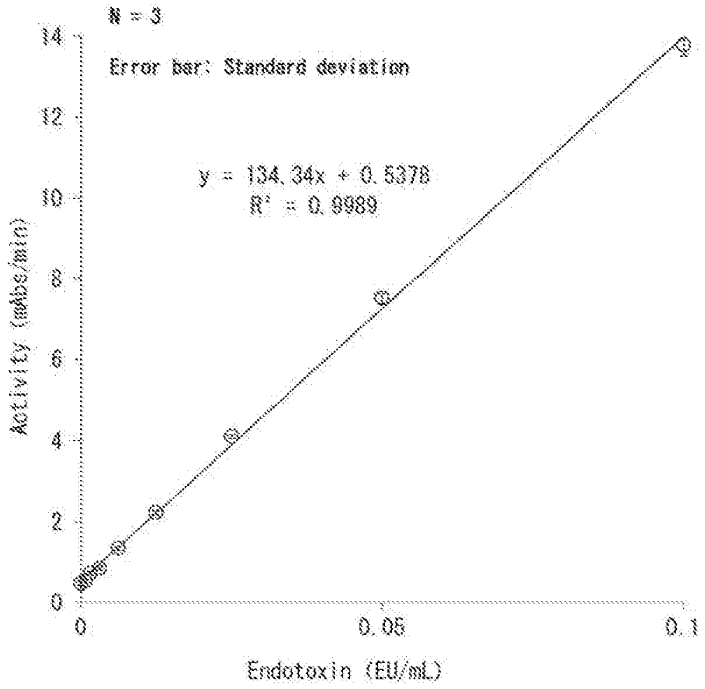

8.000
4.000
0.000
Activity (mAbs/min)
0 EU/mL
0.05 EU/mL
FC
FB
PCE
N = 3
Error bar: Standard deviation

FIG. 4


N = 3
Error bar: Standard deviation
y = 134.34x + 0.5378
R² = 0.9989
Activity (mAbs/min)
Endotoxin (EU/mL)
0
0.05
0.1
2
4
6
8
10
12
14

RECOMBINANT PROTEINS DERIVED FROM GENUS LIMULUS, AND DNA MOLECULES ENCODING SAME

TECHNICAL FIELD

[0001] The present invention relates to recombinant proteins derived from the genus *Limulus*, DNAs encoding the same, and a method for utilizing the same.

BACKGROUND ART

[0002] Horseshoe crabs are called "living fossils" and there exist only two genera and four species on Earth. As the genera, there are only "the genus *Limulus*" living only in the east coast of the North American Continent and "the genus *Tachypleus*" living only in the southeast sea area in Asia.

[0003] A distribution in which one organism group is separately distributed in regions far from each other is called "discontinuous distribution", and organisms which are separated in two regions on Earth like horseshoe crabs are very rare.

[0004] The organisms belonging to the genus *Limulus* are assigned to only one species: *Limulus polyphemus*, and the organisms belonging to the genus *Tachypleus* are assigned to only three species: *Tachypleus tridentatus*, *Tachypleus gigas*, and *Tachypleus rotundicauda* (also called *Carcinoscorpius rotundicauda*).

[0005] It is known that when an extract of amoebocytes (amoebocyte lysate) present in the blood of this organism comes into contact with an endotoxin, the extract clots. By utilizing this property, the amoebocyte lysate has been widely used for the quality control of pharmaceutical preparations or the like as a reagent for detecting an endotoxin with high sensitivity.

[0006] This reagent is called "lysate reagent". Further, the property of causing clotting by an endotoxin was found in *Limulus polyphemus* for the first time, and therefore, this reagent is sometimes called "*Limulus* reagent" or "LAL (*Limulus* amoebocyte lysate) reagent". Even a reagent using an amoebocyte lysate derived from the genus *Tachypleus* is customarily called "*Limulus* reagent" or "LAL reagent".

[0007] At present, the lysate reagent (*Limulus* reagent or LAL reagent) used by pharmaceutical manufacturers in Japan, U.S. and Europe is derived from *Limulus polyphemus* as a raw material. The number of captures of *Limulus polyphemus* is strictly limited due to extinction concerns, however, some reports also say that the population is still decreasing.

[0008] In order to protect horseshoe crabs, there has been an idea that the reagent is prepared by artificially producing proteins in the amoebocyte lysate using a recombinant technique (PTL 1 to PTL 8). Then, by utilizing recombinant proteins, products such as PyroGene (trademark) (Lonza), EndoZyme (trademark) (Hyglos), and PyroSmart (trademark) (Seikagaku Corporation) have been actually launched in the market.

[0009] However, any of these uses recombinant proteins derived from an organism of the genus *Tachypleus*, and there has been no report of a reagent using recombinant proteins derived from *Limulus polyphemus* which has been widely spread as the lysate reagent.

[0010] The reason for this is because proteins involved in a mechanism of occurrence of clotting by an endotoxin and genes thereof in *Limulus polyphemus* have not been completely identified structurally and functionally.

[0011] In the genus *Tachypleus*, for example, with respect to factor C which is one of the proteins involved in the clotting mechanism, a full gene sequence and a full amino acid sequence derived from *Tachypleus tridentatus* have been reported in 1991 (NPL 1), and a full gene sequence and a full amino acid sequence derived from *Tachypleus rotundicauda* (*Carcinoscorpius rotundicauda*) have been reported in 1995 (NPL 2).

[0012] However, in *Limulus polyphemus*, complete identification of these proteins and genes from both structural and functional aspects has not been achieved even though 20 years or more has passed since the reports regarding the genus *Tachypleus* described above were published. This is because accurate determination of the full-length sequence structures and elucidation of the expression and functions of these proteins and genes could not be achieved by general techniques due to various differences in biomolecules, diversities, etc. because of differences in genera or species of horseshoe crabs.

[0013] In fact, as part of the invertebrate genome project using a next-generation sequencer, a comprehensive analysis of genes of *Limulus polyphemus* has been carried out (http://genome.wustl.edu/genomes/detail/limulus-polyphemus/), however, the full-length sequences of all proteins and genes involved in the clotting mechanism of *Limulus polyphemus* have not been elucidated yet. This fact also supports the difficulty in accurate determination of the full-length sequence structures of these proteins and genes in *Limulus polyphemus*.

CITATION LIST

Patent Literature

[0014] PTL 1: U.S. Pat. No. 5,712,144
[0015] PTL 2: U.S. Pat. No. 5,716,834
[0016] PTL 3: U.S. Pat. No. 5,858,706
[0017] PTL 4: U.S. Pat. No. 5,985,590
[0018] PTL 5: U.S. Pat. No. 6,645,724
[0019] PTL 6: WO 2008/004674
[0020] PTL 7: JP-T-2014-510898
[0021] PTL 8: WO 2014/092079

Non Patent Literature

[0022] NPL 1: Muta T et al., Journal of Biological Chemistry, 266, 6554-6561 (1991)
[0023] NPL 2: Ding J L et al., Molecular Marine Biology and Biotechnology, 4(1), 90-103 (1995)

SUMMARY OF INVENTION

Technical Problem

[0024] An object of the present invention is to provide all full-length recombinant proteins involved in the clotting mechanism of *Limulus polyphemus*, cDNAs encoding the same, and applications thereof.

Solution to Problem

[0025] The present inventors were successful in the acquisition of full-length cDNAs encoding all protein factors involved in the clotting mechanism of *Limulus polyphemus* which no one had ever been successful for a long time, and

the expression of all recombinant proteins thereof, and further found that these can be utilized in various applications, and thus completed the present invention.

[0026] That is, the present invention includes the following embodiments.

[0027] [1]

[0028] A recombinant first protein such as a recombinant protein which is any of the following:

[0029] (A) a recombinant protein that contains the amino acid sequence represented by SEQ ID NO: 2 or 4;

[0030] (B) a recombinant protein that contains an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 2 or 4, and has factor C activity;

[0031] (C) a recombinant protein that is encoded by a DNA containing a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 1 or 3, and has factor C activity; and

[0032] (D) a recombinant protein that is encoded by a DNA hybridizing to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 1 or 3 under stringent conditions, and has factor C activity.

[0033] [2]

[0034] A recombinant second protein such as a recombinant protein which is any of the following:

[0035] (A) a recombinant protein that contains the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12;

[0036] (B) a recombinant protein that contains an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12, and has factor B activity;

[0037] (C) a recombinant protein that is encoded by a DNA containing a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 5, 7, 9, or 11, and has factor B activity; and

[0038] (D) a recombinant protein that is encoded by a DNA hybridizing to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 5, 7, 9, or 11 under stringent conditions, and has factor B activity.

[0039] [3]

[0040] A recombinant third protein such as a recombinant protein which is any of the following:

[0041] (A) a recombinant protein that contains the amino acid sequence represented by SEQ ID NO: 14, 16, 18, 20, or 22; (B) a recombinant protein that contains an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 14, 16, 18, 20, or 22, and has proclotting enzyme activity;

[0042] (C) a recombinant protein that is encoded by a DNA containing a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 13, 15, 17, 19, or 21, and has proclotting enzyme activity; and

[0043] (D) a recombinant protein that is encoded by a DNA hybridizing to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 13, 15, 17, 19, or 21 under stringent conditions, and has proclotting enzyme activity.

[0044] [1-1]

[0045] Use of the recombinant first protein for activating a protein having factor B activity (for example, a second protein such as the following protein):

[0046] (A) a protein that contains the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12;

[0047] (B) a protein that contains an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12, and has factor B activity;

[0048] (C) a protein that is encoded by a DNA containing a base sequence having a 95- or more identity with the base sequence represented by SEQ ID NO: 5, 7, 9, or 11, and has factor B activity; or

[0049] (D) a protein that is encoded by a DNA hybridizing to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 5, 7, 9, or 11 under stringent conditions, and has factor B activity.

[0050] [1-1-1]

[0051] The use according to [1-1], wherein the second protein is the recombinant second protein.

[0052] [2-1]

[0053] Use of the recombinant second protein for activating a protein having proclotting enzyme activity (for example, a third protein such as the following protein):

[0054] (A) a protein that contains the amino acid sequence represented by SEQ ID NO: 14, 16, 18, 20, or 22;

[0055] (B) a protein that contains an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 14, 16, 18, 20, or 22, and has proclotting enzyme activity;

[0056] (C) a protein that is encoded by a DNA containing a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 13, 15, 17, 19, or 21, and has proclotting enzyme activity; or

[0057] (D) a protein that is encoded by a DNA hybridizing to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 13, 15, 17, 19, or 21 under stringent conditions, and has proclotting enzyme activity.

[0058] [2-1-1]

[0059] The use according to [2-1], wherein the third protein is the recombinant third protein.

[0060] [3-1]

[0061] Use of the recombinant third protein for cleaving a substrate.

[0062] [3-1-1]

[0063] The use according to [3-1], wherein the substrate is a synthetic substrate.

[0064] [1-2]

[0065] A method for detecting an endotoxin in a specimen, including a step of bringing the recombinant first protein into contact with the specimen.

[0066] [1-2-1]

[0067] The method according to [1-2], further including a step of bringing the recombinant first protein having contacted with the specimen into contact with a protein having factor B activity such as the second protein.

[0068] [1-2-1-1]

[0069] The method according to [1-2-1], wherein the second protein is the recombinant second protein.

[0070] [1-2-2]

[0071] The method according to [1-2-1] or [1-2-1-1], further including a step of bringing the protein having factor B activity having contacted with the "recombinant first protein having contacted with the specimen" into contact with a protein having proclotting enzyme activity such as the third protein.

[0072] [1-2-2-1]

[0073] The method according to [1-2-2], wherein the third protein is the recombinant third protein.

[0074] [1-2-3]

[0075] The method according to [1-2-2] or [1-2-2-1], further including a step of bringing the protein having procloting enzyme activity having contacted with the "protein having factor B activity having contacted with the recombinant first protein having contacted with the specimen" into contact with a substrate for detection.

[0076] [1-3]

[0077] An endotoxin detecting agent, containing the recombinant first protein.

[0078] [1-3-1]

[0079] The agent according to [1-3], further containing a protein having factor B activity such as the second protein.

[0080] [1-3-1-1]

[0081] The agent according to [1-3-1], wherein the second protein is the recombinant second protein.

[0082] [1-3-2]

[0083] The agent according to [1-3-1] or [1-3-1-1], further containing a protein having procloting enzyme activity such as the third protein.

[0084] [1-3-2-1]

[0085] The agent according to [1-3-2], wherein the third protein is the recombinant third protein.

[0086] [1-4]

[0087] A cDNA which is any of the following (hereinafter also referred to as "first cDNA"):

[0088] (A) a cDNA that contains the base sequence represented by SEQ ID NO: 1 or 3;

[0089] (B) a cDNA that contains a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 1 or 3, and encodes a protein having factor C activity;

[0090] (C) a cDNA that hybridizes to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 1 or 3 under stringent conditions, and encodes a protein having factor C activity;

[0091] (D) a cDNA that encodes a protein containing the amino acid sequence represented by SEQ ID NO: 2 or 4; and

[0092] (E) a cDNA that encodes a protein containing an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 2 or 4, and having factor C activity.

[0093] [1-4-1]

[0094] A DNA construct, containing the first cDNA.

[0095] [1-4-2]

[0096] A cell, expressing the recombinant first protein by being transformed with the DNA construct according to [1-4-1].

[0097] [1-4-3]

[0098] A method for producing the recombinant first protein, including a step of culturing the cell according to [1-4-2].

[0099] [2-4]

[0100] A cDNA which is any of the following (hereinafter also referred to as "second cDNA"):

[0101] (A) a cDNA that contains the base sequence represented by SEQ ID NO: 5, 7, 9, or 11;

[0102] (B) a cDNA that contains a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 5, 7, 9, or 11, and encodes a protein having factor B activity;

[0103] (C) a cDNA that hybridizes to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 5, 7, 9, or 11 under stringent conditions, and encodes a protein having factor B activity;

[0104] (D) a cDNA that encodes a protein containing the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12; and

[0105] (E) a cDNA that encodes a protein containing an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12, and having factor B activity.

[0106] [2-4-1]

[0107] A DNA construct, containing the second cDNA.

[0108] [2-4-2]

[0109] A cell, expressing the recombinant second protein by being transformed with the DNA construct according to [2-4-1].

[0110] [2-4-3]

[0111] A method for producing the recombinant second protein, including a step of culturing the cell according to [2-4-2].

[0112] [3-4]

[0113] A cDNA which is any of the following (hereinafter also referred to as "third cDNA"):

[0114] (A) a cDNA that contains the base sequence represented by SEQ ID NO: 13, 15, 17, 19, or 21;

[0115] (B) a cDNA that contains a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 13, 15, 17, 19, or 21, and encodes a protein having procloting enzyme activity;

[0116] (C) a cDNA that hybridizes to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 13, 15, 17, 19, or 21 under stringent conditions, and encodes a protein having procloting enzyme activity;

[0117] (D) a cDNA that encodes a protein containing the amino acid sequence represented by SEQ ID NO: 14, 16, 18, 20, or 22; and

[0118] (E) a cDNA that encodes a protein containing an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 14, 16, 18, 20, or 22, and having procloting enzyme activity.

[0119] [3-4-1]

[0120] A DNA construct, containing the third cDNA.

[0121] [3-4-2]

[0122] A cell, expressing the recombinant third protein by being transformed with the DNA construct according to [3-4-1].

[0123] [3-4-3]

[0124] A method for producing the recombinant third protein, including a step of culturing the cell according to [3-4-2].

[0125] [1-5]

[0126] A method for producing an endotoxin detecting agent, including a step of artificially expressing a protein using the first cDNA.

[0127] [1-5-1]

[0128] The production method according to [1-5], further including a step of artificially expressing a protein using the second cDNA.

[0129] [1-5-2]

[0130] The production method according to [1-5-1], further including a step of artificially expressing a protein using the third cDNA.

BRIEF DESCRIPTION OF DRAWINGS

[0131] FIG. **1** is an electrophoresis photograph of a PCR product in a process for acquiring a cDNA of a protein having factor C activity of *Limulus polyphemus*.

[0132] FIG. **2** is an electrophoresis photograph of a PCR product in a process for acquiring a cDNA of a protein having factor C activity of *Limulus polyphemus*.

[0133] FIG. **3** is a figure showing the activity of cleaving a synthetic substrate by contact of each recombinant protein alone or various mixtures of recombinant proteins with an endotoxin.

[0134] FIG. **4** is a figure showing a calibration curve of an endotoxin using a mixture of three types of recombinant proteins.

DESCRIPTION OF EMBODIMENTS

<1> Recombinant First Protein and Production Method Therefor

(1) Recombinant First Protein

[0135] A recombinant first protein is a recombinant protein having factor C activity.

[0136] The recombinant first protein is specifically a recombinant protein which is either of the following:

[0137] (I) a recombinant protein that contains an amino acid sequence of the protein of *Limulus polyphemus* having factor C activity; and

[0138] (II) a recombinant protein that is a variant of the above (I) and has factor C activity.

[0139] Examples of the amino acid sequence of the protein of *Limulus polyphemus* having factor C activity include the amino acid sequence represented by SEQ ID NO: 2 or 4. Further, examples of a base sequence encoding such an amino acid sequence include the base sequence represented by SEQ ID NO: 1 or 3.

[0140] The recombinant first protein may be more specifically a recombinant protein which is any of the following:

[0141] (A) a recombinant protein that contains the amino acid sequence represented by SEQ ID NO: 2 or 4;

[0142] (B) a recombinant protein that contains an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 2 or 4, and has factor C activity;

[0143] (C) a recombinant protein that is encoded by a DNA containing a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 1 or 3, and has factor C activity; and

[0144] (D) a recombinant protein that is encoded by a DNA hybridizing to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 1 or 3 under stringent conditions, and has factor C activity.

[0145] Note that the expression of "containing an amino acid sequence" or "containing a base sequence" also includes a case of "being composed of the amino acid sequence" or a case of "being composed of the base sequence".

[0146] This protein was acquired for the first time in the world by the method disclosed in Examples of this description, and the full-length sequence structure and function thereof were elucidated for the first time. The function of the protein is a function of turning into an active form in the presence of an endotoxin and converting a protein having factor B activity such as the below-mentioned second protein into an active form (referred to as "factor C activity" in this application document). The recombinant first protein may be a protein that shows the factor C activity in combination with at least the second protein such as a recombinant second protein.

[0147] Whether having the factor C activity can be confirmed by detecting the progress of a cascade reaction in the presence of an endotoxin when combining a target recombinant protein with a protein (which is not in an active form, hereinafter referred to as "inactive form") having factor B activity such as the second protein (inactive form), a protein (inactive form) having proclotting enzyme activity such as a third protein (inactive form), and a substrate for detection.

[0148] The "cascade reaction" refers to a series of reactions in which a protein (inactive form) having factor C activity is activated by an endotoxin, a protein (inactive form) having factor B activity is activated by the protein (active form) having factor C activity, and a protein (inactive form) having proclotting enzyme activity is activated by the protein (active form) having factor B activity. The progress of the cascade reaction can be detected by cleavage of a substrate for detection.

[0149] Among the recombinant first proteins, the most preferred is the above-mentioned recombinant protein (I) such as the above-mentioned recombinant protein (A), but the recombinant protein may be a variant thereof as long as it has factor C activity.

[0150] The variant may be, for example, a recombinant protein that contains an amino acid sequence including a substitution, a deletion, an insertion, and/or an addition of one or several amino acids at one or several sites in an amino acid sequence (for example, the amino acid sequence represented by SEQ ID NO: 2 or 4) of the recombinant first protein as described above, and has factor C activity. The term "one or several" may be, for example, 1 to 20, 1 to 10, 1 to 5, or 1 to 3. The substitution, deletion, insertion, and/or addition of one or several amino acids is a conservative mutation that maintains the function of the protein normal. A representative conservative mutation is a conservative substitution. The conservative substitution is, for example, a mutation in which a substitution takes place mutually among Phe, Trp, and Tyr when the substitution site is an aromatic amino acid, among Leu, Ile, and Val when the substitution site is a hydrophobic amino acid, between Gln and Asn when the substitution site is a polar amino acid, among Lys, Arg, and His when the substitution site is a basic amino acid, between Asp and Glu when the substitution site is an acidic amino acid, and between Ser and Thr when the substitution site is an amino acid having a hydroxyl group.

[0151] Specific examples of the substitution regarded as a conservative substitution include a substitution from Ala to Ser or Thr, a substitution from Arg to Gln, His or Lys, a substitution from Asn to Glu, Gln, Lys, His, or Asp, a substitution from Asp to Asn, Glu, or Gln, a substitution from Cys to Ser or Ala, a substitution from Gln to Asn, Glu, Lys, His, Asp, or Arg, a substitution from Glu to Gly, Asn, Gln, Lys, or Asp, a substitution from Gly to Pro, a substitution from His to Asn, Lys, Gln, Arg, or Tyr, a substitution from Ile to Leu, Met, Val, or Phe, a substitution from Leu to Ile, Met, Val, or Phe, a substitution from Lys to Asn, Glu, Gln, His, or Arg, a substitution from Met to Ile, Leu, Val, or Phe, a substitution from Phe to Trp, Tyr, Met, Ile, or Leu, a

substitution from Ser to Thr or Ala, a substitution from Thr to Ser or Ala, a substitution from Trp to Phe or Tyr, a substitution from Tyr to His, Phe, or Trp, and a substitution from Val to Met, Ile, or Leu.

[0152] Further, the variant may be, for example, a recombinant protein that contains an amino acid sequence having an 80% or more, 90% or more, 95% or more, 96, or more, 97% or more, 98% or more, or 99% or more identity with an amino acid sequence (for example, the amino acid sequence represented by SEQ ID NO: 2 or 4) of the recombinant first protein as described above, and has factor C activity.

[0153] Further, the variant may be, for example, a recombinant protein that is encoded by a DNA containing a base sequence having an 80% or more, 90% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more identity with a base sequence (for example, the base sequence represented by SEQ ID NO: 1 or 3) encoding the amino acid sequence of the recombinant first protein as described above, and has factor C activity.

[0154] Further, the variant may be, for example, a recombinant protein that is encoded by a DNA hybridizing to a DNA composed of a complementary sequence to a base sequence (for example, the base sequence represented by SEQ ID NO: 1 or 3) encoding the amino acid sequence of the recombinant first protein as described above under stringent conditions, and has factor C activity. The term "stringent conditions" refers to conditions under which a so-called specific hybrid is formed and no nonspecific hybrid is formed by a general hybridization operation described in Molecular Cloning: A Laboratory Manual 2nd ed. (1989) Cold Spring Harbor Laboratory edited by T. Maniatis et al., or the like. The term "stringent conditions" specifically refers to conditions in which a sodium salt concentration is from 15 to 750 mM, preferably from 50 to 750 mM, more preferably from 300 to 750 mM, the temperature is from 25 to 70° C., preferably from 50 to 70° C., more preferably from 55 to 65° C., and a formamide concentration is from 0 to 50%, preferably from 20 to 50%, more preferably from 35 to 45%. Further, in the case of stringent conditions, washing conditions for a filter after hybridization are such that a sodium salt concentration is generally from 15 to 600 mM, preferably from 50 to 600 mM, more preferably from 300 to 600 mM, and the temperature is from 50 to 70° C., preferably from 55 to 70° C., more preferably from 60 to 65° C.

[0155] As the variant, specifically, the above-mentioned recombinant proteins (B) to (D) are preferred. The term "95% or more" in the above (B) and (C) is preferably 96% or more, more preferably 97% or more, more preferably 98% or more, and more preferably 99% or more.

[0156] Further, to the recombinant first protein, an arbitrary peptide such as a His tag or a V tag, or the like may be added as long as it has factor C activity.

[0157] Further, the term "recombinant protein" in this application document refers to a protein obtained by artificially introducing a gene encoding a protein into a host cell other than a horseshoe crab and expressing the protein. Therefore, the protein acquired from a natural horseshoe crab itself does not correspond to the "recombinant protein" in this application document.

(2) Method for Producing Recombinant First Protein

[0158] The full-length amino acid sequence of the recombinant first protein has been disclosed for the first time by the present invention, and therefore, the recombinant first protein can be produced by a genetic engineering technique using, for example, a DNA encoding this sequence. A method for producing the recombinant first protein by a genetic engineering technique is not particularly limited, and for example, a general method for producing a protein by a genetic engineering technique can be adopted. The recombinant first protein can be produced by, for example, utilizing a heterologous expression system or a cell-free protein synthesis system.

[0159] For example, the recombinant first protein can be produced by artificially introducing the below-mentioned first cDNA into a host cell other than a horseshoe crab and expressing the protein from this cDNA. The present invention also provides such a method for producing the recombinant first protein.

[0160] When artificially introducing a DNA into a host cell, a DNA construct (such as a vector) into which the DNA has been artificially integrated may be used. As such an artificial DNA construct, for example, a DNA construct into which the first cDNA has been integrated can be exemplified. Such an artificial DNA construct will be described later.

[0161] The host cell other than a horseshoe crab is not particularly limited as long as it can functionally express the protein from the first cDNA. Examples of the host cell other than a horseshoe crab include mammalian cells and insect cells. Examples of mammals include rodents and primates. Examples of the rodents include Chinese hamsters, hamsters, mice, rats, and guinea pigs. Examples of the Chinese hamster cells include a Chinese hamster ovary-derived cell line (CHO). Examples of the CHO include CHO DG44, CHO S, and CHO K1. The primates are not particularly limited, however, examples thereof include humans, monkeys, and chimpanzees. Examples of the human cells include a human embryonic kidney cell-derived cell line (HEK). Examples of the HEK include HEK 293.

[0162] The phrase "functionally expressed" refers to that the expressed recombinant first protein shows the factor C activity. Whether a protein shows the factor C activity can be confirmed by the method described in the below-mentioned Example. Further, whether the expressed protein contains a target amino acid sequence of any of the above-mentioned structures (A) to (D) or the like can be confirmed by analyzing the amino acid sequence of the expressed protein and comparing this with the target amino acid sequence.

[0163] A technique for artificially introducing the first cDNA into a host cell is also not particularly limited as long as the cDNA is in a state of being expressibly retained in the host cell. By growing the host cell into which the first cDNA has been artificially introduced, the recombinant first protein can be expressed.

[0164] The growth of the transformed host cell can be carried out by, for example, culturing the cell. The culturing conditions at this time are also not particularly limited as long as the cell can be grown or proliferated, and conditions generally used for culturing the cell can be used by being appropriately modified as needed. For example, when the host cell is a mammalian cell, a culture medium generally used for culturing the mammalian cell can be used. As such a culture medium, for example, RPMI-1640 medium (Sigma Aldrich Co. LLC.), DMEM medium (Sigma Aldrich Co. LLC.), ExpiCHO™ Expression Medium (Thermo Fisher Scientific), or the like can be used. The culturing can be carried out by, for example, static culture, suspension cul-

ture, or the like at 36° C. to 38° C. while supplying 5% to 8% CO_2. A kit dedicated to protein expression may be used.

[0165] The expressed recombinant first protein is collected as a solution fraction containing this and can be utilized in various applications described below.

[0166] The solution fraction containing the recombinant first protein can be, for example, a culture solution, a culture supernatant, a cell homogenate extract, a mixture thereof, or the like. The recombinant first protein may be used by being purified to a desired extent or may be used as such without purification.

[0167] The purification can be carried out by a known method used for purifying a protein. Examples of such a method include ammonium sulfate precipitation, gel filtration chromatography, ion exchange chromatography, hydrophobic interaction chromatography, and hydroxyapatite chromatography.

[0168] According to the present invention, it has become possible for the first time to artificially and functionally express the recombinant first protein. The thus produced recombinant first protein can be used for, for example, the application described below.

<2> Recombinant Second Protein and Production Method Therefor

[0169] The recombinant second protein is a recombinant protein having factor B activity.

[0170] The recombinant second protein is specifically a recombinant protein which is either of the following:

[0171] (I) a recombinant protein that contains an amino acid sequence of the protein of *Limulus polyphemus* having factor B activity; and

[0172] (II) a recombinant protein that is a variant of the above (I) and has factor B activity.

[0173] Examples of the amino acid sequence of the protein of *Limulus polyphemus* having factor B activity include the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12. Further, examples of a base sequence encoding such an amino acid sequence include the base sequence represented by SEQ ID NO: 5, 7, 9, or 11.

[0174] The recombinant second protein may be more specifically a recombinant protein which is any of the following:

[0175] (A) a recombinant protein that contains the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12;

[0176] (B) a recombinant protein that contains an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12, and has factor B activity;

[0177] (C) a recombinant protein that is encoded by a DNA containing a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 5, 7, 9, or 11, and has factor B activity; and

[0178] (D) a recombinant protein that is encoded by a DNA hybridizing to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 5, 7, 9, or 11 under stringent conditions, and has factor B activity.

[0179] The function of this protein is a function of turning into an active form by a protein (active form) having factor C activity such as the first protein having turned into an active form and converting a protein having proclotting enzyme activity such as the below-mentioned third protein into an active form (referred to as "factor B activity" in this application document). The recombinant second protein may be a protein that shows the factor B activity in combination with at least the first protein such as the recombinant first protein and the third protein such as a recombinant third protein.

[0180] Whether having the factor B activity can be confirmed by detecting the progress of a cascade reaction when combining a target recombinant protein with a protein (active form) having factor C activity such as the first protein (active form), a protein (inactive form) having proclotting enzyme activity such as the third protein (inactive form), and a substrate for detection. In this system, an endotoxin for converting a protein (inactive form) having factor C activity into an active form may further exist.

[0181] To the other explanation, the explanation of the above <1> can apply. That is, for example, "SEQ ID NO. 1 or 3", "SEQ ID NO. 2 or 4", "(recombinant) first protein", "first cDNA", and "factor C" in the above <1> may be replaced by "SEQ ID NO. 5, 7, 9, or 11", "SEQ ID NO. 6, 8, 10, or 12", "(recombinant) second protein", "second cDNA", and "factor B", respectively, and so on.

<3> Recombinant Third Protein and Production Method Therefor

[0182] The recombinant third protein is a recombinant protein having proclotting enzyme activity.

[0183] The recombinant third protein is specifically a recombinant protein which is either of the following:

[0184] (I) a recombinant protein that contains an amino acid sequence of the protein of *Limulus polyphemus* having proclotting enzyme activity; and

[0185] (II) a recombinant protein that is a variant of the above (I) and has proclotting enzyme activity.

[0186] Examples of the amino acid sequence of the protein of *Limulus polyphemus* having proclotting enzyme activity include the amino acid sequence represented by SEQ ID NO: 14, 16, 18, 20, or 22. Further, examples of a base sequence encoding such an amino acid sequence include the base sequence represented by SEQ ID NO: 13, 15, 17, 19, or 21.

[0187] The recombinant third protein may be more specifically a recombinant protein which is any of the following:

[0188] (A) a recombinant protein that contains the amino acid sequence represented by SEQ ID NO: 14, 16, 18, 20, or 22;

[0189] (B) a recombinant protein that contains an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 14, 16, 18, 20, or 22, and has proclotting enzyme activity;

[0190] (C) a recombinant protein that is encoded by a DNA containing a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 13, 15, 17, 19, or 21, and has proclotting enzyme activity; and

[0191] (D) a recombinant protein that is encoded by a DNA hybridizing to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 13, 15, 17, 19, or 21 under stringent conditions, and has proclotting enzyme activity.

[0192] The function of this protein is a function of turning into an active form by a protein (active form) having factor B activity such as the second protein having turned into an active form and cleaving the below-mentioned substrate (a

protein or a peptide) (referred to as "proclotting enzyme activity" in this application document). The recombinant third protein may be a protein that shows the proclotting enzyme activity in combination with at least the second protein such as the recombinant second protein.

[0193] Whether a protein has the proclotting enzyme activity can be confirmed by detecting the progress of a cascade reaction when combining a target recombinant protein with a protein (active form) having factor B activity such as the second protein (active form) and a substrate for detection. In this system, a protein (active form) having factor C activity for converting a protein (inactive form) having factor B activity into an active form may further exist. Further, in this system, an endotoxin for converting a protein (inactive form) having factor C activity into an active form may further exist.

[0194] To the other explanation, the explanation of the above <1> can apply. That is, for example, "SEQ ID NO. 1 or 3", "SEQ ID NO. 2 or 4", "(recombinant) first protein", "first cDNA", and "factor C" in the above <1> may be replaced by "SEQ ID NO. 13, 15, 17, 19, or 21", "SEQ ID NO. 14, 16, 18, 20, or 22", "(recombinant) third protein", "third cDNA", and "proclotting enzyme", respectively, and so on.

<4> Use of Recombinant First Protein

[0195] The recombinant first protein can be used for activating a protein having factor B activity such as the second protein. In other words, the present invention includes a method for activating a protein having factor B activity including a step of bringing the recombinant first protein into contact with the protein having factor B activity.

[0196] Examples of the protein having factor B activity include the second protein.

[0197] The second protein is specifically a protein which is either of the following:

[0198] (I) a protein that contains an amino acid sequence of the protein of *Limulus polyphemus* having factor B activity; and

[0199] (II) a protein that is a variant of the above (I) and has factor B activity.

[0200] The second protein may be more specifically a protein which is any of the following:

[0201] (A) a protein that contains the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12;

[0202] (B) a protein that contains an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12, and has factor B activity;

[0203] (C) a protein that is encoded by a DNA containing a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 5, 7, 9, or 11, and has factor B activity; and

[0204] (D) a protein that is encoded by a DNA hybridizing to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 5, 7, 9, or 11 under stringent conditions, and has factor B activity.

[0205] Further, examples of the protein having factor B activity also include the following proteins:

[0206] (III) a protein that contains an amino acid sequence of a protein of a horseshoe crab other than *Limulus polyphemus* having factor B activity; and

[0207] (IV) a protein that is a variant of the above (III) and has factor B activity.

[0208] To the explanation of the protein having factor B activity, the explanation of the above <2> can apply.

[0209] According to the present invention, it has been revealed for the first time that the protein (A) of the recombinant first protein has factor C activity. The protein (A) having such a function and the variant thereof are applied to the activation of the protein having factor B activity.

[0210] The protein having factor B activity used here may be a protein acquired from a natural substance or a protein produced artificially (for example, the recombinant second protein) as long as it has the above-mentioned structure and function. According to the present invention, it has become possible for the first time to artificially and functionally express the recombinant second protein and also it has become possible to stably produce the second protein with constant quality without acquiring it from native Limulus polyphemus. Therefore, the protein having factor B activity is preferably the recombinant second protein.

[0211] The activation of the protein having factor B activity using the recombinant first protein can be carried out by bringing a molecule of the recombinant first protein (active form) and a molecule of the protein (inactive form) having factor B activity into contact with each other. Incidentally, in order to convert the recombinant first protein into an active form, the recombinant first protein (inactive form) and the protein (inactive form) having factor B activity may be brought into contact with each other in the presence of an endotoxin.

[0212] The conditions during this contact are not particularly limited as long as the conditions allow the recombinant first protein (active form) to exhibit the factor C activity so as to be able to activate the protein having factor B activity (when the operation is carried out in the presence of an endotoxin, the conditions further allow the endotoxin to be able to activate the recombinant first protein). For example, reaction conditions for a known lysate reagent can be adopted.

[0213] Whether the protein having factor B activity has been activated can be confirmed by the method described in the below-mentioned Example.

<5> Use of Recombinant Second Protein

[0214] The recombinant second protein can be used for activating a protein having proclotting enzyme activity such as the third protein. In other words, the present invention includes a method for activating a protein having proclotting enzyme activity including a step of bringing the recombinant second protein into contact with the protein having proclotting enzyme activity.

[0215] Examples of the protein having proclotting enzyme activity include the third protein.

[0216] The third protein is specifically a protein which is either of the following:

[0217] (I) a protein that contains an amino acid sequence of the protein of *Limulus polyphemus* having proclotting enzyme activity; and

[0218] (II) a protein that is a variant of the above (I) and has proclotting enzyme activity.

[0219] The third protein may be more specifically a protein which is any of the following:

[0220] (A) a protein that contains the amino acid sequence represented by SEQ ID NO: 14, 16, 18, 20, or 22;

[0221] (B) a protein that contains an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 14, 16, 18, 20, or 22, and has proclotting enzyme activity;

[0222] (C) a protein that is encoded by a DNA containing a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 13, 15, 17, 19, or 21, and has proclotting enzyme activity; and

[0223] (D) a protein that is encoded by a DNA hybridizing to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 13, 15, 17, 19, or 21 under stringent conditions, and has proclotting enzyme activity.

[0224] Further, examples of the protein having proclotting enzyme activity also include the following proteins:

[0225] (III) a protein that contains an amino acid sequence of a protein of a horseshoe crab other than *Limulus polyphemus* having proclotting enzyme activity; and

[0226] (IV) a protein that is a variant of the above (III) and has proclotting enzyme activity.

[0227] To the explanation of the protein having proclotting enzyme activity, the explanation of the above <3> can apply.

[0228] The protein having proclotting enzyme activity used here may be a protein acquired from a natural substance or a protein produced artificially (for example, the recombinant third protein) as long as it has the above-mentioned structure and function.

[0229] The activation of the protein having proclotting enzyme activity using the recombinant second protein can be carried out by bringing a molecule of the recombinant second protein (active form) and a molecule of the protein (inactive form) having proclotting enzyme activity into contact with each other. Incidentally, in order to convert the recombinant second protein into an active form, the recombinant second protein (inactive form) and the protein (inactive form) having proclotting enzyme activity may be brought into contact with each other in the presence of a protein (active form) having factor C activity. Further, in order to activate this protein having factor C activity, the protein (inactive form) having factor C activity, the recombinant second protein (inactive form), and the protein (inactive form) having proclotting enzyme activity may be brought into contact with one another in the presence of an endotoxin.

[0230] The conditions during this contact are not particularly limited as long as the conditions allow the recombinant second protein (active form) to exhibit the factor B activity so as to be able to activate the protein having proclotting enzyme activity (when the operation is carried out in the presence of the protein (active form) having factor C activity, the conditions further allow the protein (active form) having factor C activity to exhibit the factor C activity so as to be able to activate the recombinant second protein, and when the operation is carried out in the presence of an endotoxin, the conditions further allow the endotoxin to be able to activate the protein having factor C activity). For example, reaction conditions for a known lysate reagent can be adopted.

[0231] Whether the third protein has been activated can be confirmed by the method described in the below-mentioned Example.

[0232] Examples of the protein having factor C activity include the first protein.

[0233] The first protein is specifically a protein which is either of the following:

[0234] (I) a protein that contains an amino acid sequence of the protein of *Limulus polyphemus* having factor C activity; and (II) a protein that is a variant of the above (I) and has factor C activity.

[0235] The first protein may be more specifically a protein which is any of the following:

[0236] (A) a protein that contains the amino acid sequence represented by SEQ ID NO: 2 or 4;

[0237] (B) a protein that contains an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 2 or 4, and has factor C activity;

[0238] (C) a protein that is encoded by a DNA containing a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 1 or 3, and has factor C activity; and

[0239] (D) a protein that is encoded by a DNA hybridizing to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 1 or 3 under stringent conditions, and has factor C activity.

[0240] Further, examples of the protein having factor C activity also include the following proteins:

[0241] (III) a protein that contains an amino acid sequence of a protein of a horseshoe crab other than *Limulus polyphemus* having factor C activity; and

[0242] (IV) a protein that is a variant of the above (III) and has factor C activity.

[0243] To the explanation of the protein having factor C activity, the explanation of the above <1> can apply.

[0244] The protein having factor C activity used here may be a protein acquired from a natural substance or a protein produced artificially (for example, the recombinant first protein) as long as it has the above-mentioned structure and function.

[0245] To the other explanation, the explanation of the above <4> can apply.

<6> Use of Recombinant Third Protein

[0246] The recombinant third protein can be used for cleaving a substrate. In other words, the present invention includes a method for cleaving a substrate including a step of bringing the recombinant third protein into contact with the substrate.

[0247] This substrate can also be used as the above-mentioned "substrate for detection" and may be a naturally derived substrate or a synthetic substrate. Such a substrate is not particularly limited as long as it is cleaved by the proclotting enzyme activity of the protein (active form) having proclotting enzyme activity such as the third protein (active form).

[0248] Examples of the substrate to be cleaved by the proclotting enzyme activity include a protein such as coagulogen and a synthetic substrate represented by a general formula: X—Y—Z (wherein X— is a protecting group bonded to Y through a covalent bond, Y is a peptide residue, and —Z is a signal substance bonded to Y through an amide bond).

[0249] The protecting group X is not particularly limited, and a known protecting group for a peptide can be used. Examples of such a protecting group include a t-butoxycarbonyl group and a benzoyl group.

[0250] The peptide residue Y is also not particularly limited, and examples thereof include Leu-Gly-Arg (LGR) and Ile-Glu-Gly-Arg (IEGR) (SEQ ID NO: 23).

[0251] The signal substance Z is also not particularly limited, and examples thereof include a dye that is detected under visible light and a fluorescent dye. Examples of such a dye include pNA (p-nitroaniline), MCA (7-methoxycoumarin-4-acetic acid), DNP (2,4-dinitroaniline), and a dansyl-type dye.

[0252] Among these, Boc-Leu-Gly-Arg-pNA (t-butoxycarbonyl-leucyl-glycyl-arginyl-p-nitroaniline, Boc-LGR-pNA) is preferred.

[0253] Whether the substrate has been cleaved by the proclotting enzyme activity can be detected by, for example, detecting "gelation" when coagulogen is used as the substrate. The gelation can be detected by detecting a decrease in fluidity by visual observation or the like.

[0254] Further, for example, when a synthetic substrate as described above is used, the signal Z is released by cleaving the amide bond between Y and Z and a signal such as a developed color or fluorescence is emitted, and therefore, by detecting this signal, whether the substrate has been cleaved by the proclotting enzyme activity can be detected. The detection of the signal may be carried out by a technique according to the type of the signal. For example, the signal of pNA can be detected by an absorbance (405 nm).

[0255] The cleavage of the substrate using the recombinant third protein can be carried out by bringing a molecule of the recombinant third protein (active form) and a molecule of the substrate into contact with each other. Incidentally, in order to convert the recombinant third protein into an active form, the recombinant third protein (inactive form) and the substrate may be brought into contact with each other in the presence of the protein (active form) having factor B activity. Further, in order to convert this protein having factor B activity into an active form, the protein (inactive form) having factor B activity, the recombinant third protein (inactive form), and the substrate may be brought into contact with one another in the presence of the protein (active form) having factor C activity. Further, in order to convert this protein having factor C activity into an active form, the protein (inactive form) having factor C activity, the protein (inactive form) having factor B activity, the recombinant third protein (inactive form), and the substrate may be brought into contact with one another in the presence of an endotoxin.

[0256] The conditions during this contact are not particularly limited as long as the conditions allow the recombinant third protein (active form) to exhibit the proclotting enzyme activity so as to be able to cleave the substrate (when the operation is carried out in the presence of the protein (active form) having factor B activity, the conditions further allow the protein (active form) having factor B activity to exhibit the factor B activity so as to be able to activate the recombinant third protein; when the operation is carried out in the presence of the protein (active form) having factor C activity, the conditions further allow the protein (active form) having factor C activity to exhibit the factor C activity so as to be able to activate the second protein; and when the operation is carried out in the presence of an endotoxin, the conditions further allow the endotoxin to be able to activate the protein having factor C activity). For example, reaction conditions for a known lysate reagent can be adopted.

[0257] To the other explanation, the explanation of the above <4> can apply.

<7> Method for Detecting Endotoxin

[0258] The method for detecting an endotoxin of the present invention is a method for detecting an endotoxin in a specimen including a step of bringing the recombinant first protein into contact with the specimen.

[0259] This method may further include a step of bringing the recombinant first protein having contacted with the specimen into contact with a protein having factor B activity such as the second protein. This protein having factor B activity may be the recombinant second protein.

[0260] Further, this method may further include a step of bringing the protein having factor B activity having contacted with the "recombinant first protein having contacted with the specimen" into contact with a protein having proclotting enzyme activity such as the third protein. This protein having proclotting enzyme activity may be the recombinant third protein.

[0261] The detection of an endotoxin in a specimen can be carried out by detecting the activation of the recombinant first protein having contacted with the specimen. Whether the recombinant first protein has been activated can be confirmed by the method described in the below-mentioned Example. Further, the activation of the recombinant first protein can also be directly detected by utilizing a substrate (substrate for detection). That is, by utilizing a substrate that emits a signal by being cleaved by the factor C activity of the activated recombinant first protein as the substrate for detection, the activation of the recombinant first protein can be directly detected. Therefore, this method may further include a step of bringing the "recombinant first protein having contacted with the specimen" into contact with the substrate for detection.

[0262] When the method further includes the step of bringing into contact with the protein having factor B activity, the detection can be carried out by detecting the activation of the protein having factor B activity by the method described in the above <4>. Further, the activation of the protein having factor B activity can also be directly detected by utilizing a substrate (substrate for detection). That is, by utilizing a substrate that emits a signal by being cleaved by the factor B activity of the activated protein having factor B activity as the substrate for detection, the activation of the protein having factor B activity can be directly detected. Therefore, this method may further include a step of bringing the "protein having factor B activity having contacted with the recombinant first protein" into contact with the substrate for detection.

[0263] When the method further includes the step of bringing into contact with the protein having proclotting enzyme activity, the detection can be carried out by detecting the activation of the protein having proclotting enzyme activity by the method described in the above <5> (the cleavage of the substrate by the method described in the above <6>). Therefore, this method may further include a step of bringing the "protein having proclotting enzyme activity having contacted with the protein having factor B activity" into contact with the substrate (substrate for detection). Examples of the substrate for detection capable of detecting the activation of the protein having proclotting enzyme activity include those exemplified in the above <6>.

[0264] These steps may be carried out sequentially or simultaneously. For example, when these steps are carried out simultaneously, the specimen, the recombinant first protein (inactive form), the protein (inactive form) having factor B activity, the protein (inactive form) having proclotting enzyme activity, and the substrate for detection may be brought into contact with one another simultaneously in the same system.

[0265] The conditions for the contact of these are the same as the conditions described in the above <4> to <6>. For example, the contact of the recombinant first protein with the specimen is not particularly limited as long as the conditions allow the endotoxin in the specimen to be able to activate the recombinant first protein. As the conditions for the contact, for example, reaction conditions for a known lysate reagent can be adopted. For example, as a pH of a reaction solution, a pH of 5 to 10, preferably 7 to 8.5 can be adopted. As a reaction temperature, a temperature of 10° C. to 80° C., preferably 20° C. to 50° C., more preferably 30° C. to 40° C. can be adopted. As the reaction temperature, for example, 37° C. is exemplified. A reaction time is also not particularly limited, and may be appropriately set according to various conditions. The reaction time may be, for example, from 5 minutes to 2 hours, preferably from 15 to 90 minutes, more preferably from 30 to 40 minutes.

[0266] The specimen is also not particularly limited as long as it is a sample requiring detection of an endotoxin. Examples of the specimen include medical water, pharmaceutical preparations, infusions, blood preparations, medical devices, medical instruments, cosmetics, foods and drinks, environmental samples, biological components, natural proteins, recombinant proteins, nucleic acids, and saccharides. The specimen can be subjected to the detection of an endotoxin by mixing, dispersing, or dissolving the specimen itself or an extract thereof or a washing solution thereof in a reaction system.

[0267] The detection of an endotoxin may be qualitative detection or quantitative detection. The quantitative detection can be carried out, for example, as follows: a plurality of reference standards having different endotoxin amounts (concentrations) are used, a relationship between the amount (concentration) of the endotoxin and the detection level according to the type of the substrate (for example, the degree of "gelation" when coagulogen is used as the substrate, and the degree of a signal when a synthetic substrate is used) is associated in advance using, for example, a calibration curve or a relational formula, and the amount (concentration) of the endotoxin is calculated by conversion from the detection level when using the actual specimen.

<8> Endotoxin Detecting Agent

[0268] The endotoxin detecting agent of the present invention is an endotoxin detecting agent containing the recombinant first protein.

[0269] This detecting agent may further contain a protein having factor B activity such as the second protein. This protein having factor B activity may be the recombinant second protein.

[0270] This detecting agent may further contain a protein having proclotting enzyme activity such as the third protein. This protein having proclotting enzyme activity may be the recombinant third protein.

[0271] These proteins can be in the form of, for example, being present in a culture solution, a culture supernatant, a cell homogenate extract, a mixture thereof, or the like as described in the above <1> to <3>. These proteins may be used by being purified to a desired extent or may be used as such without purification. For example, when these proteins are contained in a culture supernatant, the culture supernatant may be used while containing the culture medium components.

[0272] The respective amounts of the recombinant first protein, the protein having factor B activity, and the protein having proclotting enzyme activity in this detecting agent can be appropriately set according to the activity or the like of each protein, and, for example, as the final concentration of each protein in the reaction solution in a state of being mixed with the specimen, 15 to 30 μg/mL can be exemplified.

[0273] This detecting agent may further contain a substrate (substrate for detection).

[0274] This detecting agent can be produced by incorporating these materials as the constituent components. This detecting agent may further contain an additive such as an excipient, a stabilizing agent, or a buffer other than these. Further, the form of this detecting agent is also not particularly limited, and the agent can be formulated into an arbitrary form such as a solid form (for example, a lyophilized form) or a liquid form.

[0275] In this detecting agent, these materials may be collectively put in a one-unit container as the constituent components or may be put in separate containers.

[0276] Further, this detecting agent may further contain a buffer solution for dissolution, an endotoxin reference standard, a container for a reaction (for example, a tube or a microplate), or the like. This detecting agent also includes a form of a kit.

[0277] This detecting agent can be used in, for example, the method described in the above <7>.

<9> First cDNA and Utilization Thereof

(1) First cDNA

[0278] The first cDNA is a cDNA which is any of the following:

[0279] (A) a cDNA that contains the base sequence represented by SEQ ID NO: 1 or 3;

[0280] (B) a cDNA that contains a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 1 or 3, and encodes a protein having factor C activity;

[0281] (C) a cDNA that hybridizes to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 1 or 3 under stringent conditions, and encodes a protein having factor C activity;

[0282] (D) a cDNA that encodes a protein containing the amino acid sequence represented by SEQ ID NO: 2 or 4; and

[0283] (E) a cDNA that encodes a protein containing an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 2 or 4, and having factor C activity.

[0284] This cDNA was acquired for the first time in the world by the method disclosed in Examples of this description, and the full-length sequence structure thereof and the function of a protein encoded by the cDNA were elucidated for the first time. The function is "factor C activity".

[0285] Among the first cDNAs, the most preferred is the above-mentioned cDNA (A), but the cDNA may be a variant thereof as long as a protein encoded by the cDNA has factor C activity.

[0286] As the variant, specifically, the above-mentioned cDNAs (B) to (E) are preferred. The term "95% or more" in the above (B), (C), and (E) is preferably 96' or more, more preferably 97% or more, more preferably 98% or more, and more preferably 99% or more.

[0287] Further, to the meaning of the "stringent conditions" and the other explanation, the explanation of the above <1> can apply.

[0288] Further, the first cDNA may be a cDNA in which an arbitrary codon is replaced by a codon equivalent thereto. For example, a cDNA in which an arbitrary codon in the above-mentioned cDNA (A) is replaced by a codon equivalent thereto is included in the above (D).

[0289] The full-length base sequence of the first cDNA is disclosed by the present invention, and therefore, by using this sequence information, the first cDNA can be produced by a known DNA synthesis technique or a genetic engineering technique. The first cDNA was acquired for the first time by the method described in the below-mentioned Example, however, since the base sequence is disclosed in the present invention, the first cDNA can also be produced by a method other than the method described in Example.

(2) DNA Construct Containing First cDNA, Transformant, and Method for Producing Recombinant First Protein Using the Same

[0290] The present invention provides a DNA construct containing the first cDNA. As such an artificial DNA construct, for example, a vector can be exemplified. As the vector, a plasmid, a virus, or another known vector can be appropriately selected according to the purpose such as amplification, maintenance, or introduction of a recombinant DNA, library preparation, cloning, protein translation (protein expression), or the like. By introducing the first cDNA into such a vector or the like, the DNA construct containing the first cDNA can be produced.

[0291] Further, the present invention provides a cell containing the first cDNA. Such a cell may be, for example, a cell that is transformed with such a DNA construct so as to express the recombinant first protein. Further, the present invention provides a method for producing the recombinant first protein including a step of culturing such a cell.

[0292] To the method for transforming a cell (host cell) with the DNA construct and the method for producing the recombinant first protein by culturing the cell, the description of the above <1> (2) can apply.

<10> Second cDNA

(1) Second cDNA

[0293] The second cDNA is a cDNA which is any of the following:

[0294] (A) a cDNA that contains the base sequence represented by SEQ ID NO: 5, 7, 9, or 11;

[0295] (B) a cDNA that contains a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 5, 7, 9, or 11, and encodes a protein having factor B activity;

[0296] (C) a cDNA that hybridizes to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 5, 7, 9, or 11 under stringent conditions, and encodes a protein having factor B activity;

[0297] (D) a cDNA that encodes a protein containing the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12; and

[0298] (E) a cDNA that encodes a protein containing an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12, and having factor B activity.

[0299] To the other explanation, the explanation of the above <9> (1) can apply. That is, for example, "factor C" and "first cDNA" in the above <9> (1) may be replaced by "factor B" and "second cDNA", respectively, and so on.

(2) DNA Construct Containing Second cDNA, Transformant, and Method for Producing Recombinant Second Protein Using the Same

[0300] The present invention provides a DNA construct containing the second cDNA. Further, the present invention provides a cell containing the second cDNA such as a cell that is transformed with such a DNA construct so as to express the recombinant second protein. Further, the present invention provides a method for producing the recombinant second protein including a step of culturing such a cell.

[0301] To the other explanation, the explanation of the above <9> (2) can apply.

<11> Third cDNA

(1) Third cDNA

[0302] The third cDNA is a third cDNA which is any of the following:

[0303] (A) a cDNA that contains the base sequence represented by SEQ ID NO: 13, 15, 17, 19, or 21;

[0304] (B) a cDNA that contains a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 13, 15, 17, 19, or 21, and encodes a protein having procloting enzyme activity;

[0305] (C) a cDNA that hybridizes to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 13, 15, 17, 19, or 21 under stringent conditions, and encodes a protein having procloting enzyme activity;

[0306] (D) a cDNA that encodes a protein containing the amino acid sequence represented by SEQ ID NO: 14, 16, 18, 20, or 22; and

[0307] (E) a cDNA that encodes a protein containing an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 14, 16, 18, 20, or 22, and having procloting enzyme activity.

[0308] To the other explanation, the explanation of the above <9> (1) can apply. That is, for example, "factor C" and "first cDNA" in the above <9> (1) may be replaced by "procloting enzyme" and "third cDNA", respectively, and so on.

(2) DNA Construct Containing Third cDNA, Transformant, and Method for Producing Recombinant Third Protein Using the Same

[0309] The present invention provides a DNA construct containing the third cDNA. Further, the present invention provides a cell containing the third cDNA such as a cell that is transformed with such a DNA construct so as to express the recombinant third protein. Further, the present invention provides a method for producing the recombinant third protein including a step of culturing such a cell.

[0310] To the other explanation, the explanation of the above <9> (2) can apply.

<12> Method for Producing Endotoxin Detecting Agent

[0311] The present invention also provides a method for producing an endotoxin detecting agent including a step of artificially expressing a protein using the first cDNA.

[0312] This method may further include a step of artificially expressing a protein using the second cDNA. In

addition, the method may further include a step of artificially expressing a protein using the third cDNA.

[0313] A method for artificially expressing a protein using such a cDNA is also not particularly limited, however, for example, the method described in the above <9> (2), <10> (2), or <11>(2) can be used.

[0314] The recombinant first protein is expressed by being artificially expressed using the first cDNA according to such a method. Similarly, the recombinant second protein is expressed by being artificially expressed using the second cDNA, and the recombinant third protein is expressed by being artificially expressed using the third cDNA.

[0315] The expressed protein can be in the form of, for example, being present in a culture solution, a culture supernatant, a cell homogenate extract, a mixture thereof, or the like as described in the above <1> to <3>. These proteins may be used by being purified to a desired extent or may be used as such without purification. For example, when these proteins are contained in a culture supernatant, the culture supernatant may be used while containing the culture medium components.

[0316] To the other explanation, the explanation of the above <8> can apply. Therefore, the method for producing an endotoxin detecting agent of the present invention also includes the method for producing a kit.

[0317] Incidentally, in the present invention, either one or both of the second protein (such as the recombinant second protein) and the third protein (such as the recombinant third protein) may be replaced by their equivalents derived from a horseshoe crab other than *Limulus polyphemus*. The same also apply to the second cDNA and the third cDNA. Examples of the horseshoe crab other than *Limulus polyphemus* include organisms belonging to the genus *Tachypleus* such as *Tachypleus tridentatus*, *Tachypleus gigas*, and *Tachypleus rotundicauda*.

[0318] That is, in place of the second protein (such as the recombinant second protein), factor B (such as recombinant factor B) derived from a horseshoe crab (such as *Tachypleus tridentatus*) other than *Limulus polyphemus*, in place of the third protein (such as the recombinant third protein), a proclotting enzyme (such as a recombinant proclotting enzyme) derived from the same organism, in place of the second cDNA, a factor B gene (such as a cDNA) derived from the same organism, and in place of the third cDNA, a proclotting enzyme gene (such as a cDNA) derived from the same organism may be used, respectively.

[0319] These proteins and genes may all be those containing an amino acid sequence or a base sequence actually found in a horseshoe crab other than *Limulus polyphemus*, or may be variants thereof. To the variants, the explanation of the variant in the above <1> to <11> can apply. Further, these proteins and genes may be or may not be derived from the same horseshoe crab other than *Limulus polyphemus*.

[0320] Then, as apparent also from the above-mentioned description, in the method for producing an endotoxin detecting agent of the present invention, an embodiment in which each of the first cDNA, the second cDNA, and the third cDNA (with respect to the second cDNA and the third cDNA, either one or both may be replaced by a gene derived from a horseshoe crab (such as *Tachypleus tridentatus*) other than *Limulus polyphemus*) is artificially introduced into a host cell other than a horseshoe crab, the respective recombinant proteins (three types in total) are expressed from these respective cDNAs, and then, the expressed respective recombinant proteins are incorporated as the constituent components as described in the above <8> is also included.

EXAMPLES

[0321] Hereinafter, the present invention will be specifically described by way of Examples, however, these are merely examples of the present invention, and the scope of the present invention is not limited thereto.

(1) Synthesis of Primers

[0322] In order to attempt cloning of a protein having factor C activity in *Limulus polyphemus*, the following respective primers were synthesized.

<Primers Used in Cloning of Protein Having Factor C Activity>

[0323]

SK15-dT20:
(SEQ ID NO: 24)
CTGCAGGAATTCGATTTTTTTTTTTTTTTTTT

SP15-FC-S:
(SEQ ID NO: 25)
ATCGATAAGCTTGATGATCTGGGCTTGTGTGATGA

SK15-As:
(SEQ ID NO: 26)
CTGCAGGAATTCGAT

LFC-Stacm:
(SEQ ID NO: 27)
CTACACCAAGTTCCA

LFC-1st-S:
(SEQ ID NO: 28)
GTAAACCATGTGACAAACTGGAGGC

LFC-1st-As:
(SEQ ID NO: 29)
AATAAGCCTCCATCGATAGAAGTA

LFC-EcoRI-kozak-S:
(SEQ ID NO: 30)
GGGGAATTCAAGCTTGCCACCATGCTACTAGCGTCGTTC

LFC-XhoI-stop-As:
(SEQ ID NO: 31)
GGGCTCGAGTCAAATGAACTGCCGAATCCACGATA

[0324] Further, in order to attempt cloning of each of a protein having factor B activity and a protein having proclotting enzyme activity, the following respective primers were synthesized.

<Primers Used in Cloning of Protein Having Factor B Activity and Protein Having Proclotting Enzyme Activity>

[0325]

SK15-FB-S:
(SEQ ID NO: 32)
ATCGATAAGCTTGATCACATGCAAGCAAAGTTCT

SK15-FB-As:
(SEQ ID NO: 33)
CTGCAGGAATTCGATCACTGTTTAAACAAACTGAA

-continued

SK15-FC8-S:

(SEQ ID NO: 34)

ATCGATAAGCTTGATAGACCAAAGTGGTCTTTCTG

SK15-As:

(SEQ ID NO: 26)

CTCCAGGAATTCGAT

(2) Preparation of Total RNA from Blood Cells of *Limulus polyphemus*

[0326] In the preparation of total RNA, PureLink (registered trademark) RNA Mini Kit and PureLink (registered trademark) DNase kit (Thermo Fisher Scientific) were used, and the basic operation was carried out according to the accompanying instructions. A TRIzol solution was added to 2.23 g of a blood cell pool of *Limulus polyphemus*, and the cells were homogenized. Thereafter, chloroform was added thereto, followed by centrifugation, whereby an aqueous layer was obtained. After ethanol was added to the obtained aqueous layer, the resulting mixture was allowed to pass through a silica membrane cartridge included in the kit, whereby a nucleic acid component was bound to the silica membrane. To this silica membrane, DNase I included in the kit was added so as to degrade the DNA, and after the cartridge was washed, an elution buffer was added thereto, whereby total RNA (1.3 mg) was collected.

(3) Acquisition of cDNA (First cDNA) of Protein Having Factor C Activity of *Limulus polyphemus*

(3-1) First Step

[0327] Reverse transcription was carried out using the total RNA collected in the above (2) as a template and SK15-d20 (SEQ ID NO: 24) as a primer, whereby a cDNA was obtained. This primer was designed so as to selectively bind to the poly(A) sequence of mRNA and further has an addition sequence that can be utilized in a polymerase chain reaction (PCR) on the outside thereof. In this reaction, SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) was used as a reverse transcriptase, and the reaction conditions were set according to the accompanying instructions.

(3-2) Second Step

[0328] PCR was carried out using the obtained cDNA as a template, SK15-FC-S(SEQ ID NO: 25) as a sense primer, SK15-As (SEQ ID NO: 26) as an antisense primer, and Tks Gflex DNA polymerase (Takara Bio Inc.) as a polymerase.

[0329] When the PCR product was confirmed by agarose gel electrophoresis, a target DNA (LpFC1) almost could not be detected, and nonspecific DNA amplification was detected (A of FIG. 1). Therefore, the following step was attempted. Incidentally, in A of FIG. 1, the lane "1" is a size marker and the lane "2" is the PCR product. Further, the triangle mark indicates a portion where the presence of LpFC1 is expected.

(3-3) Third Step

[0330] The portion where the presence of LpFC1 was expected was cut out from the gel, and a DNA was extracted and purified. PCR was carried out using the obtained DNA as a template, SK15-FC-S(SEQ ID NO: 25) as a sense primer, SK15-As (SEQ ID NO: 26) as an antisense primer, and Tks Gflex DNA polymerase (Takara Bio Inc.) as a polymerase.

[0331] As a result of subjecting the PCR product to agarose gel electrophoresis, surprisingly, the target DNA (LpFC1) was remarkably amplified (B of FIG. 1). This LpFC1 was cut out from the gel and extracted and purified. By doing this, the acquisition of a partial fragment (LpFC1) of a DNA that may encode the protein having factor C activity of *Limulus polyphemus* was achieved. Incidentally, in B of FIG. 1, the lane "1" is a size marker and the lane "2" is the PCR product. Further, the triangle mark indicates the position of LpFC1.

(3-4) Fourth Step

[0332] LpFC1 was mixed with pBlueScript II SK (+) treated with EcoRV enzyme and introduced into a vector by recombination using In-Fusion HD Enzyme premix (Takara Bio Inc.). The reaction product was introduced into *E. coli* DH5a competent cells, and the cells were applied to an ampicillin LB medium plate containing X-Gal and IPTG, and colony selection was carried out by blue white screening.

[0333] Thereafter, DNA amplification was carried out by colony PCR for a candidate colony. At this time, M13-20 Primer was used as a sense primer, M13 Reverse Primer was used as an antisense primer, and Tks Gflex DNA polymerase (Takara Bio Inc.) was used as a polymerase. After the obtained PCR product was subjected to agarose gel electrophoresis, a DNA (LpFC1) obtained by cutting out from the gel, extraction, and purification was used as a template, and the sequence thereof was confirmed by sequence PCR. By doing this, the DNA sequence of LpFC1 was identified.

(3-5) Fifth Step

[0334] The 5' end of synthetic DNA LFC-5race (SEQ ID NO: 27) prepared based on the sequence information of LpFC1 was phosphorylated with T4 Polynucleotide Kinase (Takara Bio Inc.) in the presence of ATP. A target DNA (LpFC2) was obtained by reverse transcription using the total RNA as a template and also using the obtained phosphorylated primer and SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) as a reverse transcriptase. By doing this, a partial fragment (LpFC2) of a DNA that may encode an amino acid sequence containing a portion of the protein of *Limulus polyphemus* having factor C activity was obtained.

[0335] LpFC2 was subjected to a high-temperature treatment (95° C., 2 minutes) in the presence of RNaseA, followed by ethanol precipitation. Thereafter, the resulting material was treated with T4 RNA Ligase (New England Biolabs), and as a result, a concatenated DNA (LpFC3) in which LpFC2 is linked in series was obtained. By doing this, a partial fragment (LpFC3) of a DNA that may encode an amino acid sequence containing a portion of the protein of *Limulus polyphemus* having factor C activity was obtained.

[0336] PCR was carried out using LpFC3 as a template, LFC-1st-S (SEQ ID NO: 28) as a sense primer, LFC-1st-As (SEQ ID NO: 29) as an antisense primer, and Tks Gflex DNA polymerase (Takara Bio Inc.), whereby a target DNA (LpFC4) was amplified.

[0337] When the PCR product was confirmed by agarose gel electrophoresis, the target DNA (LpFC4) almost could

not be detected, and nonspecific DNA amplification was detected (A of FIG. **2**). Therefore, the following step was attempted. Incidentally, in A of FIG. **2**, the lane "1" is a size marker and the lane "2" is the PCR product. Further, the triangle mark indicates the position of LpFC4.

(3-6) Sixth Step

[0338] The portion where the presence of LpFC4 was expected was cut out from the gel, and a DNA was extracted and purified. PCR was carried out using the obtained DNA as a template, LFC-1st-S(SEQ ID NO: 28) as a sense primer, LFC-1st-As (SEQ ID NO: 29) as an antisense primer, and Tks Gflex DNA polymerase (Takara Bio Inc.) as a polymerase.

[0339] As a result of subjecting the PCR product to agarose gel electrophoresis, surprisingly, the target DNA (LpFC4) was remarkably amplified and detected at a very high concentration (B of FIG. **2**). This LpFC4 was cut out from the gel and extracted and purified. By doing this, the acquisition of a partial fragment (LpFC4) of a DNA that may encode an amino acid sequence containing a portion of the protein having factor C activity of *Limulus polyphemus* was achieved. Incidentally, in B of FIG. **2**, the lane "1" is a size marker and the lane "2" is the PCR product. Further, the triangle mark indicates a portion where the presence of LpFC4 is expected.

[0340] The sequence of LpFC4 was analyzed by sequence PCR using LpFC4 as a template. By doing this, the DNA sequence on the 5' side of the target protein was identified.

(3-7) Seventh Step

[0341] PCR was carried out using the cDNA obtained in the above (3-1) as a template, LFC-EcoRI-kozak-S(SEQ ID NO: 30) as a sense primer, LFC-XhoI-stop-As (SEQ ID NO: 31) as an antisense primer, and Tks Gflex DNA polymerase (Takara Bio Inc.) as a polymerase.

[0342] The PCR product was purified by phenol-chloroform extraction and ethanol precipitation, and then, treated with EcoRI enzyme and XhoI enzyme. A restriction enzyme-treated product was subjected to agarose gel electrophoresis and cut out from the gel, and a DNA was extracted and purified, whereby a target DNA (LpFC5) was obtained.

[0343] LpFC5 was mixed with an expression vector pCA7 (Takeda et al., 2005, J virol 79: 14346-14354) treated with EcoRI enzyme and XhoI enzyme, and a ligation reaction was carried out using Ligation Mix (Takara Bio Inc.). The reaction product was introduced into *E. coli* DH5α competent cells, and the cells were applied to an ampicillin-containing LB medium plate. From the obtained colonies, selection was carried out by colony PCR, and the selected colony was inoculated into an ampicillin-containing LB medium.

[0344] After culturing, plasmid purification was carried out by NucleoSpin (registered trademark) Plasmid Easy Pure (MACHEREY-NAGEL GmbH & Co. KG). The obtained two types of clones of plasmids were used as templates, and the base sequence of each clone was analyzed by sequence PCR.

[0345] By doing this, the elucidation of the full-length sequences (SEQ ID NO: 1 and SEQ ID NO: 3) of cDNAs encoding a protein that may have the factor C activity of *Limulus polyphemus*, and the full-length sequences (SEQ ID NO: 2 and SEQ ID NO: 4) of amino acids of the protein was achieved for the first time. SEQ ID NOS: 1 and 3 (SEQ ID NOS: 2 and 4) have a variant relationship and are considered to be based on polymorphism between individuals. The expression and the analysis of activity of this protein will be described later.

(4) Acquisition of cDNA (Second cDNA) of Protein Having Factor B Activity of *Limulus polyphemus*

[0346] PCR was carried out using the cDNA obtained in the above (3-1) as a template, SK15-FB-S(SEQ ID NO: 32) as a sense primer, SK15-FB-As (SEQ ID NO: 33) as an antisense primer, and Tks Gflex DNA polymerase (Takara Bio Inc.) as a polymerase. The amplified fragments were subjected to cloning according to the same procedure as in the above (3-4), and with respect to the obtained four types of clones, the base sequences thereof were analyzed.

[0347] By doing this, the full-length sequences (SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, and SEQ ID NO: 11) of cDNAs encoding a protein that may have the factor B activity of *Limulus polyphemus*, and the full-length sequences (SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, and SEQ ID NO: 12) of amino acids of the protein were elucidated. SEQ ID NOS: 5, 7, 9, and 11 (SEQ ID NOS: 6, 8, 10, and 12) have a variant relationship and are considered to be based on polymorphism among individuals. The expression and the analysis of activity of this protein will be described later.

(5) Acquisition of cDNA (Third cDNA) of Protein Having Procloting Enzyme Activity of *Limulus polyphemus*

[0348] PCR was carried out using the cDNA obtained in the above (3-1) as a template, SK15-PCE-S(SEQ ID NO: 34) as a sense primer, SK15-As (SEQ ID NO: 26) as an antisense primer, and Tks Gflex DNA polymerase (Takara Bio Inc.) as a polymerase. The amplified fragments were subjected to cloning according to the same procedure as in the above (3-4), and with respect to the obtained five types of clones, the base sequences thereof were analyzed.

[0349] By doing this, the full-length sequences (SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, and SEQ ID NO: 21) of cDNAs encoding a protein that may have the procloting enzyme activity of *Limulus polyphemus*, and the full-length sequences (SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, and SEQ ID NO: 22) of amino acids of the protein were elucidated. SEQ ID NOS: 13, 15, 17, 19, and 21 (SEQ ID NOS: 14, 16, 18, 20, and 22) have a variant relationship and are considered to be based on polymorphism among individuals. The expression and the analysis of activity of this protein will be described later.

(6) Expression of Recombinant Protein

[0350] The cDNA (SEQ ID NO: 13) obtained in the above (5) was ligated to a vector pCA7 for protein expression in mammalian cells, whereby an expression plasmid was obtained. The cDNA (SEQ ID NO: 5) obtained in the above (4) was ligated to the vector pCA7 for protein expression according to a literature (Kobayashi et al., 2015, J Biol Chem, 290: 19379-19386), whereby an expression plasmid was obtained. By using these expression plasmids and the expression plasmid (in which the cDNA of SEQ ID NO: 1 was integrated) obtained in the above (3-7), and ExpiCHO™ Expression System (Thermo Fisher Scientific), recombinant proteins thereof were obtained as culture supernatant fractions.

[0351] The transfection of the expression plasmids into cells and collection of the recombinant proteins were carried out according to the accompanying instructions.

[0352] By doing this, the expression and acquisition of the protein (SEQ ID NO: 2) that may have the factor C activity of *Limulus polyphemus*, the protein (SEQ ID NO: 6) that may have the factor B activity thereof, and the protein (SEQ ID NO: 14) that may have the proclotting enzyme activity thereof were achieved for the first time. The analysis of the activity of these proteins will be described below.

(7) Measurement of Activity

[0353] In the presence of 50 mM Tris buffer solution (pH 8.0) and a synthetic substrate (Boc-LGR-pNA), with respect to various combinations of the three types of recombinant proteins obtained in the above (6), activation of the three types of recombinant proteins depending on an endotoxin was examined using a United States Pharmacopeia reference standard endotoxin (USP-RSE, Seikagaku Corporation) (0 EU/mL and 0.05 EU/ml, EU is the endotoxin unit) as a specimen. This reaction was carried out at 37° C. for 30 minutes.

[0354] If a cascade reaction proceeds in this assay system, the protein having factor C activity is activated by the endotoxin, the protein having factor B activity is activated by this activated protein having factor C activity, the protein having proclotting enzyme activity is activated by this activated protein having factor B activity, and the synthetic substrate Boc-LGR-pNA is cleaved by this activated protein having proclotting enzyme activity, whereby pNA is released. The release of pNA was detected by measuring a change in the absorbance (A405 nm). As a result, when all the three types of recombinant proteins were included, cleavage of the synthetic substrate was observed (FIG. **3**). Incidentally, in FIG. **3**, FC denotes the protein expressed from the cDNA obtained in the above (3-7), FB denotes the protein expressed from the cDNA obtained in the above (4), and PCE denotes the protein expressed from the cDNA obtained in the above (5). The final concentrations of the proteins in the reaction solution (100 L) in a state of being mixed with the endotoxin specimen were as follows: FC: 22.8 μg/mL; FB: 20.0 μg/mL; and PCE: 23.5 μg/mL. Each assay was carried out by setting N=3, and a standard deviation was also shown in the drawing as an error bar.

[0355] From this, it was shown for the first time that the expressed protein that may have the factor C activity, the expressed protein that may have the factor B activity, and the expressed protein that may have the proclotting enzyme activity have the factor C activity, the factor B activity, and the proclotting enzyme activity, respectively, and the cascade reaction is caused by contact with an endotoxin. Further, it was shown for the first time that by utilizing these recombinant proteins, an endotoxin can be detected.

[0356] Further, when the concentration dependence of an endotoxin was examined under the conditions and a calibration curve was created, it showed favorable linearity within a concentration range from 0.001 to 0.1 EU/mL (FIG. **4**). The assay at each endotoxin concentration was carried out by setting N=3, and a standard deviation was also shown in the drawing as an error bar.

[0357] From the above results, it was shown for the first time that by using the above-mentioned respective three types of recombinant proteins, an endotoxin can also be quantitatively detected with high sensitivity and high accuracy.

(8) Comparison of Activity with Recombinant Factor C Derived from Genus *Tachypleus*

[0358] The activity was compared between recombinant factor C derived from *Limulus polyphemus* (hereinafter referred to as "LFC") and recombinant factor C derived from the genus *Tachypleus* (hereinafter referred to as "TFC").

[0359] LFC was obtained as a culture supernatant fraction by expressing LFC from the cDNA of SEQ ID NO: 1 in the same manner as in the above (6). TFC was obtained as a culture supernatant fraction by expressing TFC from a factor C gene of *Tachypleus tridentatus* (SEQ ID NO: 1 in WO 2014/092079) in the same manner as in the above (6) by the method described in Example 1. (2) in the same literature. Here, as a vector for protein expression, pCI-neo (Promega) was used for both.

[0360] With respect to LFC and TFC to be subjected to a test, Western blot was carried out using a factor C specific monoclonal antibody (2C12; Yoshiki Miura, et al., J. Biochem. 112: 476-481 (1992)). As a result of applying an equal volume of samples (3 μL each) and optically measuring the intensities of the bands, the intensity of LFC was 18036 and the intensity of TFC was 24516. That is, the relative amount (TFC/LFC) of the recombinant factor C in the equal volume of the samples was 1.4.

[0361] Subsequently, the activity of each recombinant factor C in a cascade reaction system (a system containing factor C, factor B, and a proclotting enzyme) was compared. As both the factor B and the proclotting enzyme, recombinant proteins derived from the genus *Tachypleus* were used. The recombinant proteins obtained as culture supernatant fractions by expressing the former from a factor B gene of *Tachypleus tridentatus* (SEQ ID NO: 5 in WO 2014/092079) and by expressing the latter from a proclotting enzyme gene of the same organism (SEQ ID NO: 7 in the same literature) using the method described in the same literature (Example 2. Preparation of Recombinant Factor B and Recombinant Proclotting Enzyme) were used.

[0362] By using each sample (the same volume for each) of the recombinant factor C (LFC or TFC), and the recombinant factor B (TFB) derived from *Tachypleus tridentatus* described above and the recombinant proclotting enzyme (TPCE) derived from the same organism, the activity was measured by the method described in the above (7). The conditions were set the same except for the recombinant factor C to be used.

[0363] The changes in the absorbance (mAbs/min) at the respective endotoxin concentrations of 0 EU/mL (blank) and 0.05 EU/mL in the respective cascade reaction systems were 0.53 and 14.27 in the system of LFC, and 0.51 and 9.15 in the system of TFC. That is, in this system, LFC showed the activity about 1.6 times (about 2.2 times when considering the difference in the protein amount) higher than TFC.

[0364] The disclosure of Japanese Patent Application No. 2016-204729 (filing date: Oct. 18, 2016) is hereby incorporated by reference herein in its entirety.

[0365] All publications, patent applications, and technical standards described herein are incorporated by reference herein to the same extent as if such individual publications,

patent applications, and technical standards were specifically and individually indicated to be incorporated by reference.

INDUSTRIAL APPLICABILITY

[0366] According to the present invention, all full-length recombinant proteins involved in the clotting mechanism of *Limulus polyphemus*, cDNAs encoding the same, and applications thereof can be provided. The present invention is particularly useful for detecting an endotoxin.

[Description of Sequence Listing]

[0367] SEQ ID NO: 1: base sequence of first cDNA

[0368] SEQ ID NO: 2: amino acid sequence of first protein

[0369] SEQ ID NO: 3: base sequence of first cDNA variant 1

[0370] SEQ ID NO: 4: amino acid sequence of first protein variant 1

[0371] SEQ ID NO: 5: base sequence of second cDNA

[0372] SEQ ID NO: 6: amino acid sequence of second protein

[0373] SEQ ID NO: 7: base sequence of second cDNA variant 1

[0374] SEQ ID NO: 8: amino acid sequence of second protein variant 1

[0375] SEQ ID NO: 9: base sequence of second cDNA variant 2

[0376] SEQ ID NO: 10: amino acid sequence of second protein variant 2

[0377] SEQ ID NO: 11: base sequence of second cDNA-variant 3

[0378] SEQ ID NO: 12: amino acid sequence of second protein variant 3

[0379] SEQ ID NO: 13: base sequence of third cDNA

[0380] SEQ ID NO: 14: amino acid sequence of third protein

[0381] SEQ ID NO: 15: base sequence of third cDNA variant 1

[0382] SEQ ID NO: 16: amino acid sequence of third protein variant 1

[0383] SEQ ID NO: 17: base sequence of third cDNA variant 2

[0384] SEQ ID NO: 18: amino acid sequence of third protein variant 2

[0385] SEQ ID NO: 19: base sequence of third cDNA variant 3

[0386] SEQ ID NO: 20: amino acid sequence of third protein variant 3

[0387] SEQ ID NO: 21: base sequence of third cDNA variant 4

[0388] SEQ ID NO: 22: amino acid sequence of third protein variant 4

[0389] SEQ ID NO: 23: synthetic substrate peptide IEGR

[0390] SEQ ID NO: 24 to 34: primers

SEQUENCE LISTING

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aagtggagtg gtcgaccagc aagctgtata ccagtttgtg gatggtcaga ctctccccgt   2280
tctcctttca tcgtcaatgg aaattccacc gaaataggtc agtggccgtg gcaggcagga   2340
atctccagat ggcttgcaga tcataatatg tggtttcttc agtgtggagg agctctactg   2400
aatgagaaat ggatcattac tgcagccctc tgtgtcacct actctgctac tgccgagatc   2460
attgacccaa gtcagtttaa attctacctg ggcaaatact atcgagatga cagtaaggat   2520
gatgactacg tacaagtaag agaggctatc gagatccatg tgaatcctaa ctacgatcct   2580
ggaaatcttca actttgacat agcctgatt caactgaaga cttctgttgc tctgaccaca   2640
cgagtgcaac caatatgtct gcctactgat ctcactacaa gagaaaacct gaaagaggga   2700
gcgttagcgg tggtgacagg atggggttcg aatgaaaaca acacatattc agagatgact   2760
cagcaagccg ttctgcctgt tgttgcagca agcacctgtg aacaaggata tcaggactcg   2820
ggcttgccac tgacagtgac agagaacatg ttctgtgcag gttacaagca gggggcgtat   2880
gatgcctgca gtggagacag tggaggacca ctagtgtttg ctgatgattc cggcaccgat   2940
```

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```
aggcggtggg tcctggaagg gatcgtcagc tgggggcagcc ccaatggatg tggcaagtct   3000

aacagtatg gggcttcac taaagttaac gttttctat cgtggattcg gcagttcatt   3060

tga                                                                3063


<210> SEQ ID NO 4
<211> LENGTH: 1020
<212> TYPE: PRT
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 4

Met Val Leu Ala Ser Phe Leu Val Ser Gly Leu Val Leu Gly Leu Leu
1               5                  10                  15

Ala Gln Gln Met His Pro Val Gln Ser Arg Gly Val Asp Leu Gly Leu
            20                  25                  30

Cys Asp Asp Thr Arg Phe Glu Cys Lys Cys Gly Asp Pro Gly Tyr Val
        35                  40                  45

Phe Asn Val Pro Ala Lys Gln Cys Thr Tyr Phe Tyr Arg Trp Arg Pro
    50                  55                  60

Tyr Cys Lys Pro Cys Asp Lys Leu Glu Ala Lys Asp Val Cys Pro Lys
65                  70                  75                  80

Tyr Lys Arg Cys Gln Glu Cys Arg Ala Gly Leu Asp Ser Cys Val Ser
                85                  90                  95

Cys Pro Pro Asn Lys Tyr Gly Thr Trp Cys Ser Gly Glu Cys Gln Cys
            100                 105                 110

Lys Asn Gly Gly Ile Cys Asp Gln Arg Thr Gly Ala Cys Thr Cys Arg
        115                 120                 125

Asp Arg Tyr Glu Gly Val His Cys Glu Ile Leu Gln Gly Cys Pro Leu
    130                 135                 140

Leu Gln Ser Asp Pro Gln Val Gln Glu Val Lys Asn Pro Pro Asn Asp
145                 150                 155                 160

Pro Gln Thr Ile Asp Tyr Ser Cys Ser Pro Gly Phe Lys Leu Lys Gly
                165                 170                 175

Val Ala Arg Ile Thr Cys Leu Pro Asn Gly Gln Trp Ser Ser Phe Pro
            180                 185                 190

Pro Lys Cys Ile Arg Glu Cys Ser Met Val Ser Ser Leu Glu His Gly
        195                 200                 205

Lys Val Asn Ser Pro Ser Ala Asp Leu Ile Glu Gly Ala Thr Leu Arg
    210                 215                 220

Phe Ser Cys Asp Ser Pro Tyr Tyr Leu Ile Gly Gln Glu Thr Leu Thr
225                 230                 235                 240

Cys Gln Gly Asn Gly Gln Trp Ser Gly Gln Ile Pro Gln Cys Gln Lys
                245                 250                 255

Leu Val Phe Cys Pro Asp Leu Asp Pro Ala Ser His Ala Glu His Gln
            260                 265                 270

Val Lys Ile Gly Leu Glu Gln Lys Tyr Gly Gln Phe Pro Gln Gly Thr
        275                 280                 285

Glu Val Thr Tyr Thr Cys Thr Gly Asn Tyr Phe Leu Met Gly Leu Asp
    290                 295                 300

Thr Leu Lys Cys Asn Pro Asp Gly Ser Trp Ser Gly Thr Gln Pro Ser
305                 310                 315                 320

Cys Val Lys Val Ala Asp Arg Glu Val Asn Cys Asp Ser Lys Ala Val
                325                 330                 335
```

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```
Asp Phe Leu Asp Asp Val Gly Glu Pro Val Arg Ile His Cys Pro Ala
            340                 345                 350

Gly Cys Ser Leu Thr Ala Gly Thr Val Thr Gly Thr Ala Ile Tyr His
        355                 360                 365

Glu Leu Ser Ser Val Cys Arg Ala Ala Ile His Ala Gly Lys Val Pro
    370                 375                 380

Asn Ser Gly Gly Ala Val His Val Val Asn Asn Gly Pro Tyr Ser Asp
385                 390                 395                 400

Phe Leu Ala Ser Asp Leu Asn Gly Ile Lys Ser Asp Glu Leu Lys Ser
                405                 410                 415

Leu Ala Gln Ser Phe Arg Phe Asp Tyr Val Ser Ser Ser Thr Ala Gly
            420                 425                 430

Arg Lys Ser Gly Cys Pro Asp Gly Trp Phe Glu Ile Glu Gln Asn Cys
        435                 440                 445

Val Tyr Val Thr Ser Lys Gln Arg Ala Trp Glu Arg Ala Gln Gly Val
    450                 455                 460

Cys Thr Asn Met Ala Ala Arg Leu Ala Val Leu Asp Lys Asp Val Ile
465                 470                 475                 480

Pro Ser Ser Leu Thr Glu Thr Leu Arg Gly Lys Gly Leu Ala Thr Thr
                485                 490                 495

Trp Ile Gly Leu His Arg Leu Asp Ala Asp Asn His Phe Ile Trp Glu
            500                 505                 510

Leu Met Asp Arg Ser Ser Val Ala Leu Asn Asp Ser Leu Thr Phe Trp
        515                 520                 525

Ala Pro Gly Glu Pro Gly Asn Glu Thr Asn Cys Val Tyr Leu Asp Ile
    530                 535                 540

Gln Asp Gln Leu Gln Pro Val Trp Lys Thr Lys Ser Cys Phe Gln Pro
545                 550                 555                 560

Ser Ser Phe Val Cys Met Met Asp Leu Ser Asp Lys Asn Lys Ala Lys
                565                 570                 575

Cys Lys Asp Pro Gly Pro Leu Glu Asn Gly His Ala Lys Leu His Gly
            580                 585                 590

Gln Ser Ile Asp Gly Phe Tyr Ala Gly Ser Ser Val Arg Tyr Ser Cys
        595                 600                 605

Glu Val Leu His Tyr Leu Ser Gly Thr Glu Thr Val Ser Cys Thr Ser
    610                 615                 620

Asn Gly Thr Trp Ser Ala Pro Lys Pro Arg Cys Ile Lys Val Ile Thr
625                 630                 635                 640

Cys Gln Thr Pro Pro Val Pro Ser Tyr Gly Ser Val Asp Ile Lys Pro
                645                 650                 655

Pro Ser Arg Thr Asn Ser Ile Ser Arg Val Gly Ser Pro Phe Leu Arg
            660                 665                 670

Leu Pro Arg Leu Pro Leu Pro Leu Ala Arg Ala Ala Gly Pro Pro Pro
        675                 680                 685

Lys Pro Arg Ser Ala Pro Pro Ser Thr Val Asp Leu Ser Ser Lys Val
    690                 695                 700

Lys Leu Pro Glu Gly His Tyr Arg Val Gly Ser Gln Ala Ile Tyr Thr
705                 710                 715                 720

Cys Glu Ser Arg Tyr Tyr Glu Leu Leu Gly Ser Gln Gly Arg Arg Cys
                725                 730                 735
```

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```
Asp Ser Asn Gly Lys Trp Ser Gly Arg Pro Ala Ser Cys Ile Pro Val
            740                 745                 750

Cys Gly Arg Ser Asp Ser Pro Arg Ser Pro Phe Ile Val Asn Gly Asn
        755                 760                 765

Ser Thr Gln Ile Gly Gln Trp Pro Trp Gln Ala Gly Ile Ser Arg Trp
    770                 775                 780

Leu Ala Asp His Asn Met Trp Phe Leu Gln Cys Gly Gly Ala Leu Leu
785                 790                 795                 800

Asn Glu Lys Trp Ile Ile Thr Ala Ala His Cys Val Thr Tyr Ser Ala
                805                 810                 815

Thr Ala Glu Ile Ile Asp Pro Ser Gln Phe Lys Phe Tyr Leu Gly Lys
            820                 825                 830

Tyr Tyr Arg Asp Asp Ser Lys Asp Asp Asp Tyr Val Gln Val Arg Glu
        835                 840                 845

Ala Ile Glu Ile His Val Asn Pro Asn Tyr Asp Pro Gly Asn Leu Asn
    850                 855                 860

Phe Asp Ile Ala Leu Ile Gln Leu Lys Thr Ser Val Ala Leu Thr Thr
865                 870                 875                 880

Arg Val Gln Pro Ile Cys Leu Pro Thr Asp Leu Thr Thr Arg Glu Asn
                885                 890                 895

Leu Lys Glu Gly Ala Leu Ala Val Val Thr Gly Trp Gly Leu Asn Glu
            900                 905                 910

Asn Asn Thr Tyr Ser Glu Met Ile Gln Gln Ala Val Leu Pro Val Val
        915                 920                 925

Ala Ala Ser Thr Cys Glu Gln Gly Tyr Glu Asp Ser Gly Leu Pro Leu
    930                 935                 940

Thr Val Thr Glu Asn Met Phe Cys Ala Gly Tyr Lys Gln Gly Arg Tyr
945                 950                 955                 960

Asp Ala Cys Ser Gly Asp Ser Gly Gly Pro Leu Val Phe Ala Asp Asp
                965                 970                 975

Ser Arg Thr Asp Arg Arg Trp Val Leu Glu Gly Ile Val Ser Trp Gly
            980                 985                 990

Ser Pro Asn Gly Cys Gly Lys Ser  Asn Gln Tyr Gly Gly  Phe Thr Lys
        995                 1000                1005

Val Asn  Val Phe Leu Ser Trp  Ile Arg Gln Phe Ile
    1010                1015                1020
```

<210> SEQ ID NO 5
<211> LENGTH: 1263
<212> TYPE: DNA
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 5

```
atggcgtgga tttgtgtgat aacgttgttt gttttggctt ctagtacgtt gagtaataaa     60

gttagtagag tggggatcat ctttcctaag acacagaacg atcataaaca gtgtacagca    120

aaaggtggat taaaagggtc ctgcaagtcc ctcacagact gtcctgctgt cttggctacg    180

ttgaaggata gtttcctgt cgtttgcttt tggaatggtc ggtttcagca tattgtatgt    240

tgtcttgatg cagcagcacc aagtgtaacc acaacagtta caactattgt ccctacaaaa    300

gaaacaaaga ttccaagatt acatatacca ggttgtggaa aaagaaaagt aaatgtagat    360

attacaacta ctggacgttc ggggtcaaca atacttcctc ccatacctac ttctcaagat    420
```

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```
ttgaagggtg ggagaggaat cattgctgga ggtgtagaag ctaaaattgg cgcctggcct    480

tggatggcag ctgtttttgt gaaaaatttt ggcattggca gattccattg tgctggtagc    540

ataatragta gcaagtacat tttgtctgct gcccacgctt tcctcattgg aggtcgaaag    600

ctgaccccaa ctcgcttagc tgtccgcgta ggaggccact acgtaaagat gggtcaagaa    660

tatcatgtgg aagatgtgat tatccatcct gactacgtag aaaggagaaa ttacaatgat    720

attgctatca ttgtgttaaa agaggaactg aattttactg atttggtccg tccaatctgt    780

ctcccatgacc cagaggcagt aacagattca ttaaaaggca gaaggggtgac agtagctgga    840

tgggggtgatc tggatttcgc cggtccacga agtcaagttc tgcgcgaggt tagtatcccc    900

gttgttccaa tcggtgactg taacaaagcc tatcagaagc tcaacaccct tgctctttaaa    960

aatgggataa cgaaaaagtt tatttgtgct ggattggaag aaggtgggaa agatgcttgt   1020

caaggcgatt ctggtggacc gttgatgcta gcgaacaata gtagttggat agtgacgggg   1080

gtggtgtcgt tcggacacaa gtgtgccgag gagggatttc ctggtgtgta cacgcgtgta   1140

gtgagttacc tagagtggat cgcgaaggtt acgaactcgt tagaccaggc agtcactaac   1200

tga                                                                1203
```

<210> SEQ ID NO 6
<211> LENGTH: 400
<212> TYPE: PRT
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 6

```
Met Ala Trp Ile Cys Val Ile Thr Leu Phe Ala Leu Ala Ser Ser Thr
1               5                  10                  15

Leu Ser Asn Lys Val Ser Arg Val Gly Ile Ile Phe Pro Lys Thr Gln
            20                  25                  30

Asn Asp Asn Lys Gln Cys Thr Ala Lys Gly Gly Leu Lys Gly Ser Cys
        35                  40                  45

Lys Ser Leu Thr Asp Cys Pro Ala Val Leu Ala Thr Leu Lys Asp Ser
    50                  55                  60

Phe Pro Val Val Cys Ser Trp Asn Gly Arg Phe Gln Pro Ile Val Cys
65                  70                  75                  80

Cys Pro Asp Ala Ala Ala Pro Ser Val Thr Thr Thr Val Thr Thr Ile
                85                  90                  95

Val Pro Thr Lys Glu Thr Lys Ile Pro Arg Leu His Ile Pro Gly Cys
            100                 105                 110

Gly Lys Arg Lys Val Asn Val Asp Ile Thr Thr Ile Gly Arg Ser Gly
        115                 120                 125

Ser Pro Ile Leu Pro Pro Ile Ser Thr Ser Gln Asp Leu Lys Gly Gly
    130                 135                 140

Arg Gly Ile Ile Ala Gly Gly Val Glu Ala Lys Ile Gly Ala Trp Pro
145                 150                 155                 160

Trp Met Ala Ala Val Phe Val Lys Asn Phe Gly Ile Gly Arg Phe His
                165                 170                 175

Cys Ala Gly Ser Ile Ile Ser Ser Lys Tyr Ile Leu Ser Ala Ala His
            180                 185                 190

Ala Phe Leu Ile Gly Gly Arg Lys Leu Thr Pro Thr Arg Leu Ala Val
        195                 200                 205

Arg Val Gly Gly His Tyr Val Lys Met Gly Gln Glu Tyr His Val Glu
```

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```
    210                 215                 220

Asp Val Ile Ile His Pro Asp Tyr Val Glu Arg Glu Asn Tyr Asn Asp
225                 230                 235                 240

Ile Ala Ile Ile Val Leu Lys Glu Glu Leu Asn Phe Thr Asp Leu Val
                245                 250                 255

Arg Pro Ile Cys Leu Pro Asp Pro Glu Ala Val Thr Asp Ser Leu Lys
            260                 265                 270

Gly Arg Arg Val Thr Val Ala Gly Trp Gly Asp Leu Arg Phe Ala Gly
        275                 280                 285

Pro Arg Ser Gln Val Leu Arg Glu Val Ser Ile Pro Val Val Pro Ile
    290                 295                 300

Gly Asp Cys Asn Lys Ala Tyr Gln Lys Leu Asn Thr Leu Ala Leu Lys
305                 310                 315                 320

Asn Gly Ile Thr Lys Lys Phe Ile Cys Ala Gly Leu Glu Glu Gly Gly
                325                 330                 335

Lys Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Met Leu Val Asn
            340                 345                 350

Asn Ser Ser Trp Ile Val Thr Gly Val Val Ser Phe Gly His Lys Cys
        355                 360                 365

Ala Glu Glu Gly Phe Pro Gly Val Tyr Thr Arg Val Val Ser Tyr Leu
    370                 375                 380

Glu Trp Ile Ala Lys Val Thr Asn Ser Leu Asp Gln Ala Val Thr Asn
385                 390                 395                 400


<210> SEQ ID NO 7
<211> LENGTH: 1203
<212> TYPE: DNA
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 7

atggcgtgga tttgtgtgat aacgttgttt gctttggcct ctagtacgct gagtaataaa     60

gttagtagag tggggatcat ctttcctaag acacagaacg ataataaaca gtgtacagca    120

aaaggtggat taaaagggtc ctgcaagtcc ctcacagact gtcctgctgt cttggctacg    180

ttgaaggata gtttcctgt cgtttgctct tggaatggtc ggtttcagcc tattgtatgt    240

tgtcctgatg cagcagcacc aagtgtaacc acaacagtta caactattgt ccctacaaaa    300

gaaacaaaga ttccaagatt acatatacca ggttgtggaa aaagaaaagt aaatgtagat    360

attacaacta ttggacgttc ggggtcacca atacttccta ccatatctac ttctcaagat    420

ttgaagggtg ggagaggaat cattgctgga ggtgtagaag ctaaaattgg cgcctggcct    480

tggatggcag ctgtttttgt gaaaaatttt ggcattggca gattccattg tgctggtagc    540

ataatcagta gcaagtacat tttgtctgct gcccacgctt tcctcattgg aggtcgaaag    600

ctgaccccaa ctcgcttagc tgtccgcgta ggaggccact acgtaagat gggtcaagaa    660

tatcatgtgg aagatgtgat tatccatcct gactacgtag aaagggagaa ttacaatgat    720

attgctatca ttgtgttaaa agaggaactg aattttactg atttggtccg tccaatctgt    780

ctccctgacc cagaggcagt aacagattca tcaaaaggca gaagggtgac agtagctgga    840

tgggggtgat cggatttcgc cggtccacga agtcaagttc tgcgcgaggt tagtatcccc    900

gttgttccaa tcagtgattg taacaaagcc tatcagaatc tcaacacccc tgctcttaaa    960

aatgggataa cgaaaaagtt tatttgtgct ggattggaag aaggtgggaa agatgcttgt   1020
```

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caaggcgatt ctggtggacc gttgatgcta gtgaacaata gtagttggat agtgacggga 1080

gtggtgtcgt tggacacaaa gtgtgccgag gagggatttc ctggtgtgta cacgcgtgta 1140

gtgagttacc tagagtggat cgcgaaggtt acgaactcgt tagaccaggc agtcactaac 1200

tga 1203

<210> SEQ ID NO 8
<211> LENGTH: 400
<212> TYPE: PRT
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 8

Met Ala Trp Ile Cys Val Ile Thr Leu Phe Ala Leu Ala Ser Ser Thr
1 5 10 15

Leu Ser Asn Lys Val Ser Arg Val Gly Ile Ile Phe Pro Lys Thr Gln
20 25 30

Asn Asp Asn Lys Gln Cys Thr Ala Lys Gly Gly Leu Lys Gly Ser Cys
35 40 45

Lys Ser Leu Thr Asp Cys Pro Ala Val Leu Ala Thr Leu Lys Asp Ser
50 55 60

Phe Pro Val Val Cys Ser Trp Asn Gly Arg Phe Gln Pro Ile Val Cys
65 70 75 80

Cys Pro Asp Ala Ala Ala Pro Ser Val Thr Thr Thr Val Thr Thr Ile
85 90 95

Val Pro Thr Lys Glu Thr Lys Ile Pro Arg Leu His Ile Pro Gly Cys
100 105 110

Gly Lys Arg Lys Val Asn Val Asp Ile Thr Thr Ile Gly Arg Ser Gly
115 120 125

Ser Pro Ile Leu Pro Pro Ile Ser Thr Ser Gln Asp Leu Lys Gly Gly
130 135 140

Arg Gly Ile Ile Ala Gly Gly Val Glu Ala Lys Ile Gly Ala Trp Pro
145 150 155 160

Trp Met Ala Ala Val Phe Val Lys Asn Phe Gly Ile Gly Arg Phe His
165 170 175

Cys Ala Gly Ser Ile Ile Ser Ser Lys Tyr Ile Leu Ser Ala Ala His
180 185 190

Ala Phe Leu Ile Gly Gly Arg Lys Leu Thr Pro Thr Arg Leu Ala Val
195 200 205

Arg Val Gly Gly His Tyr Val Lys Met Gly Gln Glu Tyr His Val Glu
210 215 220

Asp Val Ile Ile His Pro Asp Tyr Val Glu Arg Glu Asn Tyr Asn Asp
225 230 235 240

Ile Ala Ile Ile Val Leu Lys Glu Glu Leu Asn Phe Thr Asp Leu Val
245 250 255

Arg Pro Ile Cys Leu Pro Asp Pro Glu Ala Val Thr Asp Ser Leu Lys
260 265 270

Gly Arg Arg Val Thr Val Ala Gly Trp Gly Asp Leu Asp Phe Ala Gly
275 280 285

Pro Arg Ser Gln Val Leu Arg Glu Val Ser Ile Pro Val Val Pro Ile
290 295 300

Ser Asp Cys Asn Lys Ala Tyr Gln Asn Leu Asn Thr Leu Ala Leu Lys
305 310 315 320

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```
Asn Gly Ile Thr Lys Lys Phe Ile Cys Ala Gly Leu Glu Glu Gly Gly
                325                 330                 335

Lys Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Met Leu Val Asn
            340                 345                 350

Asn Ser Ser Trp Ile Val Thr Gly Val Val Ser Phe Gly His Lys Cys
        355                 360                 365

Ala Glu Glu Gly Phe Pro Gly Val Tyr Thr Arg Val Val Ser Tyr Leu
    370                 375                 380

Glu Trp Ile Ala Lys Val Thr Asn Ser Leu Asp Gln Ala Val Thr Asn
385                 390                 395                 400
```

<210> SEQ ID NO 9
<211> LENGTH: 1203
<212> TYPE: DNA
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 9

```
atggcgtgga tttgtgtgat aacgttgttt gctttggctt ctagtacgtt gagtaataaa     60

gttagtagag tggggatcat ctttcctaag acacagaacg atcatcaaca gtgtacagca    120

aaaggtggat taaaagggtc ctgcaagtcc ctcacagact gtcctgctgt cttggctacg    180

ttgaaggata gtttcctgt cgtttgctct tggaatggtc ggtttcagca tattgtatgt    240

tgtcctgatg cagcagcacc aagtgtaacc acaacagtta caactattgt cctacaaaa    300

gaaacaaaga ttccaagatt acatatacca ggttgtgtgg aaagaaaagt aaatgtagat    360

attacaacta ttggacgttc gggctcacca atacttccta ccatatctac ttctcaagat    420

ttgaagggtg ggagaggaat cattgctgga ggtgtagaag ctaaaattgg cgcctggcct    480

tggatggcag ctgtttttgt gaaaaatttt ggcattggca gattccattg tgctggtagc    540

atcatcagta gcaagtacat tttgtctgct gcccacgctt tcctcattgg aggtcgaaag    600

ctgaccctaa ctcgcttagc tgtccgcgta ggaggccact acgtaaagat gggtcaagaa    660

tatcatggg aagatgtgat tatccatcct gactacgtag aaaggagaa ctacaatgat    720

attgctatca ttgtgttaaa agaggaactg aatttactg atttggtccg tccaatctgt    780

atccctgacc cagaggcagt aacagattca ttaaaaggca gaagggtgac agtagctgga    840

tgggtcgatc tgatttcgc cggtccacga agtcaagttc tgcgcgaggt tagtatcccc    900

gttgttccaa tcagtgattg tagcaaagcc tatcagaatc tcaacacact tgctcttaaa    960

aatgggatca cgaacaagtt tatttgtgct ggattggaag aaggcgggaa agatgcttgt   1020

caaggcgatt ctggtggacc gttgatgcta gtgaacaata gtagctggat agtgacgggt   1080

gtggtgtcgt tcggacacaa gtgtgccgag gagggatttc ctggtgtgta cacgcgtgta   1140

gtgagttacc tagagtggat cgcgaaggtt acgaactcgt tagaccaggc agtcactaac   1200

tga                                                                 1203
```

<210> SEQ ID NO 10
<211> LENGTH: 400
<212> TYPE: PRT
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 10

```
Met Ala Trp Ile Cys Val Ile Thr Leu Phe Ala Leu Ala Ser Ser Thr
1               5                  10                  15
```

-continued

```
Leu Ser Asn Lys Val Ser Arg Val Gly Ile Ile Phe Pro Lys Thr Gln
            20                  25                  30

Asn Asp Asn Lys Gln Cys Thr Ala Lys Gly Gly Leu Lys Gly Ser Cys
        35                  40                  45

Lys Ser Leu Thr Asp Cys Pro Ala Val Leu Ala Thr Leu Lys Asp Ser
    50                  55                  60

Phe Pro Val Val Cys Ser Trp Asn Gly Arg Phe Gln Pro Ile Val Cys
65                  70                  75                  80

Cys Pro Asp Ala Ala Ala Pro Ser Val Thr Thr Thr Val Thr Thr Ile
                85                  90                  95

Val Pro Thr Lys Glu Thr Lys Ile Pro Arg Leu His Ile Pro Gly Cys
            100                 105                 110

Gly Lys Arg Lys Val Asn Val Asp Ile Thr Thr Ile Gly Arg Ser Gly
        115                 120                 125

Ser Pro Ile Leu Pro Pro Ile Ser Thr Ser Gln Asp Leu Lys Gly Gly
    130                 135                 140

Arg Gly Ile Ile Ala Gly Gly Val Glu Ala Lys Ile Gly Ala Trp Pro
145                 150                 155                 160

Trp Met Ala Ala Val Phe Val Lys Asn Phe Gly Ile Gly Arg Phe His
                165                 170                 175

Cys Ala Gly Ser Ile Ile Ser Ser Lys Tyr Ile Leu Ser Ala Ala His
            180                 185                 190

Ala Phe Leu Ile Gly Gly Arg Lys Leu Thr Pro Thr Arg Leu Ala Val
        195                 200                 205

Arg Val Gly Gly His Tyr Val Lys Met Gly Gln Glu Tyr His Val Glu
    210                 215                 220

Asp Val Ile Ile His Pro Asp Tyr Val Glu Arg Glu Asn Tyr Asn Asp
225                 230                 235                 240

Ile Ala Ile Ile Val Leu Lys Glu Glu Leu Asn Phe Thr Asp Leu Val
                245                 250                 255

Arg Pro Ile Cys Leu Pro Asp Pro Glu Ala Val Thr Asp Ser Leu Lys
            260                 265                 270

Gly Arg Arg Val Thr Val Ala Gly Trp Gly Asp Leu Asp Phe Ala Gly
        275                 280                 285

Pro Arg Ser Gln Val Leu Arg Glu Val Ser Ile Pro Val Val Pro Ile
    290                 295                 300

Ser Asp Cys Asn Lys Ala Tyr Gln Asn Leu Asn Thr Leu Ala Leu Lys
305                 310                 315                 320

Asn Gly Ile Thr Asn Lys Phe Ile Cys Ala Gly Leu Glu Glu Gly Gly
                325                 330                 335

Lys Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Met Leu Val Asn
            340                 345                 350

Asn Ser Ser Trp Ile Val Thr Gly Val Val Ser Phe Gly His Lys Cys
        355                 360                 365

Ala Glu Glu Gly Phe Pro Gly Val Tyr Thr Arg Val Val Ser Tyr Leu
    370                 375                 380

Glu Trp Ile Ala Lys Val Thr Asn Ser Leu Asp Gln Ala Val Thr Asn
385                 390                 395                 400


<210> SEQ ID NO 11
<211> LENGTH: 1203
```

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<213> TYPE: DNA
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 11

atggcgtgga tttgtgtgat aacgttgttt gctttggctt ctagtacgct gagtaataaa 60

gttagtagag tgggggatcat ctttcctaag acacagaacg atagtcaaca gtgtacagca 120

aaaggtggat taaaagggtc ctgcaagtcc ctcacagact gtcctgctgt cttggctacg 180

ttgaaggata gtttcctgt cgtttgctct tggaatggtc ggtttcagcc tattgtatgt 240

tgtcctgatg cagcagcacc aagtgtaacc acaacagtta caactattgt cctacaaaa 300

gaaacaaaga ttccaagatt acatatacca ggttgtggaa aaagaaaagt aaatgtagat 360

attacaacta ttggacgttc gggctcacca atacttccta ccatatctac ttctcaagat 420

ttgaagggtg ggagaggaat cattgctgga ggtgtagaag ctaaaattgg cgcctggcct 480

tggatggcag ctgtttttgt gaaaaatttt ggcattggca gatttcattg tgctggtagc 540

ataatcagta gcaagtacat tttgtctgct gcccacgctt tcctcattgg aggtcgaaag 600

ctgaccccaa ctcgcattagc tgtccagcgta ggaggccact acgtaaagat gggtcaagaa 660

tatcatgtgg aagatgtgat tatccatcct gactacgtag aaagggagaa ttacaatgat 720

attgctatca ttgtgttaaa agaggaactg aattttactg atttggtccg tccaatctgt 780

ctccctgacc cagaggcagt aacagattca ttaaaaggca gaagggtgac agtagctgga 840

tggggtgatc tggatttcgc cggtccacga agtcaagttc tgcgcgaggt tagtatcccc 900

gttgttccaa tcagtgattg taacaaagcc tatcagaatc tcaacacact tgctcttaaa 960

aatgggataa cgaacaagtt tatttgtgct ggattggaag aaggcgggaa agatgcttgt 1020

caaggcgatt ctggtggacc gttgatgcta gtgaacaata gtagttggat agtgacgggg 1080

gtggtgtcgt tcggacacaa gtgtgccgag gagggatttc ctggtgtgta cacgcgtgta 1140

gtgagttacc tagagtggat cgcgaaggtt acgaactcgt tagaccaggc agtcactaag 1200

tga 1203

<210> SEQ ID NO 12
<211> LENGTH: 400
<212> TYPE: PRT
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 12

Met Ala Trp Ile Cys Val Ile Thr Leu Phe Ala Leu Ala Ser Ser Thr
1 5 10 15

Leu Ser Asn Lys Val Ser Arg Val Gly Ile Ile Phe Pro Lys Thr Gln
20 25 30

Asn Asp Asn Lys Gln Cys Thr Ala Lys Gly Gly Leu Lys Gly Ser Cys
35 40 45

Lys Ser Leu Thr Asp Cys Pro Ala Val Leu Ala Thr Leu Lys Asp Ser
50 55 60

Phe Pro Val Val Cys Ser Trp Asn Gly Arg Phe Gln Pro Ile Val Cys
65 70 75 80

Cys Pro Asp Ala Ala Ala Pro Ser Val Thr Thr Thr Val Thr Thr Ile
85 90 95

Val Pro Thr Lys Glu Thr Lys Ile Pro Arg Leu His Ile Pro Gly Cys
100 105 110

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Gly Lys Arg Lys Val Asn Val Asp Ile Thr Thr Ile Gly Arg Ser Gly
        115                 120                 125

Ser Pro Ile Leu Pro Pro Ile Ser Thr Ser Gln Asp Leu Lys Gly Gly
    130                 135                 140

Arg Gly Ile Ile Ala Gly Gly Val Glu Ala Lys Ile Gly Ala Trp Pro
145                 150                 155                 160

Trp Met Ala Ala Val Phe Val Lys Asn Phe Gly Ile Gly Arg Phe His
                165                 170                 175

Cys Ala Gly Ser Ile Ile Ser Ser Lys Tyr Ile Leu Ser Ala Ala His
            180                 185                 190

Ala Phe Leu Ile Gly Gly Arg Lys Leu Thr Pro Thr Arg Leu Ala Val
        195                 200                 205

Arg Val Gly Gly His Tyr Val Lys Met Gly Gln Glu Tyr His Val Glu
    210                 215                 220

Asp Val Ile Ile His Pro Asp Tyr Val Glu Arg Glu Asn Tyr Asn Asp
225                 230                 235                 240

Ile Ala Ile Ile Val Leu Lys Glu Glu Leu Asn Phe Thr Asp Leu Val
                245                 250                 255

Arg Pro Ile Cys Leu Pro Asp Pro Glu Ala Val Thr Asp Ser Leu Lys
            260                 265                 270

Gly Arg Arg Val Thr Val Ala Gly Trp Gly Asp Leu Asp Phe Ala Gly
        275                 280                 285

Pro Arg Ser Gln Val Leu Arg Glu Val Ser Ile Pro Val Val Pro Ile
    290                 295                 300

Ser Asp Cys Asn Lys Ala Tyr Gln Asn Leu Asn Thr Leu Ala Leu Lys
305                 310                 315                 320

Asn Gly Ile Thr Asn Lys Phe Ile Cys Ala Gly Leu Glu Glu Gly Gly
                325                 330                 335

Lys Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Met Leu Val Asn
            340                 345                 350

Asn Ser Ser Trp Ile Val Thr Gly Val Val Ser Phe Gly His Lys Cys
        355                 360                 365

Ala Glu Glu Gly Phe Pro Gly Val Tyr Thr Arg Val Val Ser Tyr Leu
    370                 375                 380

Glu Trp Ile Ala Lys Val Thr Asn Ser Leu Asp Gln Ala Val Thr Lys
385                 390                 395                 400
```

<210> SEQ ID NO 13
<211> LENGTH: 1128
<212> TYPE: DNA
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 13

```
atgttggtga ataacatgtt ttcattgctg tgtttcccac tcctgatgtc tatgtttagc     60

tgcagtagtc tcggcagaca gcgtagacag tttgttttcc ccgatgatga agaatcatgc    120

tcaaacggat ttactaacga tggaatatgt aaagatgctt tgaattgcag agatcttttc    180

caaaaaaatg attataattt actgaaagaa tcaatatgcg gttttgaagg cataacaccc    240

aaagtttgtt gtccgaaaca aagtattgta aatccaataa cagaagcacc tccaaaaacc    300

actacaactg aacgaccgcc aatacggata ccatccaatc ttcctaaaca gtgtgtggaaat    360

cgtaatatta caactaccag gattacttgg aggcaggaag caacacctgg agcctggccc    420
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-continued

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tggatggctg ctgtctatat caaacaagga ggaatcagaa gtgttcagtg tggaggtgcg    480

cttgtcacca acaggcacgt gattacagca tcgcactgtg ttgtaaacag tttaggaaca    540

gacgtgatgc gagctgacgt attctcggtt cgcctaggtg aacacaattt atatagcacc    600

aatgacagtt cagatccaat tgattttgca gttacgtcag tgaaacatca tgaaaacttt    660

gtgctcgcga cgtatttgaa tgatatcgca attctgaagt taaacgacac tgttacgttt    720

acgcacaaaa ttaaaccaat ttgtctacct tatgaaagct taaggtatga ggatctagca    780

atgagaaacc catttgtcgc cggatgggga acaacagcat ttaatggccc atctagtgca    840

gtattacgag aagtgcagtt acaaatatgg ggacacgagc catgcaggca ggctacgag     900

aaggatttaa atattacaaa cgngtatarg tgngctgggt atgcagatgg cggtaaagat    960

gcttgccagg gtgattctgg aggtccaatg atgttgcctg atagaaagcgg gaacttttat   1020

ccngttggaa trgtgcattt cggaaagaaa cgcgcgttgc crggatttcc cgggctttac   1080

acaaaagtga cogaattttt agattggatt gcagtaaata tggtgtag                 1128

<210> SEQ ID NO 14
<211> LENGTH: 375
<212> TYPE: PRT
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 14

Met Leu Val Asn Asn Met Phe Ser Leu Leu Cys Phe Pro Leu Leu Met
1               5                  10                  15

Ser Met Phe Ser Cys Ser Ser Leu Gly Arg Gln Arg Arg Gln Phe Val
            20                  25                  30

Phe Pro Asp Asp Glu Glu Ser Cys Ser Asn Arg Phe Thr Asn Asp Gly
        35                  40                  45

Ile Cys Lys Asp Val Leu Asn Cys Arg Asp Leu Leu Gln Lys Asn Asp
    50                  55                  60

Tyr Asn Leu Leu Lys Glu Ser Ile Cys Gly Phe Glu Gly Ile Thr Pro
65                  70                  75                  80

Lys Val Cys Cys Pro Lys Gln Ser Ile Val Asn Pro Ile Thr Glu Ala
                85                  90                  95

Pro Pro Lys Thr Thr Thr Thr Glu Arg Pro Pro Ile Arg Ile Pro Ser
            100                 105                 110

Asn Leu Pro Lys Gln Cys Gly Asn Arg Asn Ile Thr Thr Thr Arg Ile
        115                 120                 125

Ile Gly Gly Gln Glu Ala Thr Pro Gly Ala Trp Pro Trp Met Ala Ala
    130                 135                 140

Val Tyr Ile Lys Gln Gly Gly Ile Arg Ser Val Gln Cys Gly Gly Ala
145                 150                 155                 160

Leu Val Thr Asn Arg His Val Ile Thr Ala Ser His Cys Val Val Asn
                165                 170                 175

Ser Leu Gly Thr Asp Val Met Arg Ala Asp Val Phe Ser Val Arg Leu
            180                 185                 190

Gly Glu His Asn Leu Tyr Ser Thr Asn Asp Ser Ser Asp Pro Ile Asp
        195                 200                 205

Phe Ala Val Thr Ser Val Lys His His Glu Asn Phe Val Leu Ala Thr
    210                 215                 220

Tyr Leu Asn Asp Ile Ala Ile Leu Lys Leu Asn Asp Thr Val Thr Phe
225                 230                 235                 240
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Thr His Lys Ile Lys Pro Ile Cys Leu Pro Tyr Glu Ser Leu Arg Tyr
                245                 250                 255

Glu Asp Leu Ala Met Arg Asn Pro Phe Val Ala Gly Trp Gly Thr Thr
            260                 265                 270

Ala Phe Asn Gly Pro Ser Ser Ala Val Leu Arg Glu Val Gln Leu Pro
        275                 280                 285

Ile Trp Gly His Glu Pro Cys Arg Gln Ala Tyr Glu Lys Asp Leu Asn
    290                 295                 300

Ile Thr Asn Val Tyr Met Cys Ala Gly Tyr Ala Asp Gly Gly Lys Asp
305                 310                 315                 320

Ala Cys Gln Gly Asp Ser Gly Gly Pro Met Met Leu Pro Asp Lys Ser
                325                 330                 335

Gly Asn Phe Tyr Leu Val Gly Ile Val Ser Phe Gly Lys Lys Cys Ala
            340                 345                 350

Leu Pro Gly Phe Pro Gly Val Tyr Thr Lys Val Thr Glu Phe Leu Asp
        355                 360                 365

Trp Ile Ala Val Asn Met Val
    370                 375
```

<210> SEQ ID NO 15
<211> LENGTH: 1128
<212> TYPE: DNA
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 15

```
atgctggtga ataacatgct ttcattgctg tgtttcccac tcctgatgtc tatgtttagc      60

tgcagtagtc tcggcagaca gcgtagacag tttgttttcc ccgatgatga agaatcatgc     120

tcaaaccgat ttactaacga tggaatatgt aaagatgttt tgaattgtag agatctttta     180

caaaaaaatg attataattt actgaaagaa tcaatatgcg gttttgaagg cataacaccc     240

aaagtttgtt gtccgaaaca aagtattgta aatccaataa cagaagcacc tccaaaaacc     300

actacaactg aargacegcc aatacggata ccatccaatc tcrctaaaca gtgtggaaat     360

cgtaatatta caactaccag gattattgga gggcaggaag caacacctgg agcctggccc     420

tggatggctg ctgtctatat caaacaagga ggaatcagaa gtgctcagtg tggaggtgcg     480

cttgtcacca acaggcacgt gattacagca tcgcactgtg ttgtaaaacag tttaggaaca     540

gatgtgatgc gagctgatgt attctcggtt cgcctaggtg aacacaattt atatagcacc     600

aatgacagtt cagatccaat tgattttgca gttacgtcag tgaaacatca tgaaaacttt     660

gtgcttcgcga cgtatttgaa tgatacgca attctgaagt taaacgacac tgttacgttt     720

acgcacaaaa ttaaaccaat ttgttctacct tatgaaagct taaggtatga ggattcagca     780

atgagaaacc catttgtcgc cggatgggga acaacagcat ttaatggccc atctagtgca     840

gtattacgag aagtgcagtt accaatatgg ggacacgagc cctgcaggca ggcctacgag     900

aaggatttaa atattacaaa cgtgtatacg tgtgctgggt atgcagatgg cggtaaagat     960

gcttgccagg gtgattctgg aggtccaatg atgttgcctg ataaaagcgg gaacttttat    1020

ctcgttggaa ttgtgtcttt cggaaagaaa tgcgcgttgc ctggatttcc tggggtttac    1080

acaaaagtga ccgaattttt agattggatt gcagtaaata tggtgtag                 1128
```

<210> SEQ ID NO 16

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<211> LENGTH: 375
<212> TYPE: PRT
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 16

Met Leu Val Asn Asn Met Phe Ser Leu Leu Cys Phe Pro Leu Leu Met
1 5 10 15

Ser Met Phe Ser Cys Ser Ser Leu Gly Arg Gln Arg Arg Gln Phe Val
20 25 30

Phe Pro Asp Asp Glu Glu Ser Cys Ser Asn Arg Phe Thr Asn Asp Gly
35 40 45

Ile Cys Lys Asp Val Leu Asn Cys Arg Asp Leu Leu Gln Lys Asn Asp
50 55 60

Tyr Asn Leu Leu Lys Glu Ser Ile Cys Gly Phe Glu Gly Ile Thr Pro
65 70 75 80

Lys Val Cys Cys Pro Lys Gln Ser Ile Val Asn Pro Ile Thr Glu Ala
85 90 95

Pro Pro Lys Thr Thr Thr Thr Glu Arg Pro Pro Ile Arg Ile Pro Ser
100 105 110

Asn Leu Pro Lys Gln Cys Gly Asn Arg Asn Ile Thr Thr Thr Arg Ile
115 120 125

Ile Gly Gly Gln Glu Ala Thr Pro Gly Ala Trp Pro Trp Met Ala Ala
130 135 140

Val Tyr Ile Lys Gln Gly Gly Ile Arg Ser Ala Gln Cys Gly Gly Ala
145 150 155 160

Leu Val Thr Asn Arg His Val Ile Thr Ala Ser His Cys Val Val Asn
165 170 175

Ser Leu Gly Thr Asp Val Met Arg Ala Asp Val Phe Ser Val Arg Leu
180 185 190

Gly Glu His Asn Leu Tyr Ser Thr Asn Asp Ser Ser Asp Pro Ile Asp
195 200 205

Phe Ala Val Thr Ser Val Lys His His Glu Asn Phe Val Leu Ala Thr
210 215 220

Tyr Leu Asn Asp Ile Ala Ile Leu Lys Leu Asn Asp Thr Val Thr Phe
225 230 235 240

Thr His Lys Ile Lys Pro Ile Cys Leu Pro Tyr Glu Ser Leu Arg Tyr
245 250 255

Glu Asp Leu Ala Met Arg Asn Pro Phe Val Ala Gly Trp Gly Thr Thr
260 265 270

Ala Phe Asn Gly Pro Ser Ser Ala Val Leu Arg Glu Val Gln Leu Pro
275 280 285

Ile Trp Gly His Glu Pro Cys Arg Gln Ala Tyr Glu Lys Asp Leu Asn
290 295 300

Ile Thr Asn Val Tyr Met Cys Ala Gly Tyr Ala Asp Gly Gly Lys Asp
305 310 315 320

Ala Cys Gln Gly Asp Ser Gly Gly Pro Met Met Leu Pro Asp Lys Ser
325 330 335

Gly Asn Phe Tyr Leu Val Gly Ile Val Ser Phe Gly Lys Lys Cys Ala
340 345 350

Leu Pro Gly Phe Pro Gly Val Tyr Thr Lys Val Thr Glu Phe Leu Asp
355 360 365

Trp Ile Ala Val Asn Met Val

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376 375

<210> SEQ ID NO 17
<211> LENGTH: 1128
<212> TYPE: DNA
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 17

atgttggtga ataacatgtt ttcattgcag tgtttcccac tcctgatgtc tatgtttagc 60

tgcagtagtc tcggcagaca gcgtagacaa tttgttttcc ccgatgatga agaatcatgc 120

tcaaaccgat ttactaacga agyaatatgt aaagatgttt tgaattgtag agaacttta 180

caaaaaaatg attataattt actgaaagaa tcaatatgcg gttttgaagg cataacaccc 240

aaagtttgtt gtccgaaaca aagtattgta aatccaataa cagaagcacc tccaaaaact 300

actacaactg aargaccgcc aatccgtata ccatccaatc tacctaaaca gtgtggaaat 360

cgtaatatta caactaccag gattattgga gggcaggaag caacacctgg agcctggccc 420

tggatggctg ctgtctatat caaacaagga ggaatcagaa gtgttcagtg tggaggtgcg 480

cttgtcacca araggcargt gattacagca tcgcactgtg ttgtaaacag tttaggaaca 540

gatgtgatgc gagctgacgt attctcggtt cgcctaggtg aacacaattt atatagcacc 600

aatgacagtt cagatccaat tgattttgca gttacgtcag tgaaacatca tgaaaacttt 660

gtgctcgcga cgtatttgaa tgatacgcga attctgaagt taaacgacac tgttacgttt 720

acgcacaaaa ttaaaccaat ttgtctaact tatgaaagct taaggtatga ggatctagca 780

atgagaaacc catttgtcgc cggatgggga acaacagcat ttaatggccc atctagtgca 840

gtattacgag aagtgcagtt accaatatgg ggcacgagc cctgcaggca ggcctacgag 900

aaggatttaa atattacaaa cgtgtatatg tgtgctgggt atgcagatgg cgctaaagat 960

gcttgccagg gtgattctgg aggtccaatg atgttgcctg ataaaagcgg gaacttttat 1020

ctcgttggaa ttgtgtcttt cggaaagaaa tgcgcgttgc ctggatttcc tggggtttac 1080

acaaaagtga ccgaacttct agattggatt gcagtaaata cggtgtag 1128

<210> SEQ ID NO 18
<211> LENGTH: 375
<212> TYPE: PRT
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 18

Met Leu Val Asn Asn Met Phe Ser Leu Leu Cys Phe Pro Leu Leu Met
1 5 10 15

Ser Met Phe Ser Cys Ser Ser Leu Gly Arg Gln Arg Arg Gln Phe Val
20 25 30

Phe Pro Asp Asp Glu Glu Ser Cys Ser Asn Arg Phe Thr Asn Glu Gly
35 40 45

Ile Cys Lys Asp Val Leu Asn Cys Arg Glu Leu Leu Gln Lys Asn Asp
50 55 60

Tyr Asn Leu Leu Lys Glu Ser Ile Cys Gly Phe Glu Gly Ile Thr Pro
65 70 75 80

Lys Val Cys Cys Pro Lys Gln Ser Ile Val Asn Pro Ile Thr Glu Ala
85 90 95

Pro Pro Lys Thr Thr Thr Thr Glu Arg Pro Pro Ile Arg Ile Pro Ser
100 105 110

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Asn Leu Pro Lys Gln Cys Gly Asn Arg Asn Ile Thr Thr Thr Arg Ile
        115                 120                 125

Ile Gly Gly Gln Glu Ala Thr Pro Gly Ala Trp Pro Trp Met Ala Ala
    130                 135                 140

Val Tyr Ile Lys Gln Gly Gly Ile Arg Ser Val Gln Cys Gly Gly Ala
145                 150                 155                 160

Leu Val Thr Asn Arg His Val Ile Thr Ala Ser His Cys Val Val Asn
                165                 170                 175

Ser Leu Gly Thr Asp Val Met Arg Ala Asp Val Phe Ser Val Arg Leu
            180                 185                 190

Gly Glu His Asn Leu Tyr Ser Thr Asn Asp Ser Ser Asp Pro Ile Asp
        195                 200                 205

Phe Ala Val Thr Ser Val Lys His His Glu Asn Phe Val Leu Ala Thr
    210                 215                 220

Tyr Leu Asn Asp Ile Xaa Ile Leu Lys Leu Asn Asp Thr Val Thr Phe
225                 230                 235                 240

Thr His Lys Ile Lys Pro Ile Cys Leu Pro Tyr Glu Ser Leu Arg Tyr
                245                 250                 255

Glu Asp Leu Ala Met Arg Asn Pro Phe Val Ala Gly Trp Gly Thr Thr
            260                 265                 270

Ala Phe Asn Gly Pro Ser Ser Xaa Val Leu Arg Glu Val Gln Leu Pro
        275                 280                 285

Ile Trp Gly His Glu Pro Cys Arg Gln Xaa Tyr Glu Lys Asp Leu Asn
    290                 295                 300

Xaa Thr Asn Val Tyr Met Cys Ala Gly Tyr Ala Asp Gly Gly Lys Asp
305                 310                 315                 320

Ala Cys Gln Gly Asp Ser Gly Gly Pro Met Met Leu Pro Asp Lys Ser
                325                 330                 335

Gly Asn Phe Tyr Leu Val Gly Ile Val Ser Phe Gly Lys Lys Cys Ala
            340                 345                 350

Leu Pro Gly Phe Pro Gly Val Tyr Thr Lys Val Thr Glu Phe Leu Asp
        355                 360                 365

Trp Ile Ala Val Asn Met Val
    370                 375


<210> SEQ ID NO 19
<211> LENGTH: 1128
<212> TYPE: DNA
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 19

atghtggtga staacatgtt ttcattgctg tgtttcccac tcctgatghc tatgtttagc     60

tgcagtagtc tcggcagaca grgtagacaa tttgttttcc crgatgatga agaatcatgc    120

tcaaaccgat ttactaacga tggaatatgt aaagatgttt tgaattgtag agatctttta    180

caaaaaaatg attataattt actgaaagaa tcaatatgcg gttttgaagg cataacaccc    240

aaagtttgtt gtccgaaaca aagtattgta aatccaataa cagaagcacc tccaaaaaat    300

actacaactg aacgacogcc aatccgtata ccatccaatc ttcctaaaca gtgtggaaat    360

cgtaatatta caactaccag gattattgga gggcaggaag caacacctgg agcctggccc    420

cggatggctg ctgtctatat caaacaagga ggaatcagaa gtgttcagtg tggaggtgcg    480
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cttgtcacca acaggcacgt gattacagca tcgcactgtg ttgtaaacag tttaggaaca 540

gatgtgatgc gagctgacgt attctcggtt cgcctaggtg aacacaattt atatagcacc 600

aatgacagtt cagatccaat tgattttgca gttacgtcag tgaaacatca tgaaaacttt 660

gtgctcgcga cgtatttgaa tgatatcgca attctgaagt taaacgacac tgttacgttt 720

acgcacaaaa ttaaaccaat ttgtctacct tatgaaagct taaggtatga ggatctagca 780

atgagaaacc catttgtcgc cggatgggga acaacagcat ttaatggccc atctagtgca 840

gtattacgag aagtgcagtt accaatatgg ggacacgagc cctgcaggca ggcctacgag 900

aagyatttaa atattacaaa cgtgtatatg tgtgctgggt atgcagatgg cggtaaagat 960

gcttgccagg gtgattctgg aggtccaacg atgttgcctg ataaaagcgg gaacttttat 1020

ctcgttggaa ttgtgtcttt cggaaaagaaa tgcgcgttgc ctggatttcc tggggtttac 1080

acaaaagcga ccgaacttct agattggatt gcagtaaata cggtgtag 1128

<210> SEQ ID NO 20
<211> LENGTH: 375
<212> TYPE: PRT
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 20

Met Leu Val Asn Asn Met Phe Ser Leu Leu Cys Phe Pro Leu Leu Met
1 5 10 15

Ser Met Phe Ser Cys Ser Ser Leu Gly Arg Gln Arg Arg Gln Phe Val
20 25 30

Phe Pro Asp Asp Glu Glu Ser Cys Ser Asn Arg Phe Thr Asn Asp Gly
35 40 45

Ile Cys Lys Asp Val Leu Asn Cys Arg Asp Leu Leu Gln Lys Asn Asp
50 55 60

Tyr Asn Leu Leu Lys Glu Ser Ile Cys Gly Phe Glu Gly Ile Thr Pro
65 70 75 80

Lys Val Cys Cys Pro Lys Gln Ser Ile Val Asn Pro Ile Thr Glu Ala
85 90 95

Pro Pro Lys Asn Thr Thr Thr Glu Arg Pro Pro Ile Arg Ile Pro Ser
100 105 110

Asn Leu Pro Lys Gln Cys Gly Asn Arg Asn Ile Thr Thr Thr Arg Ile
115 120 125

Ile Gly Gly Gln Glu Ala Thr Pro Gly Ala Trp Pro Trp Met Ala Ala
130 135 140

Val Tyr Ile Lys Gln Gly Gly Ile Arg Ser Val Gln Cys Gly Gly Ala
145 150 155 160

Leu Val Thr Asn Arg His Val Ile Thr Ala Ser His Cys Val Val Asn
165 170 175

Ser Leu Gly Thr Asp Val Met Arg Ala Asp Val Phe Ser Val Arg Leu
180 185 190

Gly Glu His Asn Leu Tyr Ser Thr Asn Asp Ser Ser Asp Pro Ile Asp
195 200 205

Phe Ala Val Thr Ser Val Lys His His Glu Asn Phe Val Leu Ala Thr
210 215 220

Tyr Leu Asn Asp Ile Ala Ile Leu Lys Leu Asn Asp Thr Val Thr Phe
225 230 235 240

Thr His Lys Ile Lys Pro Ile Cys Leu Pro Tyr Glu Ser Leu Arg Tyr

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                245                 250                 255

Glu Asp Leu Ala Met Arg Asn Pro Phe Val Ala Gly Trp Gly Thr Thr
            260                 265                 270

Ala Phe Asn Gly Pro Ser Ser Ala Val Leu Arg Glu Val Gln Leu Pro
        275                 280                 285

Ile Trp Gly His Glu Pro Cys Arg Gln Ala Tyr Glu Lys Asp Leu Asn
    290                 295                 300

Ile Thr Asn Val Tyr Met Cys Ala Gly Tyr Ala Asp Gly Gly Lys Asp
305                 310                 315                 320

Ala Cys Gln Gly Asp Ser Gly Gly Pro Met Met Leu Pro Asp Lys Ser
                325                 330                 335

Gly Asn Phe Tyr Leu Val Gly Ile Val Ser Phe Gly Lys Lys Cys Ala
            340                 345                 350

Leu Pro Gly Phe Pro Gly Val Tyr Thr Lys Val Thr Glu Phe Leu Asp
        355                 360                 365

Trp Ile Ala Val Asn Met Val
    370                 375
```

<210> SEQ ID NO 21
<211> LENGTH: 1128
<212> TYPE: DNA
<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 21

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atgttggtga ataacatgtt ttcattgctg tgtttctcac tcctgatgtc tatgtttagc     60

tgcagtagtc tcggcagaca gcgtagacag tttgttttcc ccgatgatga agaatcatgc    120

tcaaacngat ttactaacga tggaatatgt aaagatgttt tgaattgtag agatattta     180

caaaaaaatg attataattt actgaaagaa tcaatatgcg gttttgaagg cataacacc     240

aaagtttgtt gtccgaaaca aagtattgta aatccaataa cagaagcacc tccaaaaact    300

actacaactg aacgacngcc aatacggata ccatccaatc ttcctaaaca gtgtggaaat    360

cgtaatatta caactaccag gattattgga gggcaggaag caacacctgg agcctggccc    420

tggatggctg ctgttatat caaacaagga ggaatcagaa gtgttcagtg tggaggtgcg     480

cttgtcacca acaggcacgt gattacagca tcgcactgtg ttgtaaacag tttaggaaca    540

gatgtgatgc gagctgacgt attctcggtt cgcctaggtg aatacaattt atatagcacc    600

aatgacagtt cagatccaat tgattttgca gttacgtcag tgaaacatca tgaaaacttt    660

gtgctcgcga cgtatttgaa tgatatcgca attatgaagt taaacgacac tgttacgttt    720

aagcacaaaa ttaaaccaat ttgtctacct tatgaaagct taaggtatga ggatctagca    780

atgagaaacc catttgtcgc cggatgggga acaacagcat ttaatggccc atctagtgca    840

gtattacgag aagtgcagtt accaatatgg ggacacgagc cttgcaggca ggcctacgag    900

aaggatttaa atattacaaa cgtgtgtatg tgtgctgggt atgcagatgg cggtaaagat    960

gcttgccagg gtgattctgg aggtccaacg atgttgccng atacaaagcgg gaactttat   1020

ctcgttggaa ttgtgtcttt cggaaagaaa tgcgcgttgc ctggatttcc tggggtttac   1080

acgaaagtga ccgaattttt agattggatt gcagtaaata tggtgtag                1128
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<210> SEQ ID NO 22
<211> LENGTH: 375
<212> TYPE: PRT

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<213> ORGANISM: Limulus polyphemus

<400> SEQUENCE: 22

Met Leu Val Asn Asn Met Phe Ser Leu Leu Cys Phe Ser Leu Leu Met
1 5 10 15

Ser Met Phe Ser Cys Ser Ser Leu Gly Arg Gln Arg Arg Gln Phe Val
20 25 30

Phe Pro Asp Asp Glu Glu Ser Cys Ser Asn Arg Phe Thr Asn Asp Gly
35 40 45

Ile Cys Lys Asp Val Leu Asn Cys Arg Asp Leu Leu Gln Lys Asn Asp
50 55 60

Tyr Asn Leu Leu Lys Glu Ser Ile Cys Gly Phe Glu Gly Ile Thr Pro
65 70 75 80

Lys Val Cys Cys Pro Lys Gln Ser Ile Val Asn Pro Ile Thr Glu Ala
85 90 95

Pro Pro Lys Thr Thr Thr Thr Glu Arg Pro Pro Ile Arg Ile Pro Ser
100 105 110

Asn Leu Pro Lys Gln Cys Gly Asn Arg Asn Ile Thr Thr Thr Arg Ile
115 120 125

Ile Gly Gly Gln Glu Ala Thr Pro Gly Ala Trp Pro Trp Met Ala Ala
130 135 140

Val Tyr Ile Lys Gln Gly Gly Ile Arg Ser Val Gln Cys Gly Gly Ala
145 150 155 160

Leu Val Thr Asn Arg His Val Ile Thr Ala Ser His Cys Val Val Asn
165 170 175

Ser Leu Gly Thr Asp Val Met Arg Ala Asp Val Phe Ser Val Arg Leu
180 185 190

Gly Glu His Asn Leu Tyr Ser Thr Asn Asp Ser Ser Asp Pro Ile Asp
195 200 205

Phe Ala Val Thr Ser Val Lys His His Glu Asn Phe Val Leu Ala Thr
210 215 220

Tyr Leu Asn Asp Ile Ala Ile Leu Lys Leu Asn Asp Thr Val Thr Phe
225 230 235 240

Thr His Lys Ile Lys Pro Ile Cys Leu Pro Thr Glu Ser Leu Arg Tyr
245 250 255

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1. A recombinant protein which is any of the following:

(A) a recombinant protein that contains the amino acid sequence represented by SEQ ID NO: 2 or 4;

(B) a recombinant protein that contains an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 2 or 4, and has factor C activity;

(C) a recombinant protein that is encoded by a DNA containing a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 1 or 3, and has factor C activity; and

(D) a recombinant protein that is encoded by a DNA hybridizing to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 1 or 3 under stringent conditions, and has factor C activity.

2. Use of the recombinant protein according to claim 1 for activating the following protein:

(A) a protein that contains the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12;

(B) a protein that contains an amino acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12, and has factor B activity;

(C) a protein that is encoded by a DNA containing a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 5, 7, 9, or 11, and has factor B activity; or

(D) a protein that is encoded by a DNA hybridizing to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 5, 7, 9, or 11 under stringent conditions, and has factor B activity.

3. A method for detecting an endotoxin in a specimen, comprising a step of bringing the recombinant protein according to claim 1 into contact with the specimen.

4. An endotoxin detecting agent, comprising the recombinant protein according to claim 1.

* * * * *

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INACTIVATION OF FACTOR C BY DIMETHYL SULFOXIDE INHIBITS COAGULATION OF THE CARCINOSCORPIUS AMOEBOCYTE LYSATE

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SUMMARY: The inhibition by dimethyl sulfoxide of the coagulation of the Carcinoscorpius Amoebocyte Lysate was found to be due to the inactivation of Factor C enzyme in the coagulation cascade and not due to the inactivation of proclotting enzyme as earlier reported in studies done on Limulus (1). Kinetic studies on both purified enzymes revealed that dimethyl sulfoxide completely but reversibly inhibited the activation of Factor C by endotoxins in a non-competitive manner whereas, it did not inhibit, albeit retard the activity of proclotting enzyme. This result also explains why clotting enzyme was shown to be largely unaffected by dimethyl sulfoxide.

INTRODUCTION: The Limulus Amoebocyte Lysate (LAL) test is now a widely used test for detecting picogram quantities of endotoxin (2). It is based on a coagulation cascade involving several protease zymogens namely Factor C (3), Factor B (4), proclotting enzyme (5) and a clottable protein, coagulogen (6). By purification and characterization of such components from the Japanese horseshoe crab Tachypleus tridentatus, Japanese workers were able to elucidate the overall molecular events which are responsible for the subsequent gelation of the lysate (Fig. 1).

Recently, we have purified for the first time, Factor C and proclotting enzyme from the amoebocyte lysate (CAL) of the Singaporean species, Carcinoscorpius rotundicauda (8). These enzymes were found to be similar in their biochemical properties to those isolated from Tachypleus and Limulus. Moreover, the specificities of the different enzymes for their substrates were also found to be similar. These enzymes were subsequently used in kinetic studies involving various inhibitors.

Abbreviations: AMC, Aminomethyl coumarin; CAL, Carcinoscorpius Amoebocyte Lysate; DMSO, Dimethyl Sulfoxide; LAL, Limulus Amoebocyte Lysate; NEM, N-ethylmaleimide; p-NA, p-Nitroanilide; TAL, Tachypleus Amoebocyte Lysate.

(* to whom correspondence should be addressed)

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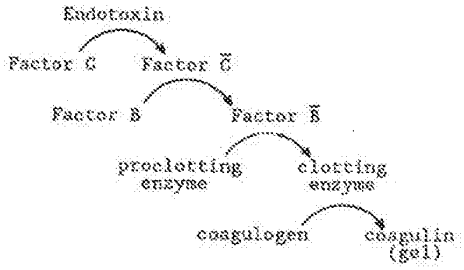


Fig. 1. Coagulation cascade of the T. tridentatus amoebocyte lysate (7).

In 1982, Liang and Liu reported that dimethyl sulfoxide inhibited the endotoxin-induced clotting activity of LAL (1). Apparently, DMSO competed with endotoxin thus inhibiting the activation of the proclotting enzyme which subsequently prevented the clotting of coagulogen. These workers also stated that DMSO was without effect on the activated clotting enzyme.

In this communication, we propose a mechanism for the inhibition by DMSO on the lysate using similar enzymes purified from a local species. Using kinetic studies, it is shown that Factor C and not the proclotting enzyme is inhibited by DMSO and that the inhibition which is dependant on DMSO concentration is complete but non-competitive.

MATERIALS AND METHODS:

Preparation of CAL: Carcinoscorpius rotundicauda were obtained from Mandai River, Northern Singapore. Amoebocyte lysate was prepared by a modification of the method of Jorgensen and Smith (9) using 3% NaCl containing 0.125% N-ethylmaleimide (Calbiochem) as anticoagulant and 3% NaCl for washing the cells. Pyrogen free water (Travenol) was used for lysing the amoebocytes through osmotic shock at 4 °C.

Purification of Factor C and proclotting Enzyme: Purification was carried out according to Navas et al (8). Briefly, whole CAL lysate was subjected to gel filtration through a pyrogen-free Sepharose CL-6B column (2.6 x 97 cm, Pharmacia) previously equilibrated with 0.05M Tris-HCl pH 8.0 containing 0.15M NaCl. Enzyme peak fractions were pooled, concentrated by freeze-drying and desalted using an Econopack 10-DG column (BioRad) packed with Sephadex G-25 previously equilibrated with pyrogen-free water. The desalted enzymes were immediately subjected to affinity chromatography on Heparin-Sepharose CL-6B column (1 x 11 cm, Pharmacia) equilibrated with the same buffer as for gel filtration. Factor C was eluted using a salt concentration of 0.5M NaCl. Unbound fractions containing proclotting enzyme were pooled and loaded onto a Benzamidine Sepharose-6B column (1.6 x 6 cm, Pharmacia). Using 0.01M sodium acetate buffer, pH 4.5 containing 0.3M NaCl, proclotting enzyme was eluted between the 9th and 15th ml into tubes containing 0.2 ml of 1M Tris-HCl pH 8.0.

Enzyme Assays: Factor C was assayed spectrofluorimetrically as described previously (10) using Boc-Val-Pro-Arg-MCA (Sigma) as substrate. Proclotting

enzyme was assayed according to the method of Harada-Suzuki (11) using Boc-Leu-Gly-Arg-pNA (Sigma) as substrate. The amount of liberated p-Nitroanilide was scanned spectrophotometrically at OD_{405}.

Effect of DMSO on whole CAL, Factor C and proclotting enzyme: Whole CAL lysate was incubated with increasing concentrations (0-50%, v/v) of DMSO (Merck). The effects of DMSO on Factor C and proclotting enzyme activities in whole CAL were monitored. Purified Factor C and proclotting enzymes were activated separately with 5 ug endotoxin, both in the absence and presence of DMSO (5,10 and 15%) while their activities were monitored at increasing concentrations of their respective synthetic substrates. The velocities of enzymatic reactions were calculated and plotted against increasing substrate concentrations as a Lineweaver-Burk plot using the Energraphics computer program (Enertronics Research, Inc.).

Other Methods: Protein concentration was determined according to the method of Lowry (12) using bovine serum albumin as standard. SDS PAGE was performed under reducing conditions according to the method of Weber and Osborn (13).

RESULTS: Figure 2 shows the effect of increasing concentrations of DMSO on both Factor C and proclotting enzyme activity in whole CAL. This result is similar to that found by Liang and Liu (1) except that complete inhibition of both enzymes was achieved only after adding 30% of DMSO as compared to 5% in LAL. From the data of Fig. 2 it is apparent that both enzymes were inhibited by DMSO. However, owing to the fact that these enzymes belong to a coagulation cascade, it is not known whether both enzymes are independently inhibited by DMSO or that only Factor C is inhibited, thereby indirectly inhibiting the proclotting enzyme. To test such a contention, it is important that the inhibitory effects of DMSO be separately investigated using purified enzymes.

Factor C and the proclotting enzymes used in this study were purified to homogeneity in three major chromatographic steps using gel filtration with Sepharose CL-6B and affinity chromatographies with Heparin-Sepharose CL-6B and Benzamidine Sepharose 6B. Figure 3 shows an electrophoretic summary of the different stages of the purification.

Factor C from CAL was purified 764-fold to a final specific activity of 122 Units/mg and a total yield of 5.6%. On reducing SDS-PAGE, the preparation was homogeneous, exhibiting a single band with an apparent Mr of 132 kDa (Fig. 3a). This was comparable to the Mr of Factor C purified from TAL and LAL (3) although Factor C from CAL was not shown to undergo any limited proteolysis even after activation with endotoxin (Fig. 4). Proclotting enzyme was purified 227-fold with a specific activity of 29 Units/mg (Fig. 3b, band A). It was found to have an apparent Mr of 54 kDa which is similar to that reported for TAL (5). However, the preparation

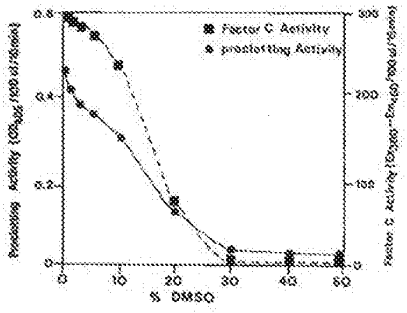


Fig 2. Effect of increasing concentrations of DMSO on Factor C and proclotting enzyme in whole CAL (30 ug) which was activated with 5 ug endotoxin. Complete inhibition of both enzyme activities was observed at 30% DMSO.

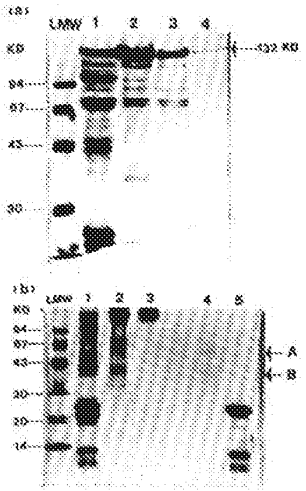


Fig 3. Reducing SDS PAGE analysis of the different stages of purification of (a) Factor C: lanes 1, whole CAL; 2, pooled first peak fractions from Sepharose CL-6B; 3, unbound fractions from Heparin-Sepharose CL-6B; 4, Factor C; and (b) proclotting enzyme: lanes 1, whole CAL, 2, unbound fractions from Heparin Sepharose CL-6B; 3, unbound fractions from Benzamidine Sepharose 6B; 4, proclotting enzyme. Lane 5 shows coagulogen derived from the third peak of the Sepharose CL-6B run (data not shown). Proclotting enzyme of 56 kDa (band A) was partially activated during purification. The appearance of the clotting enzyme (band B) reveals that some of the proclotting enzyme has undergone limited proteolysis into its smaller polypeptide fragment of 35 kDa.

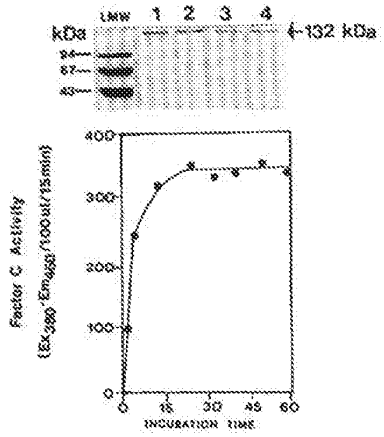


Fig 4. Time course of the activation of Factor C. Prior to the addition of 0.03 mmols substrate, Factor C (5 ug) was preincubated with 5 ug endotoxin at 5 min intervals over a period of 60 min. Maximum activation was achieved by 15 min. Electrophoretic analysis of reacted Factor C showed: Lanes 1, 0 min; 2, 15 min; 3, 30 min; 4, 45 min. Endotoxin did not effect any apparent cleavage of the 132 kDa Factor C.

obtained was partially activated during the purification process as shown by the appearance of the 35 kDa band on the gel (Fig. 3b, band B) which corresponded to the clotting enzyme (14). DMSO had no effect on the enzymatic activity of the clotting enzyme (1), thus any inhibition obtained by the addition of DMSO to this preparation is acting on the proclotting enzyme.

These results show that CAL enzymes are closely related to those of Tachypleus and Limulus. Moreover, the specificities of the enzymes from the 3 different genera to their synthetic substrates were found to be similar. Factor C purified in this study was also able to hydrolyze substrates of thrombin represented by Boc-Val-Pro-Arg-MCA (10) while proclotting enzyme was capable of hydrolyzing the synthetic substrates of the human coagulation factor, Factor Xa (15). Thus it may be assumed that kinetic data obtained from the CAL enzymes may be compared with results obtained using LAL.

Figure 5 shows a Lineweaver-Burk plot of Factor C activity against varying concentrations of its substrate in the absence and presence of 5, 10 and 15% DMSO. The lines intersected at the X-axis generating a common Km value of 1.63×10^{-4}M, which indicates a complete but non-competitive

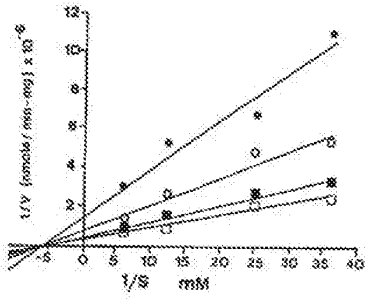


Fig 5. Lineweaver-Burk plot of Factor C against its synthetic substrate Boc-Val-Pro-Arg-MCA showing complete and non-competitive inhibition by DMSO. Factor C was incubated with 5 ug endotoxin and with 0 (□), 5 (■), 10 (○) and 15 (●)% DMSO for 1 h before reacting with 0.028, 0.04, 0.08 and 0.16 mM of substrate. Vmax was calculated at 2.3×10^{-3} umole $min^{-1}mg^{-1}$. It dropped to 1.76×10^{-3} umole $min^{-1}mg^{-1}$ in the presence of 5% DMSO, and to 6.55×10^{-4} umole $min^{-1}mg^{-1}$ at 15% DMSO. Both lines intersected at the X-axis where Km was calculated to be 1.63×10^{-4} M.

inhibition (16) of Factor C by DMSO. Moreover, no significant increase in Factor C activity was detected even with the addition of increasing amounts of enzyme or substrate to the Factor C-endotoxin-DMSO complex at 30% concentration of DMSO (Fig. 6). The inhibition was also reversible, in that Factor C activity was regained upon removal of DMSO by chromatography with Sephadex G-25.

Figure 7 shows a similar graph for proclotting enzyme against its substrate, Boc-Leu-Gly-Arg-pNA. The parallel line plots indicate uncompetitive inhibition by DMSO showing that it has no direct inhibitory effect on the catalytic site of the enzyme but has a retarding effect on the rate of reaction due to its interference on the dissociation of the enzyme-substrate complex.

DISCUSSION: It was reported that the inhibition by DMSO on the gelation of LAL was due to the inactivation of proclotting enzyme (1). This conclusion, was based on results of studies using whole lysate wherein various components of the coagulation cascade were present and as such, do not permit the identification of a particular component enzyme affected by DMSO. From the studies reported here, it is found that the inactivation of the proclotting enzyme seen in whole LAL was only apparent as a consequence of

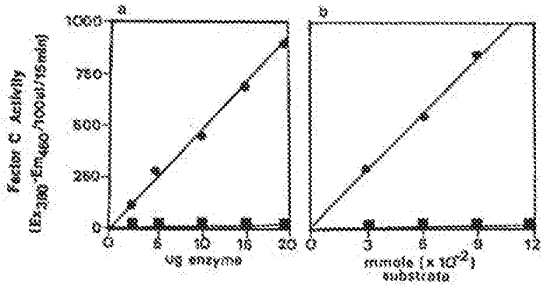


Fig 6. Effect of increasing the amounts of (a) Factor C and (b) its substrate, Boc-Val-Pro-Arg-MCA on the enzyme activity both in the absence (●) and presence (■) of 30% DMSO. In both cases, Factor C was preincubated with 5 ug endotoxin for 1 h prior to the addition of the substrate. A constant substrate concentration of 0.03 mmole was used in (a) and a constant amount of 5 ug of enzyme was used in (b). No apparent increase in enzymatic activity was detected in the presence of 30% DMSO for both instances indicating that the enzyme active site is not being inhibited by DMSO.

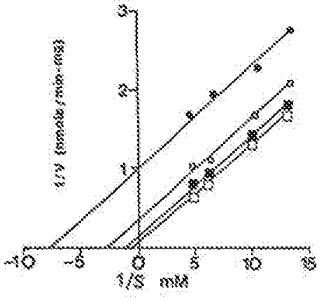


Fig 7. Lineweaver-Burk plot of preclotting enzyme activity against 0.075, 0.10, 0.15 and 0.20 mM of Boc-Leu-Gly-Arg-pNA. The enzyme was preincubated with 0 (□), 5 (■), 10 (○) and 15 (●)% DMSO prior to the addition of the substrate. All lines have a common slope of 0.055 showing uncompetitive type of inhibition. Proportional decrease of both Km and Vmax values upon addition of DMSO indicates retardation of the enzyme reaction.

the direct inactivation by DMSO of Factor C which is at the start of the cascade. Such a proposal is supported by the results of kinetic studies carried out with purified enzymes.

DMSO inhibited Factor C completely, but non-competitively, since the Km value remained the same even in the presence of the inhibitor. This non-competitive inhibition is supported by the data of Fig. 6 which show that DMSO does not compete directly for the active site of Factor C. However, this result does not discount the possibility that DMSO competes with endotoxin for the latter's binding site on Factor C. This latter possibility is in agreement with the observation (1) that greater concentrations of DMSO were needed to achieve the same degree of enzyme inhibition when the endotoxin concentration required to activate Factor C was increased. It is also a logical explanation for the observed decrease of the Vmax for Factor C measured in the presence of DMSO (Fig. 5).

This non-competitive reversible inhibition, coupled with the fact that the Factor C purified here did not undergo any proteolytic cleavage upon activation (Fig. 4), provides evidence for the allosteric nature of Factor C. Unlike the Factor C purified from TAL and LAL (3) which undergo an irreversible reaction of limited proteolysis upon activation, Factor C from CAL is putatively activated by endotoxin reversibly. This is supported by the relative stability of its primary structure on SDS-PAGE.

The following interpretation may thus be made on the mode of inhibition by DMSO on the coagulation cascade. Endotoxin activates the zymogen form of Factor C by binding to a site which causes the active site to assume a favourable conformation for binding to its substrate, Factor B. DMSO competes with endotoxin for that same binding site leading to steric changes in the active site of Factor C (refer to Fig. 1). This renders Factor C non-functional. The formation of the Factor $\bar{C}$-Factor B transition complex is thus diminished, concomittantly slowing down the whole coagulation cascade, leading to the apparent inactivation of the rest of the enzymes involved in this concatenated reaction. This deduction thus explains the lack of gel clot formation when the lysate was incubated with endotoxin in the presence of DMSO. Furthermore, it also explains the proportional decrease of both Km and Vmax observed for proclotting enzyme in the presence of DMSO. The uncompetitive inhibition (Fig. 7) is not a direct effect on the enzyme but simply a retardation of its activity as a consequence of the slowing down of the entire coagulation cascade. This

result also explains why activated clotting enzyme was found to be largely unaffected by increasing concentrations of DMSO (1).

ACKNOWLEDGEMENTS: This work was supported by NUS Grant RP 88/357 (B.H.) and Res. and Dev. Assistance Scheme RH/85/06 (J.L.D.). M.A.A.N. III is a postgraduate student supported by an NUS Research Scholarship.

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J. Biochem. 109, 150-157 (1991)

Further Studies on Lipopolysaccharide-Sensitive Serine Protease Zymogen (Factor C): Its Isolation from *Limulus polyphemus* Hemocytes and Identification as an Intracellular Zymogen Activated by α-Chymotrypsin, Not by Trypsin[1]

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An intracellular serine protease zymogen, factor C, is an initiator in the hemolymph coagulation system of horseshoe crab. We purified this zymogen from the hemocytes of the American horseshoe crab, *Limulus* (*L.*) *polyphemus*, the objective being to compare its properties with those of the Japanese horseshoe crab, *Tachypleus* (*T.*) *tridentatus*, factor C. The purified zymogen *L.*-factor C showed similar properties to those of *T.*-factor C, in terms of molecular mass (123,000), amino acid composition (1,011 residues), subunit structure (two chains), and antigenicity. Like the zymogen *T.*-factor C, this zymogen was also activated autocatalytically in the presence of bacterial lipopolysaccharide (LPS) and its synthetic lipid A analogue. A most interesting finding is that both protease zymogens are rapidly activated by α-chymotrypsin or rat mast cell chymase, but not by trypsin. The active enzyme factor C̄ showed α-thrombin-like specificity toward synthetic tripeptide substrates. This factor C̄ was also strongly inhibited by an α-thrombin inhibitor, D-Phe-Pro-Arg-chloromethyl ketone. Thus, the enzymatic properties of factor C̄ are similar to those of mammalian α-thrombin. On the other hand, the coagulation cascade system present in the hemocyte lysate was activated when chymotrypsin, free from LPS, was added to the lysate used to detect the endotoxins. The implication of our findings is that the chymotrypsin-catalyzed initiation of the horseshoe crab coagulation system is unique, since all known mammalian coagulation, fibrinolysis and complement systems are initiated by trypsin-like enzymes.

Horseshoe crab hemocytes are highly sensitive to Gram-negative bacterial endotoxins, lipopolysaccharides (LPS) (*1-3*), exposure to LPS resulting in activation of intracellular coagulation systems. In the Japanese horseshoe crab, *Tachypleus* (*T.*) *tridentatus*, the coagulation system consists of at least three intracellular serine protease zymogens, named proclotting enzyme (*4*), factor B (*5*), and factor C (*6-9*), in addition to the fibrinogen-like substance, coagulogen (*10, 11*). These zymogens and coagulogen constitute the cascade pathway responsible for the LPS-mediated activation, as shown in Fig. 1 (*6*). Factor C is an LPS-sensitive protease zymogen consisting of a single chain glycoprotein with a molecular mass of 123,000. During the LPS-mediated activation of factor C, its single chain form is converted to a two chain intermediate form comprising an 80 kDa heavy chain and a 43 kDa light chain, and the latter is subsequently transformed to a 7.9 kDa A chain and a 34 kDa B chain on cleavage of a unique Phe-Ile bond (*6, 7*). This three chain-factor C̄ has the potential to activate zymogen factor B (*5*) and to hydrolyze a synthetic tripeptide substrate, such as Boc-Val-Pro-Arg-NH-Np (*8*). The entire cDNA sequence of this factor C has been determined (manuscript submitted). The deduced amino acid sequence data suggest that the zymogen factor C is a mosaic protein composed of a Cys-rich, an EGF-like, a lectin-like, five β_3GP-I-like, and serine protease domains.

Although much progress has been made on the zymogen, factor C, isolated from *T. tridentatus* hemocytes, little is known of coagulation factors in hemocytes of the American horseshoe crab, *Limulus* (*L.*) *polyphemus*. In 1977, Liu and coworkers (*12, 13*) reported the existence of a proclotting enzyme in *L. polyphemus* hemocytes, and postulated that it is an initiation factor sensitive to LPS and closely associated with the limulus coagulation system. However, Nakamura and Levin (*14, 15*) examined *L. polyphemus* hemocytes and suggested that the lysate contains another LPS-sensitive factor differing from the known proclotting enzyme.

We attempted to shed light on these conflicting reports, and we purified and characterized a factor C-type zymogen from *L. polyphemus* hemocytes and compared its properties with those of the LPS-sensitive factor C from *T. tridentatus* hemocytes. We obtained evidence that the

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[2] To whom correspondence should be addressed.

Abbreviations: LPS, lipopolysaccharide; factor C̄, activated factor C; *T.*, *Tachypleus*; *L.*, *Limulus*; Boc, *N-tert*-butoxycarbonyl; NH-Np, *p*-nitroanilide; NH-Mec, 4-methylcoumaryl-7-amide; OBzl, O-benzyl; Z, benzyloxycarbonyl; Tris/HCl/SA buffer, 0.1 M Tris/HCl buffer, pH 8.0, containing 0.5 mg/ml of human serum albumin; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate; MUTMAC, 4-methylumbelliferyl *p*-(*N,N,N*-trimethylammonium)cinnamate.

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zymogen factor C sensitive to LPS does exist in *Limulus* hemocytes, and that the immunological and biochemical properties of the purified factor C are indistinguishable from those of factor C isolated from *Tachypleus* hemocytes. Moreover, this serine protease zymogen is unique in that it is activated by α-chymotrypsin, not by trypsin.

RESULTS

Characterization of L. polyphemus Factor C—The purity of the final preparation of *L.*-factor C was examined by SDS-PAGE. The purified zymogen factor C gave a single band of 123 kDa under nonreduced conditions, and two bands corresponding to an 80 kDa heavy chain and a 43 kDa light chain after reduction with β-mercaptoethanol, as shown in Supplementary Fig. 4. After activation with LPS, the zymogen factor C was converted to its active form, *i.e.*, factor $\bar{C}$, consisting of three chains of 80, 34, and 8 kDa, respectively, in the same manner as in the case of *T.*-factor C (data not shown). Western blotting performed with rabbit antiserum raised against *T.*-factor $\bar{C}$ indicated that the zymogen *L.*-factor C reacts with anti-*T.*-factor $\bar{C}$ antiserum, three bands corresponding to 123, 80, and 43 kDa being detected under reduced conditions (Supplementary Fig. 4, lane 3). The faint 123 kDa band seems to represent a single chain factor C (7). On Ouchterlony analysis using anti-*T.*-factor $\bar{C}$ antiserum, both preparations, *i.e.*, *T.*- and *L.*-factor Cs, formed a fusion type precipitin line (Supplementary Fig. 5).

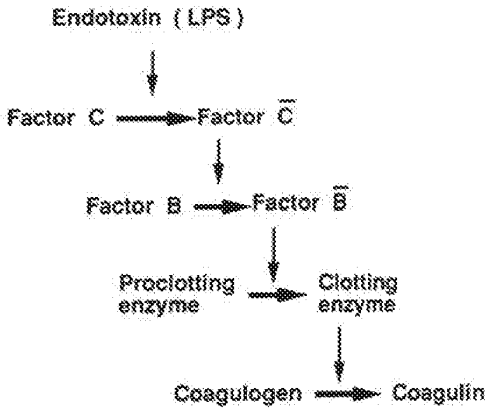


Fig. 1. **Putative mechanism for the coagulation cascade of the horseshoe crab hemocyte lysate.**

Activation of the zymogen *L.*-factor C by LPS and a synthetic *E. coli*-type lipid A analogue was examined by monitoring the generation of amidase activity of factor $\bar{C}$, using Boc-Val-Pro-Arg-NH-Np as a substrate. As shown in Supplementary Fig. 6, both LPS and the lipid A analogue activated the zymogen factor C, the activation reaction being completed within 30 min. Amidase activity did not appear to be generated in the absence of LPS.

Amino Acid Compositions and NH_2-Terminal Sequences of L.-Factor C and Its Chain Subunits—To compare the polypeptide chain structure of *L.*-factor C with that of *T.*-factor C, the purified zymogen factor C and its activated form were reduced and *S*-alkylated, and then the resulting chain subunits were separated by means of the high performance liquid chromatography used for the zymogen *T.*-factor C (6, 7). The materials thus obtained were subjected to amino acid and sequence analyses. Supplementary Table II shows the amino acid compositions of the intact *L.*-factor C and its chain subunits. *L.*-factor C was calculated to comprise a total of 1,011 residues per molecule, this number being much the same as that for *T.*-factor C (6). Furthermore, the individual amino acid compositions of the two proteins and of their chain subunits were indistinguishable. Like *T.*-factor C (6), *L.*-factor C also contained hexosamines, which were present in the acid hydrolyzate.

Supplementary Fig. 7 shows the partial NH_2-terminal

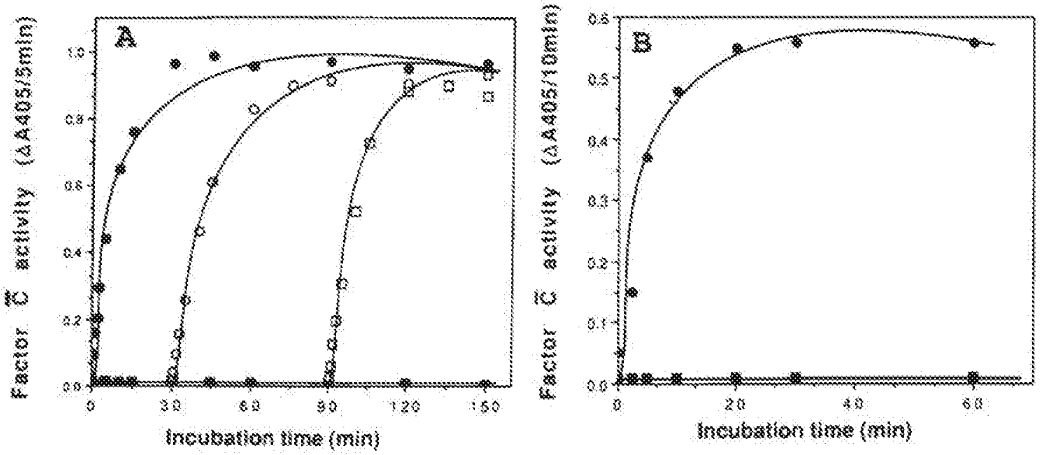


Fig. 2. **α-Chymotrypsin-mediated activation of factor C.** A: The purified *T.*-factor C was incubated, respectively, with α-chymotrypsin (●) and trypsin (■) at an enzyme to substrate weight ratio of 1 : 200. At the indicated times, a sample of the reaction mixture was withdrawn and factor $\bar{C}$ activity was measured as described under "MATERIALS AND METHODS." In other experiments, the reaction mixture containing factor C and trypsin was preincubated, respectively, for 30 (○) and 90 min (□), and then α-chymotrypsin was added after abolition of the trypsin activity with *N*-tosyl-L-lysyl chloromethyl ketone, followed by further incubation, was allowed to continue. The factor $\bar{C}$ activity generated was measured at the indicated times. B: The purified *L.*-factor C (95.6 μg/ml) was treated in the same manner as in A.

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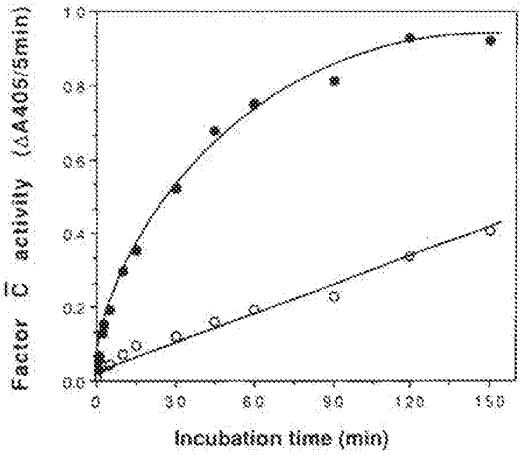


Fig. 3. **Chymase-mediated activation of factor C.** *T.*-factor C was incubated with chymase at enzyme to substrate weight ratios of 1 : 50 (●) and 1 : 200 (○), and then the factor C activity generated was measured as described under "MATERIALS AND METHODS."

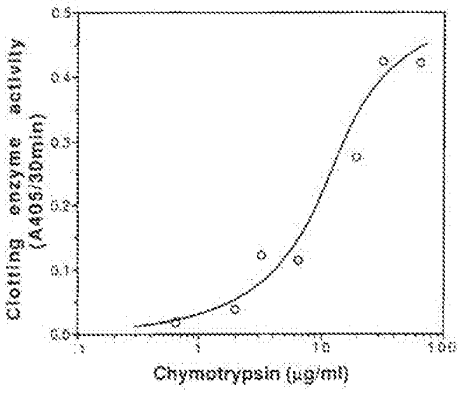


Fig. 4. **Activation of the Limulus coagulation system by α-chymotrypsin.** The toxicolor® reagent containing the hemocyte lysate of *T. tridentatus* was incubated with α-chymotrypsin for 30 min at 37°C and the clotting enzyme activity generated was measured using Boc-Leu-Gly-Arg-NH-Np as substrate. For details, see "MATERIALS AND METHODS."

TABLE I. **Kinetic parameters of *T.*-factor C.** The active site-titrated factor C̄ (2.7 nM) was used for kinetic analyses. Kinetic parameters were determined by direct linear plotting. The concentration of *p*-nitroaniline released was estimated by assuming $A^{1\%}_{405nm}$ = 10,000.

Substrates	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
P4 P3 P2 P1			
Boc- Val -Pro-Arg-NH-Np	391	54	7,240,000
Boc-Asp(OBzl) -Pro-Arg-NH-Mec	75	26	2,880,000
Boc- Gln -Pro-Arg-NH-Mec	34	66	514,000
Boc-Asp(OBzl) -Ala-Arg-NH-Mec	18	39	469,000
Boc- Val -Pro-Arg-NH-Mec	48	114	418,000
Boc- Ala -Pro-Arg-NH-Mec	72	190	379,000
Boc- Met -Ala-Arg-NH-Mec	27	88	212,000
Boc- Lys(Z) -Gly-Arg-NH-Mec	10	45	227,000
Boc- Gly -Ala-Arg-NH-Mec	29	155	190,000
Boc-Ser(OBzl) -Ala-Arg-NH-Mec	26	152	172,000
Boc- Lys -Gly-Arg-NH-Mec	19	126	149,000
Boc-Glu(OBzl) -Gly-Arg-NH-Mec	34	275	122,000
Boc- Val -Gly-Arg-NH-Mec	26	250	104,000
Boc- Ile -Gly-Arg-NH-Mec	15	189	80,000
Boc- Asp -Pro-Arg-NH-Mec	17	221	76,000
Boc- Gly -Pro-Arg-NH-Mec	8.5	167	51,000

sequences of the heavy, light and B chains isolated from *L.*-factor C and C̄, in comparison with those of *T.*-factor C. These sequences showed a close resemblance, thus the gross structure of *L.*-factor C must resemble that of known *T.*-factor C, as shown in Supplementary Fig. 8. These results indicate that the zymogen factor C's isolated from hemocytes of American and Japanese horseshoe crabs, respectively, are identical protein entities, both immunologically and biochemically. Thus, the following kinetic studies on zymogen activation and substrate specificities were performed mainly using the purified *T.*-factor C, since it was more readily available.

Time Course for Activation of the Zymogen Factor C with α-Chymotrypsin—Figure 2A shows the time course for activation of *T.*-factor C in the presence of trypsin and α-chymotrypsin. The factor C was rapidly activated at 37°C in the presence of α-chymotrypsin. However, trypsin did not activate factor C at an enzyme to substrate weight ratio of 1 : 200 or even of 1 : 20 (w/w) (data not shown). Such α-chymotrypsin-mediated activation of the *T.*-factor C was also observed for that of the purified *L.*-factor C (Fig. 2B). To determine whether or not trypsin would destroy the zymogen factor C, it was incubated with trypsin at 37°C for 30 and 90 min, and then 20 μg of *N*-tosyl-L-lysyl chloromethyl ketone was added to abolish the trypsin activity. α-Chymotrypsin, at 1 : 200 (w/w), was then added to the reaction mixture. As shown by open circles and squares in Fig. 2A, the zymogen factor C was activated by α-chymotrypsin in the same manner as with α-chymotrypsin alone, confirming again that it is not activated by trypsin. The activation of the factor C by α-chymotrypsin was also examined by SDS-PAGE under reduced conditions (Supplementary Fig. 9). After 30 and 60 min incubation, the factor C-light chain (43 kDa) was converted to a 34-kDa B chain. The factor C-heavy chain was also partially cleaved, because some bands appeared between those of the heavy and B chains upon α-chymotrypsin treatment. As rat mast cell chymase shows a similar specificity to α-chymotrypsin (*17*), we examined the chymase-mediated activation of factor C. As shown in Fig. 3, the zymogen factor C was activated by chymase at enzyme to substrate weight ratios of 1 : 200 and 1 : 50, and a plateau was reached as the same extent as in the case of α-chymotrypsin. The K_m and k_{cat} values of α-chymotrypsin toward the zymogen *T.*-factor C were estimated to be 7.4×10^{-6} M and 5.6×10^{-7} $M^{-1}\cdot s^{-1}$, respectively (data not shown). Other proteases, such as porcine elastase, lysyl endopeptidase from *Achromobacter lyticus* M497-1, *Staphylococcus aureus* V8 protease, subtilisin, and papain, did not activate the zymogen factor C (data not shown).

Activation of the Horseshoe Crab Coagulation System by α-Chymotrypsin—Figure 4 shows the α-chymotrypsin-mediated activation of horseshoe crab coagulation with the hemocyte lysate. As expected, the amidase activity generated due to the activation of proclotting enzyme (*4*) increased in a dose-dependent manner. When α-chymo-

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trypsin treated with Z-Gly-Gly-Phe-chloromethyl ketone for 15 min at 37°C or the enzyme treated at 70°C for 1 h was used, the amidase activity generated in the hemocyte lysate decreased to 73.4 and 89.5%, respectively (data not shown). These results indicate that activation of the coagulation system was not induced by LPS contaminating the α-chymotrypsin sample, but rather that it was catalyzed by α-chymotrypsin.

Substrate Specificity of Factor C̄—Since the purified factor C was activated autocatalytically in the presence of LPS through the cleavage of a Phe-Ile linkage located in the NH_2-terminal portion of the light chain (*7*), the possibility that factor C itself might have intrinsic α-chymotrypsin-like activity would have to be considered. We examined in detail the substrate specificity of factor C̄ toward various synthetic peptidyl substrates, including substrates for α-chymotrypsin. The concentration of factor C̄ was first determined by active-site titration with 4-methylumbelliferyl *p*-guanidinobenzoate (MUGB), which is used as a titrant for trypsin-type proteases. However, the rate of deacylation of the reagent was too rapid to estimate the enzyme concentration, as shown in Supplementary Fig. 10A. Therefore, 4-methylumbelliferyl *p*-(*N,N,N*-trimethylammonium)cinnamate (MUTMAC), synthesized as a suitable titrant for α-chymotrypsin (*18*), was used for the titration of factor C̄. MUTMAC proved to be an appropriate titrant for factor C̄, as deduced from the burst and stable saturation curve for the titration, as shown in Supplementary Fig. 10B. As this means that factor C probably has an intrinsic chymotrypsin-like activity, this titrant was used to determine the concentration of active factor C̄.

Table I shows the kinetic constants of factor C̄ toward various peptidyl substrates. The most effective substrate for factor C̄ was Boc-Val-Pro-Arg-NH-Np. It has a high k_{cat}/K_m value, 7,240,000, which is 17-fold higher than that of Boc-Val-Pro-Arg-NH-Mec. Among 75 peptidyl-NH-Mec substrates (*14*), Boc-Asp(OBzl)-Pro-Arg-NH-Mec was also a good substrate factor C̄, thereby indicating that the enzyme effectively cleaves substrates with a Pro residue at the P2 site. The NH-Mec substrates for α-chymotrypsin, such as succinyl-Ala-Ala-Pro-Phe-NH-Mec and succinyl-Leu-Leu-Val-Tyr-NH-Mec, were not hydrolyzed by factor C̄ (data not shown).

Inhibition of Factor C̄ by Natural and Synthetic Inhibitors—To further elucidate the enzymatic properties of factor C, the inhibitory effects of some natural or synthetic inhibitors on factor C̄ activity were examined. Natural inhibitors, such as antithrombin III–heparin complex, and synthetic inhibitors, such as diisopropylfluorophosphate, benzamidine, and leupeptin, are known to inhibit factor C̄ activity (*6*). Other natural inhibitors tested were aprotinin, soybean trypsin inhibitor, potato II inhibitor, C1 inhibitor, corn inhibitor, *Streptomyces* subtilisin inhibitor, hirudin, ovomucoid, and ovoinhibitor. However, these inhibitors had little effect on the factor C̄ activity, even in the presence of an excess molar ratio to the enzyme. Among the synthetic inhibitors, *N*-tosyl-L-lysyl chloromethyl ketone, chymostatin, Z-Gly-Gly-Phe-chloromethyl ketone, and H-Ala-Ala-Phe-chloromethyl ketone also did not inhibit factor C̄ activity, at a final concentration of 1 mM. On the other hand, D-Phe-Pro-Arg-chloromethyl ketone, an α-thrombin inhibitor, had a potent inhibitor effect on the factor C̄ activity. The inhibition constant of this inhibitor towards factor C̄ was determined by the method of Walker *et al.* (*19*), and the K_i value was estimated to be 7.4×10^{-9} M and k_3 to be 0.69 min^{-1} (data not shown).

DISCUSSION

A factor C, which is indistinguishable from those of the known *T.*-factor C in terms of immunological and biochemical properties, was proved to be present in the hemocytes of *L. polyphemus*. Both factor C's contain two molecular species with, *i.e.*, single chain and a two-chain forms. Both molecules have the same molecular mass of 123,000, but the latter consists of a heavy chain of $M_r = 80{,}000$ and a light chain of $M_r = 43{,}000$ held together by a disulfide linkage(s). The major nicked form may be produced by a trace amount of contaminating of LPS during the purification procedures, as experienced in the study on *T.*-factor C (*6*). The structural properties of the *L.*-factor C molecule, including the entire amino acid composition and the partial NH_2-terminal sequences of their chain subunits, show similarities with those of *T.*-factor C (*6, 7*). They also exhibit common antigenicity, as revealed with the immunoblotting method and the Ouchterlony test (Supplementary Figs. 4 and 5). Like the zymogen *T.*-factor C, the purified *L.*-factor C is rapidly activated in the presence of *E. coli* LPS and synthetic lipid A analogue. These observations strongly indicate that both factor C's are the same protease zymogen and function as an initiator of the coagulation cascade in the horseshoe crab hemocytes. These findings also lead to the idea that the hemocytes of *L. polyphemus* probably contain a coagulation system similar to that established for the *T. tridentatus* hemocytes (*5–7*).

The present results are the first evidence that both the purified *L.*- and *T.*-factor C's are activated by α-chymotrypsin and not by trypsin. All the serine protease zymogens associated with mammalian coagulation, fibrinolysis, and complement systems are activated by trypsin-like enzymes. Thus, the horseshoe crab factor C seems to be a novel type of serine protease zymogen susceptible to α-chymotrypsin. The necessity of cleavege of the Phe-Ile bond (*7*) implies that the zymogen factor C cannot be activated by the horseshoe crab trypsin-like proteases (*4, 5*). Thus, positive feedback activation of the clotting system, which occurs in common in the mammalian coagulation cascade reaction, probably does not occur in horseshoe crabs, only the LPS-associated factor C appearing to be the initiator of this cascade reaction.

For the LPS-mediated activation of the zymogen factor C, it is now clear that no other protease is required for its conversion to its active form, factor C̄, and only LPS-bound factor C is activated, probably through an intermolecular interaction between the two molecules (*8*), as is the case with activation of the complement C1r (*20*). The substrate specificity of factor C̄ shows a close similarity to that of α-thrombin. As shown in Table I, the most reactive substrate for factor C̄ is Boc-Val-Pro-Arg-NH-Np, with an extremely high $k_{cat}/K_m = 7{,}240{,}000$. This value is comparable to that of the sensitive fluorogenic substrate, Boc-Asp(OBzl)-Pro-Arg-NH-Mec, for bovine α-thrombin (*14*). Thus, apolar and non-aromatic residues such as Pro and Ala at the P2 site are the most favorable, as for α-thrombin (*16*). For the P3 site, bulky groups such as Val, Asp(OBzl), and Gln probably serve, as judged from their K_m values

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(Table I). Such a structural basis for a peptide substrate for factor C is also supported by the observation that D-Phe-Pro-Arg-chloromethyl ketone, which is sensitive to α-thrombin strongly inhibits factor C activity, the K_i value being 7.4×10^{-8} M. The entire cDNA sequence and deduced amino acid sequence of *T.*-factor C have been determined (manuscript submitted). Then, the factor C-derived B chain corresponding to a serine protease domain of this zymogen is the most homologous with human α-thrombin (38.5% identity) among various known mammalian serine proteases. Presumably, the serine protease domain of *T.*-factor C is closely related with α-thrombin, in terms of molecular structure and substrate specificity.

Although the active factor C shows the Arg-Xaa specificity toward synthetic peptide substrates, cleavage of the Phe-Ile bond by LPS-bound factor C is not unreasonable, because the residue preceding Phe is Pro (*7*). The reactive site of α_1-antitrypsin has been identified as Met-Ser with Met proceded by Pro (*21*). Moreover, the reactive site of heparin cofactor II is a Leu-Ser bond that is cleaved by α-thrombin. The rapid hydrolysis of MUTMAC by the LPS-bound factor C indicates that it should have intrinsic chymotrypsin-like activity. It seems likely that structural properties of horseshoe crab factor C beyond the specific cleavage site residues play an important role in the LPS-mediated activation of this zymogen. Further detailed studies on the molecular interaction of LPS and factor C are underway.

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Supplemental Materials

MATERIALS AND METHODS

Materials ..... American horseshoe crabs (Limulus polyphemus) were obtained from Taiyo Fishery Co., Ltd., Tokyo. T. tridentatus factor C (two chain form) from hemocytes of Japanese horseshoe crabs was highly purified, as described (6). The active enzyme, factor C, was prepared from the isolated factor C by treatment with LPS (6). The LPS (Escherichia coli 0111:B4), obtained from List Biochemical Laboratories, Inc., FA, was heated at 70 °C for 1 h before use. Synthetic E. coli type lipid A analog, LA-15-PP, and aprotinin were products of Daiichi Pharm., Co., Ltd., Tokyo, and Mochida Pharma., Co., Ltd., Tokyo, respectively. Bovine pancreatic α-chymotrypsin treated with N-tosyl-L-lysyl chloromethyl ketone and trypsin treated with N-tosyl-L-phenylalanyl chloromethyl ketone were obtained from Worthington Biochemical Corp., Freehold, NJ. Boc-Val-Pro-Arg-NH-Np and an endotoxin detection kit, Toxicolor® LS-1 set, which contains the T. tridentatus hemocyte lysate, were generous gifts from S. Tanaka, Seikagaku Kogyo Co., Ltd., Tokyo. Rat mast cell chymotrypsin-like enzyme, chymase (RMCP I), was kindly provided by Dr. H. Matsumoto, Institute of Enzyme Research, The University of Tokushima. Fluorogenic tripeptide substrates (14) were obtained from Peptide Institute Inc., Osaka. 4-Methylumbelliferyl p-guanidinobenzoate (MUGB); 4-methylumbelliferyl p-(N,N,N-trimethylammonium) cinnamate (MUTMAC); and soybean trypsin inhibitor were purchased from Sigma Chemical, Co., St. Louis, MO. N-Ala-Ala-Phe-chloromethyl ketone and N-Gly-Gly-Phe-chloromethyl ketone were from Bachem, Bubendorf, Switzerland, and N-Phe-Pro-Arg-chloromethyl ketone was from Calbiochem, La Jolla, CA. Sepharose CL-6B and CM-Sepharose CL-6B were purchased from Pharmacia LKB Biotechnology, Uppsala. All other chemicals were of the highest quality commercially available.

Factor C assay ..... The amidase activity of factor C generated after activation with LPS was measured using Boc-Val-Pro-Arg-NH-Np as a substrate, as described (6). The reaction mixture, comprising 10-20 µl of sample in Tris/HCl/BA buffer in a total volume of 200 µl, was preincubated at 37 °C for 30 min in the presence or absence of 4 µl of LPS (10 µg/ml). Then, 50 µl of 2 mM Boc-Val-Pro-Arg-NH-Np was added and the mixture was incubated further for 10 min. Finally, 0.8 ml of 0.6 M acetic acid was added to terminate the reaction and the absorbance at 405 nm was read. One unit of enzyme activity was defined as 1 µmol of p-nitroaniline liberated per min. The amidase activity of factor C (active form) was measured as described for factor C in the absence of LPS.

SDS-polyacrylamide gel electrophoresis and Western blotting ..... SDS-PAGE was performed according to the method of Laemmli (22). Western blotting was performed using rabbit antiserum raised against T.-factor C, as described (7). For Ouchterlony analysis, a 1 % agarose gel dissolved in 20 mM Tris-HCl, pH 8.0, and containing 0.15 M NaCl was used.

Reduction and S-alkylation ..... The sample was reduced and S-pyridylethylated and each chain was separated by reversed phase liquid chromatography on a TSK phenyl 5PW(RP column (4.6 x 75 mm), as described (7).

Amino acid and sequence analyses ..... Samples were hydrolyzed in 5.7 N HCl at 110 °C for 24, 48 and 72 h or 3 N mercaptoethanesulfonic acid for 24 h in evacuated and sealed tubes. After evaporation, the hydrolysates were analyzed using a Hitachi 835 automatic analyzer. Amino acid sequence determination was carried out with a gas-phase sequencer (Applied Biosystems 477A) and a 120A-phenylthiohydantoin analyzer on-line system (7).

Activation of factor C by trypsin, α-chymotrypsin and chymase ..... Factor C (206 µg/ml) was incubated at 37 °C with 1/200 (w/w) of trypsin, α-chymotrypsin or chymase, in a total volume of 150 µl of 0.1 M Tris/HCl buffer, pH 8.0. At the indicated times, a 10 µl sample was taken and mixed with 50 µg/ml of aprotinin for α-chymotrypsin and trypsin, or 50 µg/ml of soybean trypsin inhibitor for chymase. These protease inhibitors were dissolved in a total 200 µl of Tris/HCl/BA buffer. Each mixture was incubated for a further 5 min to inhibit the protease activity and finally the factor C activity generated was measured.

Activation of the horseshoe crab coagulation system by α-chymotrypsin ..... α-Chymotrypsin-mediated activation of horseshoe crab coagulation system was measured using Toxicolor® reagent, LS-1 set, obtained from Seikagaku Kogyo, Ltd., Tokyo, according to the manufacturer's manual. The LS-1 set contained Tachypleus hemocyte lysate and Boc-Leu-Gly-Arg-NH-Np, the latter of which is an appropriate substrate for the clotting enzyme generated during activation of the coagulation system. The LS-1 set (100 µl) and various concentrations of α-chymotrypsin dissolved in 100 µl of 20 mM Tris/HCl buffer, pH 8.0, containing 0.15 M NaCl, were mixed and incubated for 30 min at 37 °C, and then 0.8 ml of 0.6 M acetic acid was added and the absorbance at 405 nm was read.

Kinetic analysis ..... The factor C activity was measured at 37 °C in 50 mM Tris/HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.1 mg/ml of bovine serum albumin (BSA), with various fluorogenic peptide substrates. The initial rate of hydrolysis was measured with a Hitachi 650 fluorometer with excitation at 380 nm and emission at 440 nm at 37 °C, as described in detail elsewhere (14). Substrate concentrations of 1 µM to 2 mM and 2.7 nM factor C were used. Kinetic parameters were determined by direct linear plotting (23). The concentration of active factor C was determined with MUTMAC from the initial burst of 4-methylumbelliferone in 0.1 M sodium barbiturate buffer, pH 8.3, containing 0.1 mg/ml of BSA at 25 °C (18). Kinetics analysis for irreversible inhibitors, such as N-Phe-Pro-Arg-chloromethyl ketone, was performed by the method of Walker et al. (15).

Inhibitory effects of natural and synthetic inhibitors on factor C ..... The screening of various inhibitors was performed with an inhibitor concentration of 0.2 mM and a factor C concentration of 10 nM in Tris/HCl/BA buffer. Each mixture was incubated at 37 °C for 10 min, in a total volume of 200 µl, and then the remaining factor C activity was measured.

RESULTS

Purification of L. polyphemus factor C ..... Purification of L.-factor C was carried out under sterilized conditions according to the method of Nakamura et al. (6). The lysate (595 ml) was first applied to a Dextran-sulfate Sepharose CL-6B column (7.6 x 24.0 cm). Stepwise elution was performed with NaCl concentrations of from 50 mM, 0.25 M, 0.5 M and 2 M, as shown in Supplementary Fig. 1. Factor C in the 0.5 M NaCl fractions was collected, pooled and concentrated by Diaflo ultrafiltration. The concentrated fraction was then applied to a Sepharose CL-6B column (2.6 x 141 cm), equilibrated with 20 mM acetate buffer, pH 5.6, containing 0.1 M NaCl (Supplementary Fig. 2). The pooled factor C fraction was finally applied to a CM-Sepharose CL-6B column (2.6 x 24.0 cm), equilibrated with the same buffer as used in Supplementary Fig. 2 and eluted with a linear gradient of NaCl (0.1-0.35 M) in 20 mM acetate buffer, pH 5.6 (Supplementary Fig. 3). A summary of the purification of factor C is shown in Supplementary Table 1. The yield of purified factor C was 5.3 mg from 80 g hemocytes (wet weight), 18.7-fold purification from the Dextran-sulfate Sepharose CL-6B fraction (Supplementary Fig. 1) being achieved (6).

Supplementary Table I

Summary of the purification procedure for L. polyphemus factor C.

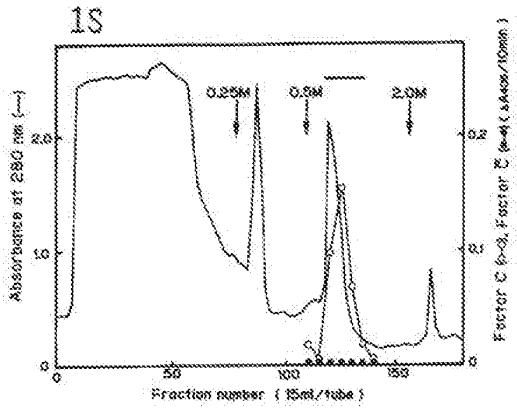
Step	Volume	Total protein	Total activity	Specific activity	Purification	Yield
	ml	mg	units	units/mg	-fold	%
Hemocyte lysate	298	—	—	—	—	—
Dextran-sulfate-Sepharose CL-6B	138	148	397	2.8	1	100
Sepharose CL-6B	90	17.3	421	24.3	8.7	106
CM-Sepharose CL-6B	138	5.2	214	41.2	14.7	54

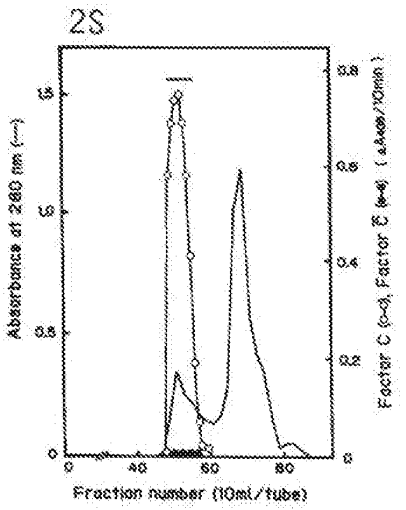
Supplementary Table II

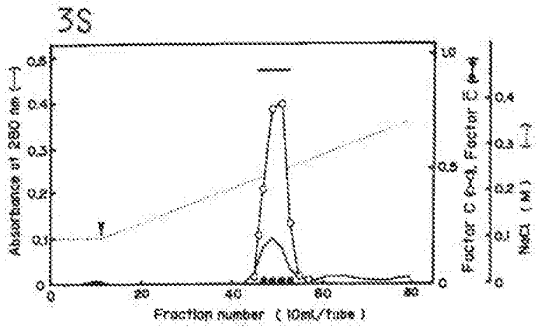
Amino acid compositions of L. polyphemus factor C and its S-alkylated derivatives[a].

Amino acid	Factor C			Factor C	
	Whole	H chain	L chain	A chain	B chain
	Residues/molecule				
Asp	101.0 (101)	69.4 (67)	39.4 (34)	4.8 (5)	34.6 (29)
Thr	53.6 (59)	36.7 (37)	20.5 (22)	2.2 (2)	18.7 (20)
Ser	88.0 (82)	59.7 (55)	31.5 (27)	11.2 (13)	25.1 (14)
Glu	92.1 (89)	60.7 (58)	34.1 (31)	5.8 (5)	30.8 (26)
Pro	60.8 (69)	47.4 (52)	23.7 (17)	6.9 (6)	20.1 (11)
Gly	89.9 (88)	55.4 (57)	32.6 (29)	7.7 (7)	27.6 (22)
Ala	57.4 (56)	34.7 (35)	22.1 (21)	3.0 (3)	18.5 (18)
Val	61.3 (64)	44.7 (42)	23.8 (22)	3.9 (4)	20.4 (18)
Met	9.3 (10)	6.3 (7)	3.5 (3)	— (0)	3.2 (3)
Ile	39.5 (41)	22.9 (23)	16.5 (18)	2.2 (2)	13.3 (16)
Leu	69.0 (71)	45.6 (47)	25.4 (24)	3.9 (4)	22.2 (20)
Tyr	36.3 (37)	22.1 (22)	14.6 (15)	4.2 (4)	11.4 (11)
Phe	28.4 (28)	20.4 (20)	12.3 (8)	1.9 (1)	14.6 (7)
Lys	54.7 (50)	37.8 (38)	15.9 (12)	3.4 (2)	15.0 (10)
His	17.7 (17)	13.1 (12)	5.3 (5)	1.1 (1)	5.1 (4)
Trp	16.9 (23)	11.9 (12)	8.9 (11)	1.6 (1)	8.0 (10)
Pe-Cys	n.d.[b] (56)	37.5 (45)	13.5 (11)	3.4 (4)	12.3 (7)
Arg	48.4 (53)	25.6 (34)	15.8 (19)	6.8 (8)	11.8 (11)
Total	920.4 (994)	651.7 (865)	338.9 (329)	74.0 (72)	312.5 (257)
$GlcNH_2$	+	+	+	-	+
$GalNH_2$	+	+	+	-	+

a: Numbers in parentheses are taken from the sequence data for T.-factor C (manuscript summitted).
b: not determined.





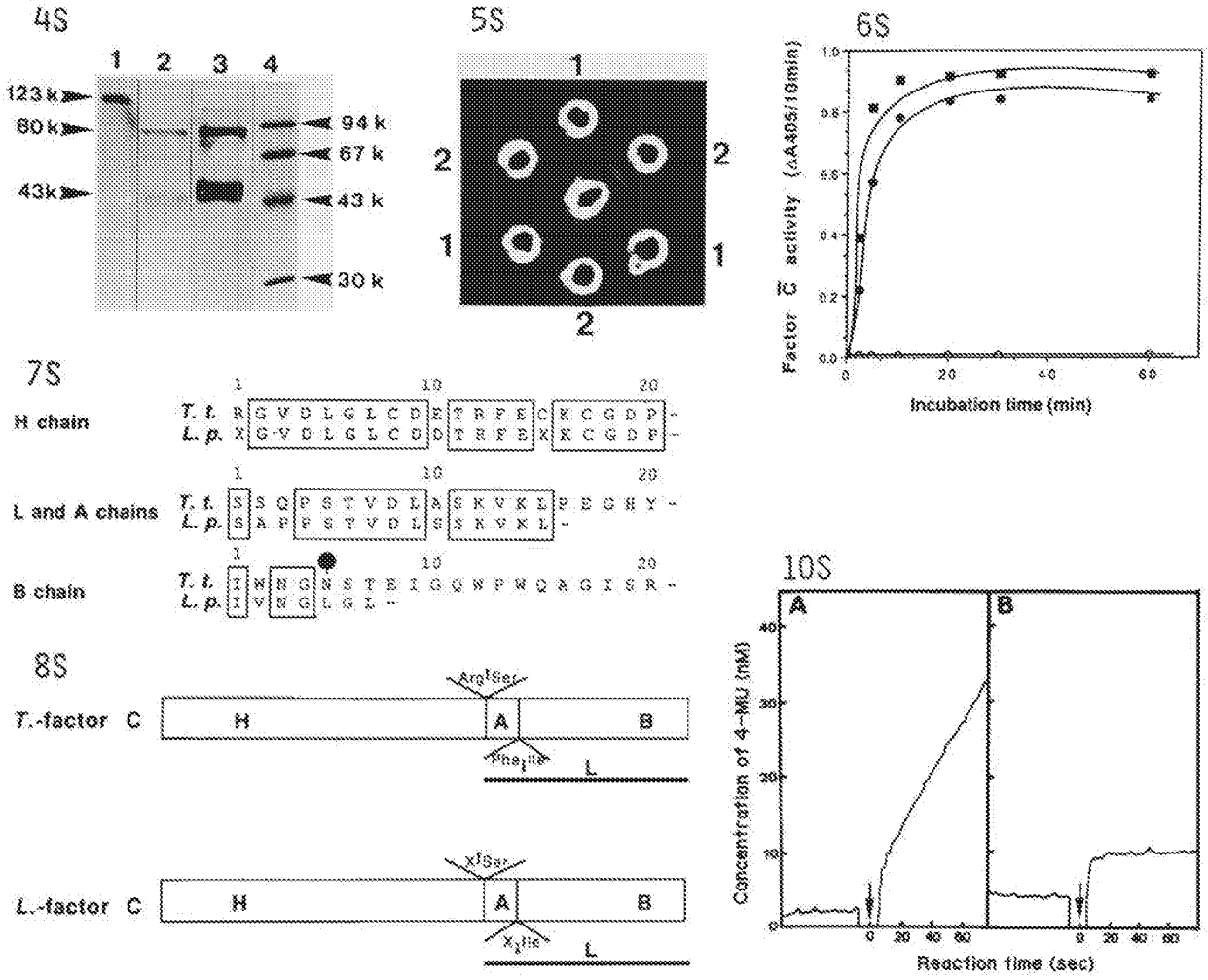


Supplementary Fig. 1. Dextran-sulfate Sepharose CL-6B chromatography of the L. polyphemus hemocyte lysate. Details of the procedure are given under Methods. The fractions indicated by a solid bar were pooled and concentrated. The activities of factor C (○) and its active form, factor C (●), were assayed, using Boc-Val-Pro-Arg-NH-Np as a substrate. The absorbance at 280 nm (——) was measured.

Supplementary Fig. 2. Sepharose CL-6B gel filtration of the L.-factor C fraction obtained from a dextran-sulfate Sepharose CL-6B column. The zymogen factor C (○) and the activity of factor C (●) were assayed, using Boc-Val-Pro-Arg-NH-Np as a substrate. The absorbance at 280 nm (——) was measured.

Supplementary Fig. 3. CM-Sepharose CL-6B chromatography of the L.-factor C fraction obtained from a Sepharose CL-6B column. The zymogen factor C (○) and the activity of factor C (●) were assayed, using Boc-Val-Pro-Arg-NH-Np as a substrate. The absorbance at 280 nm (——) was measured and gradient elution (- - -) with NaCl was started at the position indicated by the arrowhead.

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Supplementary Fig. 4. SDS-PAGE and Western blotting of the purified L.-factor C. Ten µg of L.-factor C was subjected to SDS-PAGE in the absence (lane 1) and presence (lane 2) of 2-mercaptoethanol. Western blotting (lane 3) of the reduced L.-factor C was also performed using rabbit anti-T.-factor C serum as the 1st antibody, and the positions of co-electrophoresed standards of known molecular weight (lane 4) are indicated.

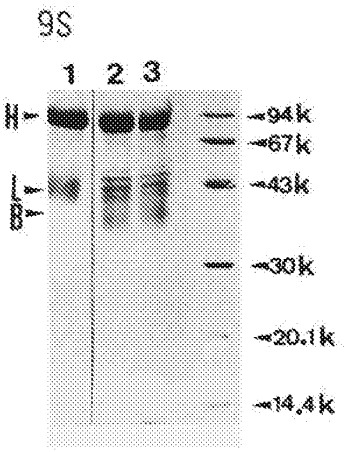
Supplementary Fig. 5. Immunodiffusion of the purified L.-factor C (1) and T.-factor C (2) against rabbit anti-T.-factor C antiserum (center well).

Supplementary Fig. 6. Time course of activation of the L.-factor C in the presence of LPS and a lipid A analogue. The purified L.-factor C (105.6 µg/ml) was incubated at 37°C in the presence (■) and absence (○) of LPS (10 µg/ml). E. coli type-synthetic lipid A analogue, (A-18-PP ; ● , 10 µg/ml), was also used. At the indicated times, 20 µl aliquots were withdrawn and assayed for factor C activity.

Supplementary Fig. 7. Comparison of the NH$_2$-terminal sequences of the heavy, light and B chains derived from each of L.-factor C and T.-factor C. Residues identical in the two factor C's are boxed. The solid circle indicates the carbohydrate attachment site (7).

Supplementary Fig. 8. Cross structure of T.- and L.- factor C's. H, A, B and L denote the heavy, A, B and light chains of factor C. The arrowheads and solid bars indicate the cleavage sites and the locations of the light chains of the factor C's, respectively.

Supplementary Fig. 9. SDS-PAGE of T.-factor C activated by α-chymotrypsin. Samples of the zymogen factor C (lane 1) and the factor C treated with α-chymotrypsin (weight ratio of enzyme/substrate = 1/200) for 30 min (lane 2) and 60 min (lane 3) were subjected to SDS-PAGE. H, L and B denote the heavy, light and B chains of the factor C, respectively.



Supplementary Fig. 10. Active site titration of T.-factor C. A, 4-methylumbelliferyl p-guanidinobenzoate (MUGB) and B, 4-methylumbelliferyl p-(N,N,N-trimethylammonium) cinnamate (MUTMAC) were used as titrants, being added, respectively, to the reaction mixture containing T.-factor C at the times indicated by arrows after preincubation for 60 sec.

Electronic Acknowledgement Receipt

EFS ID:	40586079
Application Number:	16399386
International Application Number:	
Confirmation Number:	9293
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Customer Number:	23373
Filer:	Hui Chen Wauters/Sharon Jones
Filer Authorized By:	Hui Chen Wauters
Attorney Docket Number:	Q246419
Receipt Date:	17-SEP-2020
Filing Date:	30-APR-2019
Time Stamp:	12:57:48
Application Type:	Utility under 35 USC 111(a)

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Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		Q246419Amendment111.pdf	183944 c4e7d09a9922fa6e338640e47f1a120ff7eb776c	yes	14

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	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Specification	2	2
	Claims	3	7
	Applicant Arguments/Remarks Made in an Amendment	8	14

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	Information Disclosure Statement (IDS) Form (SB08)	3	4

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3	Non Patent Literature	Q246419NPLREFFinalOfficeAction08042020.pdf	6680772 c91e5b604ac40a19677e479d33129f1b00afa871	no	34
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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.

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Doc Code: DIST.E.FILE **Document Description: Electronic Terminal Disclaimer - Filed**	PTO/SB/25 PTO/SB/26 U.S. Patent and Trademark Office Department of Commerce

Electronic Petition Request	**TERMINAL DISCLAIMER TO OBVIATE A PROVISIONAL DOUBLE PATENTING REJECTION OVER A PENDING "REFERENCE" APPLICATION AND TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT**
Application Number	16399386
Filing Date	30-Apr-2019
First Named Inventor	Hikaru MIZUMURA
Attorney Docket Number	Q246419
Title of Invention	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

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Owner	Percent Interest
SEIKAGAKU CORPORATION	100 %

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15983725 filed on 05/18/2018

16331810 filed on 03/08/2019

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10144923

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○ I certify, in accordance with 37 CFR 1.4(d)(4), that the terminal disclaimer fee under 37 CFR 1.20(d) required for this terminal disclaimer has already been paid in the above-identified application.

Applicants claims the following fee status:

○ Small Entity

○ Micro Entity

◉ Regular Undiscounted

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THIS PORTION MUST BE COMPLETED BY THE SIGNATORY OR SIGNATORIES

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 is of record in this application

Registration Number 57426

○ A sole inventor

○ A joint inventor; I certify that I am authorized to sign this submission on behalf of all of the inventors as evidenced by the power of attorney in the application

○ A joint inventor; all of whom are signing this request

Signature	/Hui C. Wauters/

Name	Hui C. Wauters

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).
Form PTO/SB/96 may be used for making this certification. See MPEP § 324.

Electronic Patent Application Fee Transmittal

Application Number:	16399386
Filing Date:	30-Apr-2019
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Filer:	Hui Chen Wauters/Sharon Jones
Attorney Docket Number:	Q246419

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
STATUTORY OR TERMINAL DISCLAIMER	1814	1	160	160
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**160**

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Document Description: Electronic Terminal Disclaimer – Approved

Application No.: 16399386

Filing Date: 30-Apr-2019

Applicant/Patent under Reexamination: MIZUMURA

Electronic Terminal Disclaimer filed on September 17, 2020

☒ APPROVED

This patent is subject to a terminal disclaimer

☐ DISAPPROVED

Approved/Disapproved by: Electronic Terminal Disclaimer automatically approved by EFS-Web

U.S. Patent and Trademark Office

Electronic Acknowledgement Receipt	
EFS ID:	40585872
Application Number:	16399386
International Application Number:	
Confirmation Number:	9293
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Customer Number:	23373
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Filer Authorized By:	Hui Chen Wauters
Attorney Docket Number:	Q246419
Receipt Date:	17-SEP-2020
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Payment Type	CARD
Payment was successfully received in RAM	$160
RAM confirmation Number	E20209GC48473249
Deposit Account	194880
Authorized User	Sharon Jones
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.21 (Miscellaneous fees and charges) 37 CFR 1.19 (Document supply fees)	

37 CFR 1.17 (Patent application and reexamination processing fees)

37 CFR 1.16 (National application filing, search, and examination fees)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Terminal Disclaimer-Filed (Electronic)	eTerminal-Disclaimer.pdf	37413 eb5e98e0df7284d0975a3a1a65353e0503db1400	no	3
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30837 c8cfea61929966bad7297426c480ff9f3e6a2457	no	2
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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.

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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.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/399,386	04/30/2019	Hikaru MIZUMURA	Q246419	9293

23373 7590 06/30/2020

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EXAMINER
STEADMAN, DAVID J

ART UNIT	PAPER NUMBER
1656	

NOTIFICATION DATE	DELIVERY MODE
06/30/2020	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):

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USPTO@sughrue.com
sughrue@sughrue.com

Office Action Summary	**Application No.** 16/399,386	**Applicant(s)** MIZUMURA et al.	
	Examiner DAVID J STEADMAN	**Art Unit** 1656	**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 17 June 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) 1-18 is/are pending in the application.

5a) Of the above claim(s) 14-18 is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-13 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 30 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. 14/650,767.

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 CORRESPONDENCE

Status of the Application

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Claims 1-18 are pending in the application.

Election/Restriction

Applicant's election without traverse of Group I, claims 1-13, in the reply filed on June 17, 2020 is acknowledged.

Claims 14-18 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to nonelected inventions, there being no allowable generic or linking claim. Election was made without traverse in the reply filed on June 17, 2020.

Priority

This application is a continuation application of U.S. non-provisional application no. 15/983,725, filed on May 18, 2018, which is a continuation application of U.S. non-provisional application no. 14/650,767, filed on June 9, 2015, now U.S. Patent 10,144,923, which is filed under 35 U.S.C. 371 as a national stage of international application PCT/JP2013/083082, filed on December 10, 2013, which claims foreign priority under 35 USC § 119(a)-(d) to Japanese application no. 2012-269840, filed on December 10, 2012. A certified copy of the foreign priority document has been filed in the prior-filed application no. 14/650,767 on June 9, 2015.

Information Disclosure Statement

The information disclosure statements (IDSs) submitted on April 30, 2019, March 5, 2020, and June 17, 2020 are in compliance with the provisions of 37 CFR 1.97. Accordingly, the IDSs are being considered by the examiner.

Specification/Informalities

The applicant is requested to update the status of prior-filed application no. 14/650,767 in the disclosure of the specification at p. 1 under "Cross-Reference to Related Applications". According to USPTO records, the application no. 14/650,767 has issued as U.S. Patent 10,144,923.

Claim Objections

Claims 1, 2, and 12 are objected to in the recitation of "HEK" and in the interest of improving claim form, it is suggested that the entire phrase for which the abbreviation is used, *i.e.,* "human embryonic kidney", be recited at least once in the claims.

Claim Rejections - 35 USC § 112(b)

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.

Claims 11 and 12 are rejected under 35 U.S.C. 112(b) as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor regards as the invention.

Claims 11 and 12 are indefinite in the recitation of "a corresponding native horseshoe crab Factor C protein and/or a corresponding horseshoe crab Factor C protein recombinantly expressed using Sf9 as a host cell" and "the horseshoe crab Factor C", respectively. Claim 2 (from which claims 11 and 12 depend) encompasses Factor C variants of SEQ ID NO: 2 or 4 that do not have a "corresponding native horseshoe crab Factor C protein" or "a corresponding horseshoe crab Factor C protein" (for claim 11) and encompasses variants that are not a horseshoe crab Factor C (for claim 12). In the interest of compact prosecution, it is suggested that the noted phrase of claim 11 be amended to recite, *e.g.,* "a corresponding Factor C protein recombinantly expressed using Sf9 as a host cell" and the noted phrase of claim 12 be amended to recite, *e.g.,* "the Factor C protein".

Claim Rejections - 35 USC § 112(a)

The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

Claims 2-9, 12, and 13 are rejected under 35 U.S.C. 112(a) as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, at the time the application was filed, had possession of the claimed invention.

MPEP 2163.II.A.2.(a).i) states, "Whether the specification shows that applicant was in possession of the claimed invention is not a single, simple determination, but rather is a factual determination reached by considering a number of factors. Factors to be considered in determining whether there is sufficient evidence of possession include the level of skill and knowledge in the art, partial structure, physical and/or chemical properties, functional characteristics alone or coupled with a known or disclosed correlation between structure and function, and the method of making the claimed invention".

For claims drawn to a genus, MPEP § 2163 states the written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice, reduction to drawings, or by disclosure of relevant, identifying characteristics, *i.e.,* structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus. See *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406.

MPEP § 2163 further states that "[s]atisfactory disclosure of a 'representative number' depends on whether one of skill in the art would recognize that the applicant was in possession of the necessary common attributes or features possessed by the members of the genus in view of the species disclosed. For inventions in an unpredictable art, adequate written description of a genus which embraces widely variant species cannot be achieved by disclosing only one species within the genus…Instead, the disclosure must adequately reflect the structural diversity of the

claimed genus, either through the disclosure of sufficient species that are 'representative of the full variety or scope of the genus,' or by the establishment of 'a reasonable structure-function correlation.' Such correlations may be established 'by the inventor as described in the specification,' or they may be 'known in the art at the time of the filing date.'"

The factors considered in the Written Description requirement are (1) level of skill and knowledge in the art, (2) partial structure, (3) physical and/or chemical properties, (4) functional characteristics alone or coupled with a known or disclosed correlation between structure and function, and the (5) method of making the claimed invention. Disclosure of any combination of such identifying characteristics that distinguish the claimed invention from other materials and would lead one of skill in the art to the conclusion that the applicant was in possession of the claimed species is sufficient." MPEP § 2163.

The claims are drawn to or recite a Factor C protein having activity of Factor C,

wherein the Factor C protein comprises an amino acid sequence selected from the group consisting of:

(A) the amino acid sequence of SEQ ID NO: 2;

(B) an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:2, and having activity of Factor C;

(C) the amino acid sequence of SEQ ID NO: 4; and

(D) an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:4, and having activity of Factor C,

wherein the Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or HEK cell, and

wherein the Factor C protein exhibits a residual activity of 10% or higher in the presence of 21 mM sodium citrate.

The specification discloses an actual reduction to practice of the following representative species of the genus of recited recombinant horseshoe crab Factor C proteins – a recombinant *Tachypleus tridentatus* or *Carcinoscorpius rotundicauda* Factor C protein expressed in Chinese Hamster Ovary (CHO) cells or human embryonic kidney (HEK) cells, which Factor C protein has Factor C activity and contains (α-2,3)-linked terminal sialic acid in a greater amount as compared with a corresponding native horseshoe crab Factor C protein and/or a corresponding horseshoe crab Factor C protein recombinantly expressed using Sf9 as a host cell. Other than these disclosed representative species, the specification fails to describe any other species of the recited genus of recombinant horseshoe crab Factor C polypeptides that exhibit a residual activity of 10% or higher in the presence of 21 mM sodium citrate. Regarding a residual activity of 10% or higher in the presence of 21 mM sodium citrate, the specification discloses the correlation between expressing a *Tachypleus tridentatus* or *Carcinoscorpius rotundicauda* Factor C protein in Chinese Hamster Ovary (CHO) cells or human embryonic kidney (HEK) cells, which results in the Factor C protein containing (α-2,3)-linked terminal sialic acid in a greater amount as compared with a corresponding native horseshoe crab Factor C protein and/or a corresponding horseshoe crab Factor C protein recombinantly expressed using Sf9 as a host cell, and the characteristic of having a residual activity of 10% or higher in the presence of 21 mM sodium citrate, and

the additional characteristics recited in claims 3-9. Although the claims recite being expressed in a Chinese Hamster Ovary (CHO) DG44 cell or HEK cell, the claims do not recite the feature of containing (α-2,3)-linked terminal sialic acid in a greater amount as compared with a corresponding native horseshoe crab Factor C protein and/or a corresponding horseshoe crab Factor C protein recombinantly expressed using Sf9 as a host cell. The recitation of "wherein the Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or HEK cell" is a product-by-process and given a broadest reasonable interpretation, the claims encompass additional production steps or post-translational modifications, *e.g.,* treatment of the Factor C polypeptide expressed in a CHO DG44 cell or HEK cell with a glycosidase to remove (α-2,3)-linked sialic acid (which interpretation is supported by the applicant's specification at Example 9 beginning at p. 94). However, according to the results shown in the applicant's Figure 7, the residual activity of Factor C expressed in Sf9 (with no (α-2,3)-linked sialic acid) or native Factor C exhibits almost no residual activity in 21 mM sodium citrate (see No. 5 of Figure 7; 10% sodium citrate corresponds to 21 mM sodium citrate). Other than a recombinant *Tachypleus tridentatus* or *Carcinoscorpius rotundicauda* Factor C protein expressed in Chinese Hamster Ovary (CHO) cells or human embryonic kidney (HEK) cells, which Factor C protein has Factor C activity and contains (α-2,3)-linked terminal sialic acid in a greater amount as compared with a corresponding native horseshoe crab Factor C protein and/or a corresponding horseshoe crab Factor C protein, there is no disclosed or art-recognized correlation between the structure of the recited Factor C protein and the characteristic of residual activity of 10% or higher in the presence of 21 mM sodium citrate and the

additional characteristics recited in claims 3-9. In view of the limited disclosed structure-function correlation and the disclosure of only two representative species and the limited disclosed structure-function correlation, one of skill in the art would not accept the disclosure of a recombinant *Tachypleus tridentatus* or *Carcinoscorpius rotundicauda* Factor C protein expressed in Chinese Hamster Ovary (CHO) cells or human embryonic kidney (HEK) cells, which Factor C protein has Factor C activity and contains (α-2,3)-linked terminal sialic acid in a greater amount as compared with a corresponding native horseshoe crab Factor C protein and/or a corresponding horseshoe crab Factor C protein recombinantly expressed using Sf9 as a host cell as being representative of other Factor C proteins as encompassed by the claims. As such, the specification fails to satisfy the written description requirement of 35 U.S.C. 112(a).

Claim Rejections - 35 USC § 103

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.

Claims 1-13 are rejected under 35 U.S.C. 103 as being unpatentable over Ding et al. (US 5,858,706; cited on the IDS filed on April 30, 2019; hereafter “Ding”) in view of *Current Protocols in Molecular Biology* (1993) 16.23.1-16.23.13, John Wiley and Sons, Inc., 2002 (cited on the IDS filed on June 17, 2020; hereafter “Di”) and as evidenced by

Mizumura et al. (WO 2012/118226 A1; cited on the IDS filed on April 30, 2019; hereafter "Mizumura").

Claims 1 and 10 are drawn to a horseshoe crab Factor C protein having activity of Factor C, wherein the horseshoe crab is selected from the group consisting of Tachypleus tridentatus, Limulus polyphemus, and Carcinoscorpius rotundicauda, and wherein the horseshoe crab Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or HEK cell.

Claims 2-9 and 11 are drawn to a Factor C protein having activity of Factor C,

wherein the Factor C protein comprises an amino acid sequence selected from the group consisting of:

(A) the amino acid sequence of SEQ ID NO: 2;

(B) an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:2, and having activity of Factor C;

(C) the amino acid sequence of SEQ ID NO: 4; and

(D) an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:4, and having activity of Factor C,

wherein the Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or HEK cell, and

wherein the Factor C protein exhibits a residual activity of 10% or higher in the presence of 21 mM sodium citrate.

Claim 12 is drawn to a method for producing the Factor C protein of claim 2, the method comprising culturing a CHO DG44 cell or HEK cell recombinantly expressing

said Factor C protein under conditions suitable for the expression of the horseshoe crab Factor C to produce the Factor C protein.

Claim 13 is drawn to an endotoxin assay kit comprising the Factor C protein according to claim 2 and one or more elements.

Regarding claims 1, 2, and 12, the reference of Ding discloses a mammalian host cell, including a Chinese Hamster Ovary (CHO) cell, transformed with a recombinant vector for expressing a cDNA encoding a Factor C (column 9, Example 1). Ding discloses cDNAs for expressing a horseshoe crab Factor C polypeptide, including the representative examples of *T. tridentatus* and *C. rotundicauda* Factor C polypeptides (column 3, lines 14-20). Ding discloses a method for producing the Factor C polypeptide by culturing the host cell and recovering the Factor C protein (column 9, Example 1). Ding discloses activation of the Factor C by endotoxin (column 1, lines 44-47).

Regarding claim 2, Ding does not disclose the amino acid sequence of *T. tridentatus* factor C has the amino acid sequence of SEQ ID NO: 2. However, the evidentiary reference of Mizumura is cited to show that the amino acid sequence of *T. tridentatus* factor C is identical to SEQ ID NO: 2 of this application.

Regarding claim 3, Ding discloses solubilization of the factor C (column 7, lines 456-53) and thus Ding's Factor C is considered to be water-soluble.

Regarding claim 13, Ding discloses the recombinant Factor C for use in an assay for endotoxin detection and teaches elements for conducting such an assay (column 8, lines 50-60), which is considered to be an endotoxin assay kit as encompassed by claim 13.

Ding does not disclose CHO DG44 cells as an expression host.

The reference of Current Protocols generally teaches a method for using CHO cells as an expression host for amplifying and expressing a desired gene (see whole document). Current Protocols teaches CHO DG44 as a cell line that greatly increases the ease of gene amplification (p. 16.23.11).

It would have been obvious to one of ordinary skill in the art before the effective filing date to combine the teachings of Ding and Current Protocols to modify Ding to use CHO DG44 cell line of Current Protocol as an expression host. One would have been motivated to do this because Ding discloses using CHO cells as an expression host and Current Protocol teaches a method for expressing a desired gene using a CHO cell line, noting that CHO DG44 cell line greatly increases the ease of gene amplification. One would have had a reasonable expectation of success to modify Ding to use the CHO DG44 cell line of Current Protocol as an expression host because Ding teaches cDNAs for expressing a horseshoe crab Factor C polypeptide using CHO cells and Current Protocol teaches a method for expressing a desired gene using a CHO DG44 cell line.

Regarding claim 2, the combination of Ding and Current Protocols does not teach or suggest the activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells exhibits a residual activity of 10% or higher in the presence of 21 mM sodium citrate. However, Figure 7 of this application shows that horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells exhibits a residual activity of 10% or higher in the presence of 10% sodium citrate with a dilution factor of 8, which, according to the specification is about 21 mM sodium citrate (p. 88). As such, since horseshoe crab Factor C recombinantly

expressed and recovered from CHO cells exhibits a residual activity of 10% or higher in the presence of 21 mM sodium citrate, it follows that Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells necessarily exhibits a residual activity of 10% or higher in the presence of 21 mM sodium citrate.

Regarding claims 4 and 5, the combination of Ding and Current Protocols does not teach or suggest the activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells has a molecular weight as recited in the claims. However, the applicant's specification discloses that *T. tridentatus* factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells has a molecular weight of 128 kDa by SDS-PAGE under non-reducing conditions (p. 92, paragraph [0144] and p. 94, Table 1). As such, since Ding's activated *T. tridentatus* factor C polypeptide is recombinantly expressed and recovered from CHO cells, it follows that Ding's activated Factor C polypeptide expressed from CHO DG44 cells necessarily has a molecular weight of 128 kDa by SDS-PAGE under non-reducing conditions.

Regarding claim 6, the combination of Ding and Current Protocols does not teach or suggest the activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells exhibits a residual activity of 25% or higher in the presence of 52 mM sodium hydrogencarbonate. However, Figure 7 of this application shows that horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells exhibits a residual activity of 25% or higher in the presence of about 7% sodium citrate with a dilution factor of 8, which, according to the

specification is about 52 mM sodium hydrogencarbonate (specification at p. 88). As such, since horseshoe crab Factor C recombinantly expressed and recovered from CHO cells recombinantly expressed and recovered from CHO cells exhibits a residual activity of 25% or higher in the presence of 52 mM sodium hydrogencarbonate, it follows that Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells necessarily exhibits a residual activity of 25% or higher in the presence of 52 mM sodium hydrogenate.

Regarding claim 7, the combination of Ding and Current Protocols does not teach the activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells exhibits a residual activity of 25% or higher in the presence of 214 mM sodium chloride. However, Figure 7 of this application shows that horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO cells exhibits a residual activity of 25% or higher in the presence of about 10% sodium citrate with a dilution factor of 4, which, according to the specification is about 214 mM sodium chloride (specification at p. 88). As such, since horseshoe crab Factor C recombinantly expressed and recovered from CHO cells recombinantly expressed and recovered from CHO cells exhibits a residual activity of 25% or higher in the presence of 214 mM sodium chloride, it follows that Ding2's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells necessarily exhibits a residual activity of 25% or higher in the presence of 214 mM sodium chloride.

Regarding claim 8, the combination of Ding2 and Current Protocols does not teach the activated horseshoe crab Factor C polypeptide recombinantly expressed and

recovered from CHO DG44 cells exhibits a residual activity of 15% or higher in the presence of 16 mM magnesium sulfate. However, Figure 7 of this application shows that horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO cells exhibits a residual activity of 15% or higher in the presence of about 0.5 M magnesium sulfate with a dilution factor of 16, which, according to the specification is about 16 mM magnesium sulfate (specification at p. 89). As such, since horseshoe crab Factor C recombinantly expressed and recovered from CHO cells recombinantly expressed and recovered from CHO cells exhibits a residual activity of 15% or higher in the presence of 16 mM magnesium sulfate, it follows that Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells necessarily exhibits a residual activity of 15% or higher in the presence of 16 mM magnesium sulfate.

Regarding claim 9, Ding does not teach the activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells exhibits a residual activity of 35% or higher in the presence of 2.5 mM calcium chloride. However, Figure 7 of this application shows that horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO cells exhibits a residual activity of 35% or higher in the presence of 0.5 M calcium chloride with a dilution factor of 100, which, according to the specification is about 2.5 mM calcium chloride (specification at p. 89). As such, since horseshoe crab Factor C recombinantly expressed and recovered from CHO cells recombinantly expressed and recovered from CHO cells exhibits a residual activity of 35% or higher in the presence of 2.5 mM calcium chloride, it follows that Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed

and recovered from CHO DG44 cells necessarily exhibits a residual activity of 35% or higher in the presence of 2.5 mM calcium chloride.

Regarding claims 10 and 11, the combination of Ding and Current Protocol does not teach or suggest the activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells has (α-2,3) linked terminal sialic acid in a greater amount as compared with a horseshoe crab Factor C expressed in Sf9 as a host cell. However, the applicant's specification discloses that horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO cells has (α-2,3) linked terminal sialic acid, while a native horseshoe crab Factor C polypeptide expressed in Sf9 as a host cell does not have any (α-2,3) linked terminal sialic acid (p. 98, paragraph [0154]). As such, since horseshoe crab Factor C recombinantly expressed and recovered from CHO cells has (α-2,3)-linked terminal sialic acid and a native horseshoe crab Factor C polypeptide expressed in Sf9 as a host cell does not have any (α-2,3) linked terminal sialic acid, it follows that Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells necessarily has a greater amount of (α-2,3)-linked terminal sialic acid than horseshoe crab Factor C polypeptide expressed in Sf9 as a host cell. The combination of Ding and Current Protocols also does not teach or suggest the activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells has (α-2,3) linked terminal sialic acid in a greater amount as compared with a native horseshoe crab Factor C. However, the applicant's specification discloses that horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO cells has greater (α-2,3) linked terminal sialic acid than a native horseshoe crab Factor C

polypeptide (paragraph [0153]). As such, since horseshoe crab Factor C recombinantly expressed and recovered from CHO cells has greater (α-2,3)-linked terminal sialic acid than a native horseshoe crab Factor C polypeptide, it follows that Ding's activated horseshoe crab Factor C polypeptide recombinantly expressed and recovered from CHO DG44 cells necessarily has a greater amount of (α-2,3)-linked terminal sialic acid than a native horseshoe crab Factor C polypeptide.

Therefore, the invention of claims 1-13 would have been obvious to one of ordinary skill in the art at the time of the invention.

Claim Rejections - Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may

be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Claims 1-13 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-8 and 13-20 of U.S. Patent No. 10,144,923 B2 (cited on Form PTO-892; hereafter "patent").

Claims 1 and 10 of this application are anticipated by claim 1 of the patent.

Claims 2 and 11 of this application are anticipated by claims 2, 4, and 18 of the patent.

Claim 3 of this application is anticipated by claim 5 of the patent.

Claim 4 of this application is anticipated by claim 3 of the patent.

Claim 5 of this application is anticipated by claim 13 of the patent.

Claim 6 of this application is anticipated by claim 14 of the patent.

Claim 7 of this application is anticipated by claim 15 of the patent.

Claim 8 of this application is anticipated by claim 16 of the patent.

Claim 9 of this application is anticipated by claim 17 of the patent.

Claim 10 of this application is anticipated by claim 4 of the patent.

Claim 12 of this application is anticipated by claims 6 and 7 of the patent.

Claim 13 of this application is anticipated by claims 8, 19, and 20 of the patent.

Claims 1-13 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1, 2, 4, 9, and 12-15 of copending Application No. 15/983,725 (reference application).

Claims 1 and 10 of this application are anticipated by claims 1 and 9 of the reference application.

Claims 2, 3, 11, and 12 of this application are anticipated by claims 2 and 12 of the reference application.

Claim 6 of this application is anticipated by claims 4 and 13 of the reference application.

Claim 7 of this application is anticipated by claim 14 of the reference application.

Claim 8 of this application is anticipated by claim 15 of the reference application.

Claim 13 of this application is anticipated by claim 9 of the reference application.

Regarding claims 4, 5, and 9 of this application, the claims of the reference application do not recite the limitations of claims 4, 5, and 9 of this application. However, these characteristics are considered to be inherent features of the Factor C as recited in claims 2 and 12 of the reference application (see MPEP 2112.01.I).

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

Claims 1-13 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1 and 4 of copending Application No. 16/331,810 (reference application) in view of Ding (*supra*) and Current Protocols (*supra*).

Claim 1 of the reference application is drawn to a recombinant protein which is any of the following: (A) a recombinant protein that contains the amino acid sequence represented by SEQ ID NO: 2 or 4; (B) a recombinant protein that contains an amino

acid sequence having a 95% or more identity with the amino acid sequence represented by SEQ ID NO: 2 or 4, and has factor C activity; (C) a recombinant protein that is encoded by a DNA containing a base sequence having a 95% or more identity with the base sequence represented by SEQ ID NO: 1 or 3, and has factor C activity; and (D) a recombinant protein that is encoded by a DNA hybridizing to a DNA composed of a complementary sequence to the base sequence represented by SEQ ID NO: 1 or 3 under stringent conditions, and has factor C activity.

Claim 4 of the reference application is drawn to an endotoxin detecting agent, comprising the recombinant protein according to claim 1.

The amino acid sequences of SEQ ID NO: 2 and 4 of the reference application are the amino acid sequences of *Limulus polyphemus* Factor C and have 93.4% sequence identity to the amino acid sequence of SEQ ID NO: 2 of this application and have 92.5% sequence identity to the amino acid sequence of SEQ ID NO: 4 of this application.

The differences between the claims of this application and the claims of the reference application is that the claims do not recite the recombinant protein is produced using CHO DG44 cells as an expression host and the claims of the reference application do not recite the characteristics of exhibiting a residual activity of 10% or higher in the presence of 21 mM sodium citrate, being water soluble, having a molecular weight of 115 kDa to 140 kDa as measured by SDS-PAGE under non-reducing condition, having a molecular weight of 127 kDa±5 kDa, exhibiting a residual Factor C activity of 25% or higher in the presence of 52 mM sodium hydrogen carbonate, exhibiting a residual Factor C activity of 25% or higher in the presence of 214 mM

sodium chloride, exhibiting a residual Factor C activity of 15% or higher in the presence of 16 mM magnesium sulfate, exhibiting a residual Factor C activity of 35% or higher in the presence of 2.5 mM calcium chloride, containing (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a corresponding native horseshoe crab Factor C protein and/or a corresponding horseshoe crab Factor C protein recombinantly expressed using Sf9 as a host cell, and a method for producing a Factor C protein.

The reference of Ding discloses a mammalian host cell, including a Chinese Hamster Ovary (CHO) cell, transformed with a recombinant vector for expressing a cDNA encoding a Factor C (column 9, Example 1). Ding discloses cDNAs for expressing a horseshoe crab Factor C polypeptide, including the representative examples of *T. tridentatus* and *C. rotundicauda* Factor C polypeptides (column 3, lines 14-20). Ding discloses a method for producing the Factor C polypeptide by culturing the host cell and recovering the Factor C protein (column 9, Example 1). Ding discloses activation of the Factor C by endotoxin (column 1, lines 44-47). Ding discloses solubilization of the factor C (column 7, lines 456-53) and thus Ding's Factor C is considered to be water-soluble.

The reference of Current Protocols generally teaches a method for using CHO cells as an expression host for amplifying and expressing a desired gene (see whole document). Current Protocols teaches CHO DG44 as a cell line that greatly increases the ease of gene amplification (p. 16.23.11).

It would have been obvious to one of ordinary skill in the art before the effective filing date to produce the recombinant protein of the claims of the reference application using CHO DG44 cell line of Current Protocol as an expression host. One would have

been motivated to do this because Ding discloses using CHO cells as an expression host for producing a horseshoe crab Factor C and Current Protocol teaches a method for expressing a desired gene using a CHO cell line, noting that CHO DG44 cell line greatly increases the ease of gene amplification. One would have had a reasonable expectation of success to produce the recombinant protein of the claims of the reference application using CHO DG44 cell line of Current Protocol as an expression host because Ding teaches methods for expressing a horseshoe crab Factor C polypeptide using CHO cells and Current Protocol teaches a method for expressing a desired gene using a CHO DG44 cell line.

Regarding the characteristics of exhibiting a residual activity of 10% or higher in the presence of 21 mM sodium citrate, being water soluble, having a molecular weight of 115 kDa to 140 kDa as measured by SDS-PAGE under non-reducing condition, having a molecular weight of 127 kDa±5 kDa, exhibiting a residual Factor C activity of 25% or higher in the presence of 52 mM sodium hydrogen carbonate, exhibiting a residual Factor C activity of 25% or higher in the presence of 214 mM sodium chloride, exhibiting a residual Factor C activity of 15% or higher in the presence of 16 mM magnesium sulfate, exhibiting a residual Factor C activity of 35% or higher in the presence of 2.5 mM calcium chloride, and containing (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a corresponding native horseshoe crab Factor C protein and/or a corresponding horseshoe crab Factor C protein recombinantly expressed using Sf9 as a host cell, as explained in detail above, these characteristics are inherent to the recombinant horseshoe crab Factor C protein produced using CHO DG44 cell line.

As such, claims 1-13 of this application are unpatentable over claims 1 and 4 of the reference application in view of Ding (*supra*) and Current Protocols (*supra*).

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

Examiner Comment

The applicant is advised that withdrawal of the restriction requirement between the claims of the elected invention of Group I and the claims of the non-elected and patentably distinct invention of Group II may necessitate a nonstatutory double patenting rejection based on the claims of co-pending application 16/399,575.

Conclusion

Status of the claims:

Claims 1-18 are pending.

Claims 14-18 are withdrawn from consideration.

Claims 1-13 are rejected.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to DAVID J STEADMAN whose telephone number is (571)272-0942. The examiner can normally be reached on Monday to Friday, 7:30 AM to 4:00 PM.

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, MANJUNATH N. RAO can be reached on 571-272-0939. 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.

/David Steadman/
Primary Examiner, Art Unit 1656

Notice of References Cited	Application/Control No. 16/399,386	Applicant(s)/Patent Under Reexamination MIZUMURA et al.	
	Examiner DAVID J STEADMAN	Art Unit 1656	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-10144923-B2	12-2018	Mizumura; Hikaru	C12N9/6408	1/1
	B					
	C					
	D					
	E					
	F					
	G					
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	I					
	J					
	K					
	L					
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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.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/399,386	MIZUMURA et al.
	Examiner	Art Unit
	DAVID J STEADMAN	1656

CPC - Searched*		
Symbol	Date	Examiner
NONE		

CPC Combination Sets - Searched*		
Symbol	Date	Examiner
NONE		

US Classification - Searched*			
Class	Subclass	Date	Examiner
NONE			

* 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
PALM inventor name search, all inventors searched	06/24/2020	/DJS/
Reviewed prosecution history for applications 14/650,767, 15/983,725, and 16/399575	06/24/2020	/DJS/
STIC search of SEQ ID NO: 2 and 4 against amino acid and nucleic acid prior and interference databases, see SCORE for search results	06/24/2020	/DJS/
EAST, STN database keyword search, see attached search history printouts for search strategy and databases searched	06/24/2020	/DJS/

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

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     (FILE 'HOME' ENTERED AT 16:09:03 ON 22 APR 2020)
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     FILE 'CAPLUS' ENTERED AT 16:09:16 ON 22 APR 2020
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L1               24 SEA "MIZUMURA HIKARU"/AU
                E ODA TOSHIO/AU 25
L2               90 SEA "ODA TOSHIO"/AU
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L3              137 SEA "KAWABATA SHUN ICHIRO"/AU
L4                5 SEA "KAWABATA SHUN"/AU
L5              241 SEA L1 OR L2 OR L3 OR L4
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L8               13 SEA L7 AND "FACTOR C"
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     16:39:25 ON 22 APR 2020
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L10             249 SEA L9 AND "FACTOR C"
L11             122 SEA L10 AND ENDOTOXIN
L12              68 SEA L11 AND (RECOMBINANT? OR TRANSFORM? OR TRANSFECT?)
L13               1 SEA L12 AND SIAL?
                D IBIB ABS
L14              18 SEA L12 AND CASCADE
L15              11 DUP REM L14 (7 DUPLICATES REMOVED)
                D IBIB ABS 1-
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16/399,386
	Confirmation Number	9293
	Filing Date	April 30, 2019
	First Named Inventor	Hikaru MIZUMURA
	Art Unit	1656
	Examiner Name	STEADMAN, DAVID J
	Attorney Docket Number	Q246419

U.S. PATENTS

Examiner Initials*	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

U.S. PATENT APPLICATION PUBLICATIONS

Examiner Initials*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

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NON-PATENT LITERATURE DOCUMENTS

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, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1.	Bram Blomme et al., "Alteration of protein glycosylation in liver diseases", Journal of Hepatology, 2009, Vol. 50, pages 592-603 (12 pages total)	
	2.	Office Action dated February 12, 2020 issued in US Patent Application No. 15/983,725	
	3.	Stephanie Holst et al., "Profiling of different pancreatic cancer cells used as models for metastatic behaviour shows large variation in their N-glycosylation" Scientific Reports, November 30, 2017, Vol. 7, No. 16623, Pages 1-15 (15 pages total)	

EXAMINER SIGNATURE

Examiner Signature	/David Steadman/	Date Considered	06/24/2020

*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.

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	Art Unit	1656
	Examiner Name	STEADMAN, DAVID J
	Attorney Docket Number	Q246419

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).

☑ That each item of information contained in the Information Disclosure Statement filed concurrently herewith: (i) was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement; or (ii) is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement. See 37 CFR 1.704(d).

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

☑ The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

☐ A certification statement is not submitted herewith.

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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	/markboland/	Date (YYYY-MM-DD)	2020-03-05
Name/Print	Mark Boland	Registration Number	32,197

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Examiner Name	Not Yet Assigned
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U.S. PATENTS

Examiner Initials*	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.	5712144	A	1998-01-27	Ding et al.	
	2.	5795962	A	1998-08-18	Iwanaga et al.	
	3.	5858706	A	1999-01-12	Ding et al.	
	4.	6077946	A	2000-01-20	Iwanaga et al.	
	5.	5716834	A	1998-02-	Ding, et al.	

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	6.	20030054432	A1	2003-03-20	Chen et al.	
	7.	20040235080	A1	2004-11-25	Chen et al.	
	8.	20080131929	A1	2008-06-05	Prentice et al.	
	9.	20090208995	A1	2009-08-20	Tamura et al.	
	10.	20100086967	A1	2010-04-08	Kaufmann et al.	
	11.	20130295613	A1	2013-11-07	Kishishita et al.	
	12.	20140024062	A1	2014-01-23	Mizumura et al.	
	13.	20140249082	A1	2014-09-04	Cottingham et al.	

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	14.	2005-500520	JP	A	2005-01-06	CAMBREX BIO SCIENCE WALKERVILLE INC		Corresponds to cites 6 and 7; Cited in cite 31
	15.	2006-87435	JP	A	2006-04-06	SEIKAGAKU KOGYO CO LTD		English Abstract; Corresponds to cites 3 and 4; Cited in cite 31
	16.	2008/004674	WO	A1	2008-01-10	SEIKAGAKU CORPORATION		English Abstract; Corresponds to cite 5; Cited in cite 31
	17.	2008520250	JP	A	2008-06-19	BIOGEN IDEC MA INC.		Corresponds to US 20080131929; Cited in JP comm. dated 8/7/18 in 2014-552048

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	18.	2008-520250	JP	A	2008-06-19	BIOGEN IDEC MA INC.,		Cited in JPOA 8/7/18 in no. 2014-552048; Corresponds to US 2008/0131929 A1
	19.	2010516264	JP	A	2010-05-20	BOEHRINGER INGELHEIM PHARMA GMBH & CO. KG		Corresponds to US 2010086967; Cited in JP comm. dated 8/7/18 in 2014-552048
	20.	2010-516264	JP	A	2010-05-20	BOEHRINGER INGELHEIM PHARMA GMBH & CO. KG,		Cited in JPOA 8/7/18 in no. 2014-552048; Corresponds to US 2010/0086967 A1
	21.	2012/118226	WO	A1	2012-09-07	SEIKAGAKU CORPORATION		Corresponds to cite 8; Cited in cite 31
	22.	2012091124	WO	A1	2012-07-05	CHUGAI SEIYAKU KABUSHIKI KAISHA		Abstract; Corresponds to US 20130295613; Cited in JP comm. dated 8/7/18 in 2014-552048
	23.	94673	SG	A1	2003-03-18	NATIONAL UNIVERSITY OF SINGAPORE		Cited in cite 31
	24.	9915676	WO	A1	1999-04-01	National University of Singapore		

NON-PATENT LITERATURE DOCUMENTS

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, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	25.	Communication dated April 19, 2017, from the State Intellectual Property Office of the P.R.C., in counterpart Chinese application no. 201380064649.3	Translation
	26.	Communication dated August 7, 2018 issued by the Japanese Patent Office in counterpart application no. 2014-552048	Machine Translation
	27.	Communication dated August 7, 2018, from the Japanese Patent Office in counterpart application no. 2014-552048	
	28.	Communication dated July 29, 2016 from the State Intellectual Property Office of the P.R.C. in counterpart application no. 201380064649.3	
	29.	Communication dated July 5, 2016, from the European Patent Office in counterpart European Application No. 13862466.3	
	30.	Extended European Search Report dated January 10, 2019 issued by the European Patent Office in counterpart application no. 18190686.8	

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	Art Unit	Not Yet Assigned
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	Q246419

NON-PATENT LITERATURE DOCUMENTS			
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, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	31.	International Search Report for PCT/JP2013/083082 dated February 10, 2012	English Translation
	32.	JEAK L. DING et al., "A new era in pyrogen testing", TRENDS in Biotechnology, August 2001, pp. 277-281, Vol. 19, No. 8	
	33.	Jeak Ling Ding et al., "Chapter 9, Endotoxin Detection - from Limulus Amebocyte Lysate to Recombinant Factor C ", Endotoxins: Structure, Function and Recognition SERIES : SUBCELLULAR BIOCHEMISTRY IN: SUBCELLULAR BIOCHEMISTRY: REVIEWS AND ESSAYS DEALING WITH THE FUNCTION, GENETICS, BIOGENESIS AND EVOLUTION OF SUB-CELLULAR COMPONENTS, vol. 53, January 1, 2010, XP009510157, 22 pages total	
	34.	JING WANG et al., "Functional expression of full length Limulus Factor C in stably transformed Sf9 cells", Biotechnology Letters, 2001, pp. 71-76, Vol. 23	
	35.	JING WANG et al., "Modular Arrangement and Secretion of a Multidomain Serine Protease", The Journal of Biological Chemistry, September 27, 2002, pp. 36363-36372, Vol. 277, No. 39	
	36.	Mizumura, et al., Genetic engineering approach to develop next-generation reagents for endotoxin quantification; Innate Immunity, vol. 23, no. 2, pp. 136-146 , 2017	
	37.	Nakamura T. et al. "Lipopolysaccharide-sensitive serine-protease zymogen (factor C) found in Limulus hemocytes" European Journal of Biochemistry vol. 154, 1986; pages 511-521	
	38.	Office Action issued in the corresponding European Application No. 13862466.3 dated November 30, 2017	
	39.	ROOPASHREE S. DWARAKANATH et al., "Recombinant COS-1 cells espress Carcinoscorpius rotundicauda Factor C", Biotechnology Letters, April 1997, pp. 357-361, Vol. 19, No. 4	
	40.	SADAAKI IWANAGA, "The limulus clotting reaction", Current Opinion in Immunology 1993, pp. 74-82, Vol. 5	
	41.	Shengwu Wuli Jinzhan Vol.31, No.8, p. 736-740, 2004	
	42.	TAKANORI NAKAMURA et al., "Purification and Properties of Intracellular Clotting Factor, Factor B, from Horseshoe Crab (Tachypleus tridentatus) Hemocytes", J. Biochem, 1986, Vol. 99, No. 3	
	43.	Tokunaga F. et al. "Lipopolysaccharide-sensitive serine-protease zymogen factor C of horseshoe crab hemocytes identification and alignment of proteolytic fragments produced during the activation show that it is a novel type of serine protease" European Journal of Biochemistry vol. 167 no. 3, 1987; pages 405-416	
	44.	XIANGJUN et al., "Role of domains in factor C from Tachypleus tridentatus in neutralization endotoxin", "Accession:2006:239467" File CAPLUS , 2003	
	45.	XIANGJUN et al., "Role of domains in factor C from Tachypleus tridentatus in neutralization endotoxin", "Accession:2006:239467" File CAPLUS [online],Retrived from the STN on the Web, [retrieved on 2014-01-29], Shengwu Huaxue Yu Shengwu Wuli Jinzhan Vol.31, No.8, p736-740, 2004	

EXAMINER SIGNATURE			
Examiner Signature	/David Steadman/	Date Considered	06/24/2020

*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. /DJS/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	Not Yet Assigned
	Confirmation Number	Not Yet Assigned
	Filing Date	April 30, 2019
	First Named Inventor	Hikaru MIZUMURA
	Art Unit	Not Yet Assigned
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	Q246419

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).

☐ That each item of information contained in the Information Disclosure Statement filed concurrently herewith: (i) was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement; or (ii) is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement. See 37 CFR 1.704(d).

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

☑ The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

☐ A certification statement is not submitted herewith.

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

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	/markboland/	Date (YYYY-MM-DD)	April 30, 2019
Name/Print	Mark Boland	Registration Number	32,197

*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. /DJS/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16/399,386
	Confirmation Number	9293
	Filing Date	April 30, 2019
	First Named Inventor	Hikaru MIZUMURA
	Art Unit	1656
	Examiner Name	STEADMAN, DAVID J
	Attorney Docket Number	Q246419

U.S. PATENTS

Examiner Initials*	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.	5840510		1998-11-24	Tanaka et al.	

U.S. PATENT APPLICATION PUBLICATIONS

Examiner Initials*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No	Foreign Document Number[3]	Country Code[2]	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]

NON-PATENT LITERATURE DOCUMENTS

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, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	2.	Office Action dated April 27, 2020 issued by the USPTO in U.S. Patent Application No. 16/399,575	
	3.	Office Action dated May 21, 2020 issued by the USPTO in U.S. Patent Application No. 15/983,725	
	4.	Strategies for Protein Purification Handbook, GE Healthcare, September 2010, 167 pages	
	5.	KOSHIBA et al., "A Structural Perspective on the Interaction between Lipopolysaccharide and Factor C, a Receptor Involved in Recognition of Gram-negative Bacteria", J. Biol. Chem., 282, 3962-3967, 2007 (13 pages in total)	
	6.	Current Protocols in Molecular Biology (1993) 16.23.1-16.23.13, John Wiley and Sons, Inc., 2002	
	7.	SLADE et al., "Identifying the CHO Secretome using Mucin-type O-Linked Glycosylation and Click-chemistry", J. Prot. Res., 11,:6175-6186, 2012	
	8.	International Preliminary Report on Patentability in International Application No. PCT/JP2013/083082 issued June 16, 2015 and mailed June 25, 2015	
	9.	MIZUMURA et al., "Genetic engineering approach to develop next-generation reagents for endotoxin quantification", Innate Immunity, vol. 23, no. 2, pp. 136-146, **2017**	
	10.	Written Opinion for PCT/JP2013/083082 dated February 10, 2014	
	11.	International Search Report for PCT/JP2013/083082 dated **February 10, 2014**	

EXAMINER SIGNATURE

Examiner Signature	/David Steadman/	Date Considered	06/24/2020

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[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. /DJS/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	16/399,386
	Confirmation Number	9293
	Filing Date	April 30, 2019
	First Named Inventor	Hikaru MIZUMURA
	Art Unit	1656
	Examiner Name	STEADMAN, DAVID J
	Attorney Docket Number	Q246419

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OR

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

☑ The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

☑ A certification statement is not submitted herewith.

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23373
CUSTOMER NUMBER

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	/Hui C. Wauters/	Date (YYYY-MM-DD)	2020-06-17
Name/Print	Hui C. Wauters	Registration Number	57,426

*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. /DJS/

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S5	7,687	(horseshoe adj crab) OR limulus	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:46
S6	13,415	factor adj "c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S7	13,415	"factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S8	335	S5 same S6	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S9	212	S8 and recombinant	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S10	89	S8 same recombinant	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:47
S11	26	S10 and mammalian	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 09:48
S12	9	S10 and ((chinese adj hamster) OR CHO)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:02

EAST Search History (Prior Art)

S13	2	S10 and (hek OR (embryonic adj kidney))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:02
S14	6	S10 and (hek$ OR (embryonic adj kidney))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:03
S15	4	S14 not S13	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:03
S16	2	"6,645,724".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:04
S17	12	"2012118226"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 10:35
S18	4	"5716834".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/12 15:58
S19	2,454	seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:23
S20	18,057	(mizumura-h$ OR oda-t$ OR kawabata-s$). in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:23

EAST Search History (Prior Art)

S21	20,466	S19 or S20	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:25
S22	91	S21 and (horseshoe adj crab)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:25
S23	66	S21 and tridentatus	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:25
S24	41	S21 and rotundicauda	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:26
S25	108	S22 or S23 or S24	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:26
S26	40	S25 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 09:26
S27	23	("5,858,706" OR "5,712,144" OR "5,795,962" OR "6,077,946" OR "20090208995" OR "20030054432" OR "20040235080" OR "20140024062").pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/13 11:34
S28	4	"5,716,834".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/05 18:17

EAST Search History (Prior Art)

S29	4	"5,858,706".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 11:27
S30	2	"6,645,724".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 15:42
S31	2	"20140249082".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 16:45
S32	2,474	seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:07
S33	18,751	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:07
S34	21,180	S32 or S33	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:07
S35	19	S33 AND (tridentatus OR polyphemus OR rotundicauda OR gigas)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:08
S36	13	S35 and "factor C"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:08

EAST Search History (Prior Art)

S37	2	S36 and (sialic OR sialyl$)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/04/06 17:08
S38	14,664	"factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:53
S39	218	S38 and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:53
S40	7	S38 same (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:54
S41	218	S39 and (cho OR "chinese hamster ovary")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:54
S42	805	"factor c".clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:54
S43	2	S42 and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:55
S44	5,676	(cho OR "chinese hamster ovary") same (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:55

EAST Search History (Prior Art)

S45	211	S44 and S38	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:55
S47	7	S45 and horseshoe	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/30 15:56
S48	20	horseshoe and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:45
S49	218	"factor c" and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:47
S50	211	S49 not S48	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:47
S51	0	S50 and horseshoe	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:48
S52	2	"factor c".clm. and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:48
S53	6	"factor c".ti. and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:49

EAST Search History (Prior Art)

S54	7	"factor c".ab. and (dg44 OR dg-44 OR dg/44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 11:49
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S56	2	"9133495".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:36
S57	19,132	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45
S58	6	S57 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45
S59	2,485	seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45
S60	3	S59 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45
S61	7	S58 or S60	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/10/31 15:45

EAST Search History (Prior Art)

S62	19,414	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S63	6	S62 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S64	2,493	seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S65	3	S64 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S66	7	S63 or S65	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/04/12 15:58
S67	19,635	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/08/14 09:19
S68	6	S67 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/08/14 09:19
S80	3	("20080131929"|"20100086967"|"20130295613").PN.	US-PGPUB; USPAT	OR	OFF	2018/09/07 10:58
S81	19,653	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/09/07 11:43

EAST Search History (Prior Art)

S82	6	S81 AND (dg44 OR dg-44 OR chodg44 OR cho-dg44)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/09/07 11:43
S94	6	"10144923".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/10/01 09:23
S96	6	(US-20080131929-$ | US-20100086967-$ | US-20130295613-$).DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/10/01 10:53
S97	20,399	(mizumura-h$ OR oda-t$ OR kawabata-s$).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/10/02 15:15
S98	40	S97 AND (tridentatus OR polyphemus OR rotundicauda OR gigas)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/10/02 15:15
S99	23,093	(mizumura-h$ OR oda-t$ OR kawabata-s$).in. OR seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/02/07 10:15
S100	95	S99 AND (tridentatus OR polyphemus OR rotundicauda OR gigas)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/02/07 10:16
S101	54	S100 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/02/07 10:16

EAST Search History (Prior Art)

S102	24	S101 and ("chinese hamster" OR CHO OR human OR HEK)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/02/07 10:16
S103	627	tridentatus OR rotundicauda	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S104	389	S103 and endotoxin	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S105	263	S104 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S106	224	S103 same "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S107	216	S105 and S106	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:41
S108	140	S107 and "37"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:42
S109	1,050,017	"2 hours"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:42

EAST Search History (Prior Art)

S110	114	S108 and S109	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:42
S113	349,173	cascade	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:44
S114	169	S107 and S113	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 14:45
S115	468	limulus adj test	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 15:30
S116	1	S115 same cation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/20 15:31
S117	3	"6,645,724".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 12:19
S118	13	("20030054432"|"20040235080"|"20080131929"|"20090208995"|"20100086967"|"20130295613"|"20140024062"|"20140249082"|"5712144"|"5716834"|"5795962"|"5858706"|"6077946").PN.	US-PGPUB; USPAT	OR	OFF	2020/04/22 14:32
S119	23,218	(mizumura-h$ OR oda-t$ OR kawabata-s$).in. OR seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 14:37
S120	85	S119 AND (tridentatus OR rotundicauda)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 14:37

EAST Search History (Prior Art)

S121	54	S120 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 14:38
S122	33	S121 and endotoxin.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/22 14:39
S123	23,351	(mizumura-h$ OR oda-t$ OR kawabata-s$). in. OR seikagaku.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:10
S124	95	S123 AND (tridentatus OR rotundicauda OR polyphemus)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:10
S125	55	S124 and "factor c"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:11
S126	10	S125 and (cho OR "chinese hamster")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:11
S127	8	S125 and (hek OR (human adj embryo$))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:12
S128	10	S126 or S127	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/06/24 15:12

EAST Search History (Interference)

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S69	2,075	(dg44 OR dg-44 OR chodg44 OR cho-dg44)	USPAT	OR	OFF	2018/08/14 09:27
S70	5,851	"factor c"	USPAT	OR	OFF	2018/08/14 09:27
S71	92	S69 and S70	USPAT	OR	OFF	2018/08/14 09:27
S72	17,574	horseshoe OR crab	USPAT	OR	OFF	2018/08/14 09:28
S73	2	S71 and S72	USPAT	OR	OFF	2018/08/14 09:28
S74	40	(dg44 OR dg-44 OR chodg44 OR cho-dg44).clm.	USPAT	OR	OFF	2018/08/14 09:30
S75	380	"factor c".clm.	USPAT	OR	OFF	2018/08/14 09:30
S76	0	S74 and S75	USPAT	OR	OFF	2018/08/14 09:30
S77	2,254	(horseshoe OR crab).clm.	USPAT	OR	OFF	2018/08/14 09:31
S78	0	S74 and S77	USPAT	OR	OFF	2018/08/14 09:31
S79	0	(dg44 OR dg-44 OR chodg44 OR cho-dg44) same "factor c"	USPAT	OR	OFF	2018/08/14 09:31
S83	2,086	(dg44 OR dg-44 OR chodg44 OR cho-dg44)	USPAT	OR	OFF	2018/09/07 11:44
S84	5,873	"factor c"	USPAT	OR	OFF	2018/09/07 11:44
S85	92	S83 and S84	USPAT	OR	OFF	2018/09/07 11:44
S86	17,635	horseshoe OR crab	USPAT	OR	OFF	2018/09/07 11:44
S87	2	S85 and S86	USPAT	OR	OFF	2018/09/07 11:44
S88	41	(dg44 OR dg-44 OR chodg44 OR cho-dg44).clm.	USPAT	OR	OFF	2018/09/07 11:44
S89	383	"factor c".clm.	USPAT	OR	OFF	2018/09/07 11:44
S90	0	S88 and S89	USPAT	OR	OFF	2018/09/07 11:44
S91	2,260	(horseshoe OR crab).clm.	USPAT	OR	OFF	2018/09/07 11:44
S92	0	S88 and S91	USPAT	OR	OFF	2018/09/07 11:44
S93	0	(dg44 OR dg-44 OR chodg44 OR cho-dg44) same "factor c"	USPAT	OR	OFF	2018/09/07 11:44

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Docket No: Q246419
Hikaru MIZUMURA, et al.	
Appln. No.: 16/399,386	Group Art Unit: 1656
Confirmation No.: 9293	Examiner: STEADMAN, DAVID J
Filed: April 30, 2019	

For: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

RESPONSE TO RESTRICTION AND ELECTION OF SPECIES REQUIREMENTS

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

Sir:

This responds to the Restriction and Election of Species Requirements dated April 17, 2020. In response to the Restriction Requirement, Applicant elects Group I, claims 1-13, for examination. This election is made without traverse, subject to Applicant's right to request rejoinder as may be appropriate.

The Election of Species Requirement only relates to Group III. Since Applicant does not elect the invention of Group III, no species needs to be elected.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880.

Please also credit any overpayments to said Deposit Account.

Respectfully submitted,

/Hui C. Wauters/

Hui C. Wauters
Registration No. 57,426

SUGHRUE MION, PLLC
Telephone: 202.293.7060
Facsimile: 202.293.7860

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

Date: June 17, 2020

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Docket No: Q246419
Hikaru MIZUMURA, et al.	
Appln. No.: 16/399,386	Group Art Unit: 1656
Confirmation No.: 9293	Examiner: STEADMAN, DAVID J
Filed: April 30, 2019	

For: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

INFORMATION DISCLOSURE STATEMENT
UNDER 37 C.F.R. §§ 1.97 and 1.98

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

Sir:

In accordance with the duty of disclosure under 37 C.F.R. § 1.56, Applicant hereby notifies the U.S. Patent and Trademark Office of the documents which are listed on the attached PTO/SB/08 (modified) form and/or listed herein.

One copy of each of the listed documents is submitted herewith, except for U.S. patents.

The present Information Disclosure Statement is being filed: (1) No later than three months from the application's filing date; (2) Before the mailing date of the first Office Action on the merits (whichever is later); or (3) Before the mailing date of the first Office Action after filing a request for continued examination (RCE) under §1.114, and therefore, no Statement under 37 C.F.R. § 1.97(e) or fee under 37 C.F.R. § 1.17(p) is required.

Applicant notes that Cite Nos. 9 and 11 on the attached SB/08 Form were submitted to correct the date for Cite Nos. 36 and 31 on the SB/08 Form submitted April 30, 2019.

The submission of the listed documents is not intended as an admission that any such document constitutes prior art against the claims of the present application. Applicant does not waive any right to take any action that would be appropriate to antedate or otherwise remove any listed document as a competent reference against the claims of the present application.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,

/Hui C. Wauters/

Hui C. Wauters
Registration No. 57,426

SUGHRUE MION, PLLC
Telephone: 202.293.7060
Facsimile: 202.293.7860

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

Date: June 17, 2020

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16/399,386
Confirmation Number	9293
Filing Date	April 30, 2019
First Named Inventor	Hikaru MIZUMURA
Art Unit	1656
Examiner Name	STEADMAN, DAVID J
Attorney Docket Number	Q246419

U.S. PATENTS

Examiner Initials*	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.	5840510		1998-11-24	Tanaka et al.	

U.S. PATENT APPLICATION PUBLICATIONS

Examiner Initials*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No	Foreign Document Number[3]	Country Code[2]	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]

NON-PATENT LITERATURE DOCUMENTS

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, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	2.	Office Action dated April 27, 2020 issued by the USPTO in U.S. Patent Application No. 16/399,575	
	3.	Office Action dated May 21, 2020 issued by the USPTO in U.S. Patent Application No. 15/983,725	
	4.	Strategies for Protein Purification Handbook, GE Healthcare, September 2010, 167 pages	
	5.	KOSHIBA et al., "A Structural Perspective on the Interaction between Lipopolysaccharide and Factor C, a Receptor Involved in Recognition of Gram-negative Bacteria", J. Biol. Chem., 282, 3962-3967, 2007 (13 pages in total)	
	6.	Current Protocols in Molecular Biology (1993) 16.23.1-16.23.13, John Wiley and Sons, Inc., 2002	
	7.	SLADE et al., "Identifying the CHO Secretome using Mucin-type O-Linked Glycosylation and Click-chemistry", J. Prot. Res., 11,:6175-6186, 2012	
	8.	International Preliminary Report on Patentability in International Application No. PCT/JP2013/083082 issued June 16, 2015 and mailed June 25, 2015	
	9.	MIZUMURA et al., "Genetic engineering approach to develop next-generation reagents for endotoxin quantification", Innate Immunity, vol. 23, no. 2, pp. 136-146, **2017**	
	10.	Written Opinion for PCT/JP2013/083082 dated February 10, 2014	
	11.	International Search Report for PCT/JP2013/083082 dated **February 10, 2014**	

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	16/399,386
	Confirmation Number	9293
	Filing Date	April 30, 2019
	First Named Inventor	Hikaru MIZUMURA
	Art Unit	1656
	Examiner Name	STEADMAN, DAVID J
	Attorney Docket Number	Q246419

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).

☐ That each item of information contained in the Information Disclosure Statement filed concurrently herewith: (i) was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement; or (ii) is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement. See 37 CFR 1.704(d).

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

☑ The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

☑ A certification statement is not submitted herewith.

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

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	/Hui C. Wauters/	Date (YYYY-MM-DD)	2020-06-17
Name/Print	Hui C. Wauters	Registration Number	57,426

*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.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2013/083082

A. CLASSIFICATION OF SUBJECT MATTER
C12N15/09(2006.01)i, *C12N9/64*(2006.01)i, *C12P21/02*(2006.01)i, *C12Q1/37*(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)
C12N15/09, C12N9/64, C12P21/02, C12Q1/37

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho	1922-1996	Jitsuyo Shinan Toroku Koho	1996-2014
Kokai Jitsuyo Shinan Koho	1971-2014	Toroku Jitsuyo Shinan Koho	1994-2014

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAplus/MEDLINE/EMBASE/BIOSIS(STN), GenBank/EMBL/DDBJ/GeneSeq, UniProt/GeneSeq, JSTPlus/JMEDPlus/JST7580(JDreamIII), PubMed

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 5858706 A (National University of Singapore), 12 January 1999 (12.01.1999), abstract; example 1 & US 5858706 A & US 5985590 A & US 5712144 A & SG 42456 A & SG 107125 A	1-9,11-18, 20-29,32-42 19
X Y	SG 94673 A1 (National University of Singapore), 18 March 2003 (18.03.2003), example 1 (Family: none)	1-9,11-18, 20-29,32-42 19

☒ 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
29 January, 2014 (29.01.14)	10 February, 2014 (10.02.14)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2013/083082

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 5712144 A (National University of Singapore), 27 January 1998 (27.01.1998), columns 19 to 20 & US 5712144 A & US 5858706 A & US 5985590 A & SG 42456 A & SG 107125 A & SG 107124 A	1-9,11-18, 20-29,32-42 19
X Y	WO 2008/004674 A1 (Seikagaku Corp.), 10 January 2008 (10.01.2008), paragraph [0105]; claims & US 2009/0208995 A1 & EP 2050820 A1 & EP 2495321 A1 & EP 2495322 A1 & CN 101454448 A & CN 103060349 A	1-4,7-11, 29-42 19
X Y	JP 2005-500520 A (Cambrez Bio Science Walkersville, Inc.), 06 January 2005 (06.01.2005), claims 16, 17 & JP 2005-500520 A & US 2003/0054432 A1 & US 2004/0235080 A1 & EP 1409984 A & WO 2003/002976 A2 & DE 60233142 D & BR 210681 A & CN 1529711 A & AT 437886 T & DK 1409984 T & ES 2329664 T & PT 1409984 E & AU 2002330860 B & MX PA03012032 A	1-4,7-11 19
X Y	WO 2012/118226 A1 (SEIKAGAKU CORP.), 07 September 2012 (07.09.2012), claims (Family: none)	1-4,7-11, 29-42 19
X	Xiangjun et al, Role of domains in factor C from Tachypleus tridentatus in neutralization endotoxin, "Accession:2006:239467" File CAPLUS [online],Retrived from the STN on the Web, [retrieved on 2014-01-29], Shengwu Huazue Yu Shengwu Wuli Jinzhan Vol.31,No.8,p736-740,2004	1-8,11-15, 20-29
A	JP 2006-87435 A (Seikagaku Corp.), 06 April 2006 (06.04.2006), paragraphs [0002], [0014] & JP 3781771 B & US 5795962 A & US 6077946 A & EP 669396 A1 & WO 1995/001432 A1 & DE 69435098 D & CA 2143621 A & CN 1111909 A	1-42

Electronic Acknowledgement Receipt

EFS ID:	39746655
Application Number:	16399386
International Application Number:	
Confirmation Number:	9293
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Customer Number:	23373
Filer:	Hui Chen Wauters/Sharon Jones
Filer Authorized By:	Hui Chen Wauters
Attorney Docket Number:	Q246419
Receipt Date:	17-JUN-2020
Filing Date:	30-APR-2019
Time Stamp:	14:25:44
Application Type:	Utility under 35 USC 111(a)

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	Response to Election / Restriction Filed	Q246419ResponsetoRestrictionandElectionofSpeciesRequirements.pdf	94665 594630ad47ec53e8e02a774e71865921c00da325	no	2

Warnings:

Information:					
2		Q246419IDS.pdf	184805 2ff038904b6c58c2d5704ee1a480f5098376846d	yes	4
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Transmittal Letter		1	2	
	Information Disclosure Statement (IDS) Form (SB08)		3	4	
Warnings:					
Information:					
3	Non Patent Literature	Q246419NPLREFUSOfficeAction04272020.pdf	6899349 6c54e35bacf78b426e738d59660c1a8319009192	no	33
Warnings:					
Information:					
4	Non Patent Literature	Q246419NPLREFUSOfficeAction05212020.pdf	3329204 92d83ef1c0a9c76943da83cf69df2e9aa006b947	no	17
Warnings:					
Information:					
5	Non Patent Literature	Q246419NPLREFKoshibaetal.pdf	4339055 56b9f9f9d298ad9f3392a67cc72952f992409fc2	no	13
Warnings:					
Information:					
6	Non Patent Literature	Q246419NPLREFCurrentProtocolsinMolecularBiology.pdf	6515641 45ecd8dab8d5b1e2beefe24fd976b1087d87bf76	no	13
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Information:					
7	Non Patent Literature	Q246419NPLREFSladeetal.pdf	1887094 c3d08c473a9f7a2d859e73c64923b20ac5c1ea12	no	12
Warnings:					
Information:					

8	Non Patent Literature	Q246419NPLREFInternationalPreliminaryReportonPatentability.pdf	630540 f301b086419d3ac941ea86e6530ad500c6f1d889	no	7
Warnings:					
Information:					
9	Non Patent Literature	Q246419NPLREFMizumuraetal.pdf	1965670 85f396510faca9d7420dc015b7b84e7ab9af3391	no	11
Warnings:					
Information:					
10	Non Patent Literature	Q246419NPLREFWrittenOpinion_2.pdf	521208 664d69c35b64e9b3dd812ab5ebad80a2c3f253ac	no	5
Warnings:					
Information:					
11	Non Patent Literature	Q246419NPLREFISR.pdf	398979 405187255273fc5498b39d76e3004124105c1563	no	2
Warnings:					
Information:					
12	Non Patent Literature	Q246419NPLREFStrategiesforProteinPurificationHandbookpage1thru60.pdf	10206298 329936663c964aec093af2b0b6eebdaebe62445d	no	60
Warnings:					
Information:					
13	Non Patent Literature	Q246419NPLREFStrategiesforProteinPurificationHandbookpage61thru110.pdf	9324625 82149af9b0d9360aed0b2fadbc360feefadc1e78	no	50
Warnings:					
Information:					
14	Non Patent Literature	Q246419NPLREFStrategiesforProteinPurificationHandbookpage111thru167.pdf	8979738 4f5ef22b253ce7b88556f3cd577c3750665c00a5	no	57
Warnings:					
Information:					
		Total Files Size (in bytes):	55276871		

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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United States Patent and Trademark Office
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P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/399,386	04/30/2019	Hikaru MIZUMURA	Q246419	9293

23373 7590 04/17/2020

SUGHRUE MION, PLLC
2000 PENNSYLVANIA AVENUE, N.W.
SUITE 900
WASHINGTON, DC 20006

EXAMINER
STEADMAN, DAVID J

ART UNIT	PAPER NUMBER
1656	

NOTIFICATION DATE	DELIVERY MODE
04/17/2020	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):

PPROCESSING@SUGHRUE.COM
USPTO@sughrue.com
sughrue@sughrue.com

Office Action Summary	**Application No.** 16/399,386	**Applicant(s)** MIZUMURA et al.	
	Examiner DAVID J STEADMAN	**Art Unit** 1656	**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 2 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 30 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) 1-18 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) _____ is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☑ Claim(s) 1-18 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 CORRESPONDENCE

Status of the Application

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Claims 1-18 are pending in the application.

Election/Restrictions

Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claims 1-13, drawn to a horseshoe crab Factor C protein, a Factor C protein, a method for producing a Factor C protein, and an endotoxin assay kit, classified in CPC C12N9/6424.

II. Claims 14-17, drawn to a method for measuring endotoxin, classified in CPC G01N33/579.

III. Claim 18, drawn to a product subjected to an endotoxin assay, classified in CPC A61K9/0026.

The inventions are distinct, each from the other because:

The protein of Group I and the method of Group II are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product. See MPEP § 806.05(h). In the instant case the protein of Group I can be used as an antigen in a method for producing an antibody.

The protein of Group I and the product of Group III are related by the protein of Group I. The related inventions are distinct if: (1) the inventions as claimed are either not capable of use together or can have a materially different design, mode of operation, function, or effect; (2) the inventions do not overlap in scope, i.e., are mutually exclusive; and (3) the inventions as claimed are not obvious variants. See MPEP § 806.05(j). In the instant case, the inventions of Groups I and III are distinct products and are considered to have a materially different design, mode of operation, function, or effect. Furthermore, the inventions as claimed do not encompass overlapping subject matter and there is nothing of record to show them to be obvious variants.

The product of Group III and the method of Group II are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product. See MPEP § 806.05(h). In the instant case the product of Group I can be used as a pharmaceutical in a method for disease treatment.

Restriction for examination purposes as indicated is proper because all these inventions listed in this action are independent or distinct for the reasons given above and there would be a serious search and examination burden if restriction were not required because of at least the following reasons: the inventions have acquired a separate status in the art in view of their different classifications and the inventions

require a different field of search (*e.g.,* searching different classes/subclasses or electronic resources, or employing different search strategies or search queries).

Applicant is advised that the reply to this requirement to be complete must include (i) an election of an invention to be examined even though the requirement may be traversed (37 CFR 1.143) and (ii) identification of the claims encompassing the elected invention.

The election of an invention may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected invention.

If claims are added after the election, applicant must indicate which of these claims are readable upon the elected invention.

Should applicant traverse on the ground that the inventions are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention.

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

Election of Species

If applicant elects the invention of Group III, the following species election is also required under 35 U.S.C. 121. This application contains claims directed to the following patentably distinct species of products:

A) the group of water for medical use, a pharmaceutical product, an infusion solution, a blood product, and a biological specimen;

B) the group of a medical apparatus and a medical instrument;

C) the group of a native protein and a recombinant protein;

D) a nucleic acid; and

E) a saccharide.

The species are independent or distinct because each of the species of A) to E) is a distinct product. In addition, these species are not obvious variants of each other based on the current record.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species, or a single grouping of patentably indistinct species, for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claim 18 is generic.

There is a search and/or examination burden for the patentably distinct species as set forth above because at least the following reason(s) apply: a separate text-based keyword search is required for each of the species of A) to E).

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a species to be examined even though the requirement may be traversed (37 CFR 1.143) and (ii) identification of the claims encompassing the elected species or grouping of patentably indistinct species, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

The election may be made with or without traverse. To preserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the election of species requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected species or grouping of patentably indistinct species.

Should applicant traverse on the ground that the species, or groupings of patentably indistinct species from which election is required, are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing them to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the species unpatentable over the prior art, the

evidence or admission may be used in a rejection under 35 U.S.C. 103 or pre-AIA 35 U.S.C. 103(a) of the other species.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which depend from or otherwise require all the limitations of an allowable generic claim as provided by 37 CFR 1.141.

Notice of Potential Rejoinder

The examiner has required restriction between product or apparatus claims and process claims. Where applicant elects claims directed to the product/apparatus, and all product/apparatus claims are subsequently found allowable, withdrawn process claims that include all the limitations of the allowable product/apparatus claims should be considered for rejoinder. All claims directed to a nonelected process invention must include all the limitations of an allowable product/apparatus claim for that process invention to be rejoined.

In the event of rejoinder, the requirement for restriction between the product/apparatus claims and the rejoined process claims will be withdrawn, and the rejoined process claims will be fully examined for patentability in accordance with 37 CFR 1.104. Thus, to be allowable, the rejoined claims must meet all criteria for patentability including the requirements of 35 U.S.C. 101, 102, 103 and 112. Until all claims to the elected product/apparatus are found allowable, an otherwise proper restriction requirement between product/apparatus claims and process claims may be maintained. Withdrawn process claims that are not commensurate in scope with an allowable product/apparatus claim will not be rejoined. See MPEP § 821.04.

Additionally, in order for rejoinder to occur, applicant is advised that the process claims should be amended during prosecution to require the limitations of the product/apparatus claims. Failure to do so may result in no rejoinder. Further, note that the prohibition against double patenting rejections of 35 U.S.C. 121 does not apply where the restriction requirement is withdrawn by the examiner before the patent issues. See MPEP § 804.01.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to DAVID J STEADMAN whose telephone number is (571)272-0942. The examiner can normally be reached on Monday to Friday, 7:30 AM to 4:00 PM.

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, MANJUNATH N. RAO can be reached on 571-272-0939. 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.

/David Steadman/
Primary Examiner, Art Unit 1656

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P.O. Box 1450
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BIB DATA SHEET

CONFIRMATION NO. 9293

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
16/399,386	04/30/2019 RULE	435	1656	Q246419

APPLICANTS

SEIKAGAKU CORPORATION, Tokyo, JAPAN;

INVENTORS

Hikaru MIZUMURA, Tokyo, JAPAN;
Toshio ODA, Tokyo, JAPAN;
Shun-ichiro KAWABATA, Fukuoka, JAPAN;

**** CONTINUING DATA ***************************

This application is a CON of 15/983,725 05/18/2018
which is a CON of 14/650,767 06/09/2015 PAT 10144923
which is a 371 of PCT/JP2013/083082 12/10/2013

**** FOREIGN APPLICATIONS ***************************

JAPAN 2012-269840 12/10/2012

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

05/10/2019

Foreign Priority claimed ☑ Yes ☐ No 35 USC 119(a-d) conditions met ☑ Yes ☐ No Verified and Acknowledged /DAVID J STEADMAN/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		JAPAN	10	18	2

ADDRESS

SUGHRUE MION, PLLC
2000 PENNSYLVANIA AVENUE, N.W.
SUITE 900
WASHINGTON, DC 20006
UNITED STATES

TITLE

NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

FILING FEE RECEIVED		
1720	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

BIB (Rev. 05/07).

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	16/399,386
Confirmation Number	9293
Filing Date	April 30, 2019
First Named Inventor	Hikaru MIZUMURA
Art Unit	1656
Examiner Name	STEADMAN, DAVID J
Attorney Docket Number	Q246419

U.S. PATENTS

Examiner Initials*	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

U.S. PATENT APPLICATION PUBLICATIONS

Examiner Initials*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No	Foreign Document Number[3]	Country Code[2]	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]

NON-PATENT LITERATURE DOCUMENTS

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, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1.	Bram Blomme et al., "Alteration of protein glycosylation in liver diseases", Journal of Hepatology, 2009, Vol. 50, pages 592-603 (12 pages total)	
	2.	Office Action dated February 12, 2020 issued in US Patent Application No. 15/983,725	
	3.	Stephanie Holst et al., "Profiling of different pancreatic cancer cells used as models for metastatic behaviour shows large variation in their N-glycosylation" Scientific Reports, November 30, 2017, Vol. 7, No. 16623, Pages 1-15 (15 pages total)	

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	16/399,386
	Confirmation Number	9293
	Filing Date	April 30, 2019
	First Named Inventor	Hikaru MIZUMURA
	Art Unit	1656
	Examiner Name	STEADMAN, DAVID J
	Attorney Docket Number	Q246419

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).

☑ That each item of information contained in the Information Disclosure Statement filed concurrently herewith: (i) was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement; or (ii) is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement. See 37 CFR 1.704(d).

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

☑ The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

☐ A certification statement is not submitted herewith.

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

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	/markboland/	Date (YYYY-MM-DD)	2020-03-05
Name/Print	Mark Boland	Registration Number	32,197

*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.

Electronic Acknowledgement Receipt

EFS ID:	38780590
Application Number:	16399386
International Application Number:	
Confirmation Number:	9293
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Customer Number:	23373
Filer:	John Michael Bird/Jessica White
Filer Authorized By:	John Michael Bird
Attorney Docket Number:	Q246419
Receipt Date:	05-MAR-2020
Filing Date:	30-APR-2019
Time Stamp:	15:15:10
Application Type:	Utility under 35 USC 111(a)

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	Non Patent Literature	Q246419REF1NPLBramBlommeetalAlterationofprotein.pdf	1695605 e6a92a5359f7c03e0f57be5dc1189c6ea7ae9cc2	no	12

Warnings:

Information:					
2	Non Patent Literature	Q246419REF2USOA15983725.pdf	1946694 631d92903b5df6d5e747d380d6a16f7d6c4934d1	no	34
Warnings:					
Information:					
3	Non Patent Literature	Q246419REF3NPLStephanieHolstetalProfilingofdifferent.pdf	2958563 864bdfb48d3fbe5ff6dfc02b76f74397bdc9991d	no	15
Warnings:					
Information:					
4		Q246419IDSasFiled.pdf	53216 9520fa84be9732aabbeab530fa8a2fa4c51ce487	yes	4

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Transmittal Letter	1	2
	Information Disclosure Statement (IDS) Form (SB08)	3	4

Warnings:	
Information:	
Total Files Size (in bytes):	6654078

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

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Docket No: Q246419
Hikaru MIZUMURA, et al.	
Appln. No.: 16/399,386	Group Art Unit: 1656
Confirmation No.: 9293	Examiner: STEADMAN, DAVID J
Filed: April 30, 2019	

For: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

INFORMATION DISCLOSURE STATEMENT
UNDER 37 C.F.R. §§ 1.97 and 1.98

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

Sir:

In accordance with the duty of disclosure under 37 C.F.R. § 1.56, Applicant hereby notifies the U.S. Patent and Trademark Office of the documents which are listed on the attached PTO/SB/08 (modified) form and/or listed herein and which the Examiner may deem material to patentability of the claims of the above-identified application.

One copy of each of the listed documents is submitted herewith. The citations are in English.

The present Information Disclosure Statement is being filed: (1) No later than three months from the application's filing date; (2) Before the mailing date of the first Office Action on the merits (whichever is later); or (3) Before the mailing date of the first Office Action after filing a request for continued examination (RCE) under §1.114, and therefore, no Statement

under 37 C.F.R. § 1.97(e) or fee under 37 C.F.R. § 1.17(p) is required. However, since a Statement can be made, one is submitted herewith.

The submission of the listed documents is not intended as an admission that any such document constitutes prior art against the claims of the present application. Applicant does not waive any right to take any action that would be appropriate to antedate or otherwise remove any listed document as a competent reference against the claims of the present application.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,

/markboland/

Mark Boland
Registration No. 32,197

SUGHRUE MION, PLLC
Telephone: 202.293.7060
Facsimile: 202.293.7860

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

Date: March 5, 2020

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	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/399,386	04/30/2019	Hikaru MIZUMURA	Q246419

CONFIRMATION NO. 9293

23373
SUGHRUE MION, PLLC
2000 PENNSYLVANIA AVENUE, N.W.
SUITE 900
WASHINGTON, DC 20006

PUBLICATION NOTICE

OC000000110895114

Title:NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

Publication No.US-2019-0270977-A1
Publication Date:09/05/2019

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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United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
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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
16/399,386	04/30/2019	1653	1720	Q246419	18	2

CONFIRMATION NO. 9293

FILING RECEIPT

23373
SUGHRUE MION, PLLC
2100 PENNSYLVANIA AVENUE, N.W.
SUITE 800
WASHINGTON, DC 20037

OC000000108484459

Date Mailed: 05/29/2019

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)

Hikaru MIZUMURA, Tokyo, JAPAN;
Toshio ODA, Tokyo, JAPAN;
Shun-ichiro KAWABATA, Fukuoka, JAPAN;

Applicant(s)

SEIKAGAKU CORPORATION, Tokyo, JAPAN;

Assignment For Published Patent Application

SEIKAGAKU CORPORATION, Tokyo, JAPAN

Power of Attorney: The patent practitioners associated with Customer Number 23373

Domestic Priority data as claimed by applicant

This application is a CON of 15/983,725 05/18/2018
which is a CON of 14/650,767 06/09/2015 PAT 10144923
which is a 371 of PCT/JP2013/083082 12/10/2013

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.)
JAPAN 2012-269840 12/10/2012 No Access Code Provided

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: 05/10/2019

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/399,386**

Projected Publication Date: 09/05/2019

Non-Publication Request: No

Early Publication Request: No

Title

NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

Preliminary Class

435

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

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Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 16/399,386

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	660
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))	18 minus 20 =	*			OR	x 100 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2 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	1720

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	RATE($)	ADDITIONAL FEE($)	OR	RATE($)	ADDITIONAL FEE($)
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.

RAW SEQUENCE LISTING

Loaded by SCORE, no errors detected.

Application Serial Number: 16399386
Source: OPAP
Date Processed by SCORE: 05/03/2019

ENTERED

SEQUENCE LISTING

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<120> Novel Recombinant Factor C and Method for Producing the Same, and
Method for Measuring Endotoxin

<130> Q220395

<140> US 16/399,386
<141> 2019-04-30

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2112
Pro Arg Ser Ser Gln Pro Ser Thr Val Asp Leu Ala Ser Lys Val Lys
    690                 695                 700
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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	April 30, 2019		
First Named Inventor	Hikaru MIZUMURA		
Title	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN		
Art Unit	Not Yet Assigned		
Examiner Name	Not Yet Assigned		
Attorney Docket Number	Q246419		
SIGNATURE of Applicant or Patent Practitioner			
Signature	/markboland/	Date (Optional)	April 30, 2019
Name	Mark Boland	Registration Number	32,197
Title (if Applicant is a juristic entity)			
Applicant Name (if Applicant is a juristic entity)			
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 1 forms are submitted.			

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I hereby appoint:

Patent Practitioner(s) associated with the following Customer Number:

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

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The address associated with the above-mentioned Customer Number.

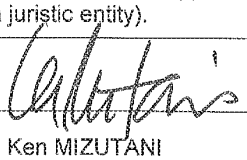
I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

SEIKAGAKU CORPORATION

- ☐ 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)	May, 22, 2015
Name	Ken MIZUTANI		
Title	President		

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 1 forms are submitted.

Electronic Acknowledgement Receipt

EFS ID:	35880009
Application Number:	16399386
International Application Number:	
Confirmation Number:	9293
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Customer Number:	23373
Filer:	Arun Shome/Veronica Gardner
Filer Authorized By:	Arun Shome
Attorney Docket Number:	Q246419
Receipt Date:	30-APR-2019
Filing Date:	
Time Stamp:	17:09:19
Application Type:	Utility under 35 USC 111(a)

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	Power of Attorney	Q246419POATransmittalwithPOAExecuted.pdf	262170 8c6c8c99ad15a8a613712c226c65d1374c54a3fe	no	2

Warnings:

Information:	
Total Files Size (in bytes):	262170

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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

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Electronic Patent Application Fee Transmittal

Application Number:	16399386
Filing Date:	
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Filer:	Arun Shome/Veronica Gardner
Attorney Docket Number:	Q246419

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY APPLICATION FILING	1011	1	300	300
UTILITY SEARCH FEE	1111	1	660	660
UTILITY EXAMINATION FEE	1311	1	760	760
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**1720**

Electronic Acknowledgement Receipt	
EFS ID:	35879906
Application Number:	16399386
International Application Number:	
Confirmation Number:	9293
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Correspondence Address:	SUGHRUE MION, PLLC - 2100 PENNSYLVANIA AVENUE, N.W. SUITE 800 WASHINGTON DC 20037 US 202-293-7060 PPROCESSING@SUGHRUE.COM
Filer:	Arun Shome/Veronica Gardner
Filer Authorized By:	Arun Shome
Attorney Docket Number:	Q246419
Receipt Date:	30-APR-2019
Filing Date:	
Time Stamp:	17:05:26
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$1720

RAM confirmation Number	050119INTEFSW17060900
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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	Fee Worksheet (SB06)	fee-info.pdf	35415 a23ca5ae2e87fc4370bb797daa165939670b66d2	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	35415		

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National Stage of an International Application under 35 U.S.C. 371
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	Not Yet Assigned
Confirmation Number	Not Yet Assigned
Filing Date	April 30, 2019
First Named Inventor	Hikaru MIZUMURA
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	Q246419

U.S. PATENTS

Examiner Initials*	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.	5712144	A	1998-01-27	Ding et al.	
	2.	5795962	A	1998-08-18	Iwanaga et al.	
	3.	5858706	A	1999-01-12	Ding et al.	
	4.	6077946	A	2000-01-20	Iwanaga et al.	
	5.	5716834	A	1998-02-	Ding, et al.	

U.S. PATENT APPLICATION PUBLICATIONS

Examiner Initials*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, where Relevant Passages or Relevant Figures Appear
	6.	20030054432	A1	2003-03-20	Chen et al.	
	7.	20040235080	A1	2004-11-25	Chen et al.	
	8.	20080131929	A1	2008-06-05	Prentice et al.	
	9.	20090208995	A1	2009-08-20	Tamura et al.	
	10.	20100086967	A1	2010-04-08	Kaufmann et al.	
	11.	20130295613	A1	2013-11-07	Kishishita et al.	
	12.	20140024062	A1	2014-01-23	Mizumura et al.	
	13.	20140249082	A1	2014-09-04	Cottingham et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No	Foreign Document Number[3]	Country Code[2]	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]
	14.	2005-500520	JP	A	2005-01-06	CAMBREX BIO SCIENCE WALKERVILLE INC		Corresponds to cites 6 and 7; Cited in cite 31
	15.	2006-87435	JP	A	2006-04-06	SEIKAGAKU KOGYO CO LTD		English Abstract; Corresponds to cites 3 and 4; Cited in cite 31
	16.	2008/004674	WO	A1	2008-01-10	SEIKAGAKU CORPORATION		English Abstract; Corresponds to cite 5; Cited in cite 31
	17.	2008520250	JP	A	2008-06-19	BIOGEN IDEC MA INC.		Corresponds to US 20080131929; Cited in JP comm. dated 8/7/18 in 2014-552048

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[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	Not Yet Assigned
Confirmation Number	Not Yet Assigned
Filing Date	April 30, 2019
First Named Inventor	Hikaru MIZUMURA
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	Q246419

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	18.	2008-520250	JP	A	2008-06-19	BIOGEN IDEC MA INC.,		Cited in JPOA 8/7/18 in no. 2014-552048; Corresponds to US 2008/0131929 A1
	19.	2010516264	JP	A	2010-05-20	BOEHRINGER INGELHEIM PHARMA GMBH & CO. KG		Corresponds to US 2010086967; Cited in JP comm. dated 8/7/18 in 2014-552048
	20.	2010-516264	JP	A	2010-05-20	BOEHRINGER INGELHEIM PHARMA GMBH & CO. KG,		Cited in JPOA 8/7/18 in no. 2014-552048; Corresponds to US 2010/0086967 A1
	21.	2012/118226	WO	A1	2012-09-07	SEIKAGAKU CORPORATION		Corresponds to cite 8; Cited in cite 31
	22.	2012091124	WO	A1	2012-07-05	CHUGAI SEIYAKU KABUSHIKI KAISHA		Abstract; Corresponds to US 20130295613; Cited in JP comm. dated 8/7/18 in 2014-552048
	23.	94673	SG	A1	2003-03-18	NATIONAL UNIVERSITY OF SINGAPORE		Cited in cite 31
	24.	9915676	WO	A1	1999-04-01	National University of Singapore		

NON-PATENT LITERATURE DOCUMENTS

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, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	25.	Communication dated April 19, 2017, from the State Intellectual Property Office of the P.R.C., in counterpart Chinese application no. 201380064649.3	Translation
	26.	Communication dated August 7, 2018 issued by the Japanese Patent Office in counterpart application no. 2014-552048	Machine Translation
	27.	Communication dated August 7, 2018, from the Japanese Patent Office in counterpart application no. 2014-552048	
	28.	Communication dated July 29, 2016 from the State Intellectual Property Office of the P.R.C. in counterpart application no. 201380064649.3	
	29.	Communication dated July 5, 2016, from the European Patent Office in counterpart European Application No. 13862466.3	
	30.	Extended European Search Report dated January 10, 2019 issued by the European Patent Office in counterpart application no. 18190686.8	

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[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	Not Yet Assigned
	Confirmation Number	Not Yet Assigned
	Filing Date	April 30, 2019
	First Named Inventor	Hikaru MIZUMURA
	Art Unit	Not Yet Assigned
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	Q246419

NON-PATENT LITERATURE DOCUMENTS			
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, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	31.	International Search Report for PCT/JP2013/083082 dated February 10, 2012	English Translation
	32.	JEAK L. DING et al., "A new era in pyrogen testing", TRENDS in Biotechnology, August 2001, pp. 277-281, Vol. 19, No. 8	
	33.	Jeak Ling Ding et al., "Chapter 9, Endotoxin Detection - from Limulus Amebocyte Lysate to Recombinant Factor C ", Endotoxins: Structure, Function and Recognition SERIES : SUBCELLULAR BIOCHEMISTRY IN: SUBCELLULAR BIOCHEMISTRY: REVIEWS AND ESSAYS DEALING WITH THE FUNCTION, GENETICS, BIOGENESIS AND EVOLUTION OF SUB-CELLULAR COMPONENTS, vol. 53, January 1, 2010, XP009510157, 22 pages total	
	34.	JING WANG et al., "Functional expression of full length Limulus Factor C in stably transformed Sf9 cells", Biotechnology Letters, 2001, pp. 71-76, Vol. 23	
	35.	JING WANG et al., "Modular Arrangement and Secretion of a Multidomain Serine Protease", The Journal of Biological Chemistry, September 27, 2002, pp. 36363-36372, Vol. 277, No. 39	
	36.	Mizumura, et al., Genetic engineering approach to develop next-generation reagents for endotoxin quantification; Innate Immunity, vol. 23, no. 2, pp. 136-146	
	37.	Nakamura T. et al. "Lipopolysaccharide-sensitive serine-protease zymogen (factor C) found in Limulus hemocytes" European Journal of Biochemistry vol. 154, 1986; pages 511-521	
	38.	Office Action issued in the corresponding European Application No. 13862466.3 dated November 30, 2017	
	39.	ROOPASHREE S. DWARAKANATH et al., "Recombinant COS-1 cells espress Carcinoscorpius rotundicauda Factor C", Biotechnology Letters, April 1997, pp. 357-361, Vol. 19, No. 4	
	40.	SADAAKI IWANAGA, "The limulus clotting reaction", Current Opinion in Immunology 1993, pp. 74-82, Vol. 5	
	41.	Shengwu Wuli Jinzhan Vol.31, No.8, p. 736-740, 2004	
	42.	TAKANORI NAKAMURA et al., "Purification and Properties of Intracellular Clotting Factor, Factor B, from Horseshoe Crab (Tachypleus tridentatus) Hemocytes", J. Biochem, 1986, Vol. 99, No. 3	
	43.	Tokunaga F. et al. "Lipopolysaccharide-sensitive serine-protease zymogen factor C of horseshoe crab hemocytes identification and alignment of proteolytic fragments produced during the activation show that it is a novel type of serine protease" European Journal of Biochemistry vol. 167 no. 3, 1987; pages 405-416	
	44.	XIANGJUN et al., "Role of domains in factor C from Tachypleus tridentatus in neutralization endotoxin", "Accession:2006:239467" File CAPLUS	
	45.	XIANGJUN et al., "Role of domains in factor C from Tachypleus tridentatus in neutralization endotoxin", "Accession:2006:239467" File CAPLUS [online],Retrived from the STN on the Web, [retrieved on 2014-01-29], Shengwu Huaxue Yu Shengwu Wuli Jinzhan Vol.31, No.8, p736-740, 2004	

EXAMINER SIGNATURE			
Examiner Signature		Date Considered	

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[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	Not Yet Assigned
	Confirmation Number	Not Yet Assigned
	Filing Date	April 30, 2019
	First Named Inventor	Hikaru MIZUMURA
	Art Unit	Not Yet Assigned
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	Q246419

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).

☐ That each item of information contained in the Information Disclosure Statement filed concurrently herewith: (i) was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement; or (ii) is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement. See 37 CFR 1.704(d).

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

☑ The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

☐ A certification statement is not submitted herewith.

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

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	/markboland/	Date (YYYY-MM-DD)	April 30, 2019
Name/Print	Mark Boland	Registration Number	32,197

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[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.

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Hikaru MIZUMURA, et al.

Docket No: Q246419

Appln. No.: Not Yet Assigned

Group Art Unit: Not Yet Assigned

Confirmation No.: Not Yet Assigned

Examiner: Not Yet Assigned

Filed: April 30, 2019

For: NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN

INFORMATION DISCLOSURE STATEMENT UNDER 37 C.F.R. §§ 1.97 and 1.98

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

Sir:

In accordance with the duty of disclosure under 37 C.F.R. § 1.56, Applicant hereby notifies the U.S. Patent and Trademark Office of the documents which are listed on the attached PTO/SB/08 A & B form which are all the references of record in parent application **No. 15/983,725, filed May 18, 2018**. Applicant is not submitting duplicate copies of these references but requests that they be listed on the face of any patent granted on the above application. (See 37 CFR §1.98(d)).

The submission of the listed documents is not intended as an admission that any such document constitutes prior art against the claims of the present application. Applicant does not waive any right to take any action that would be appropriate to antedate or otherwise remove any listed document as a competent reference against the claims of the present application.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,

/markboland/

Mark Boland
Registration No. 32,197

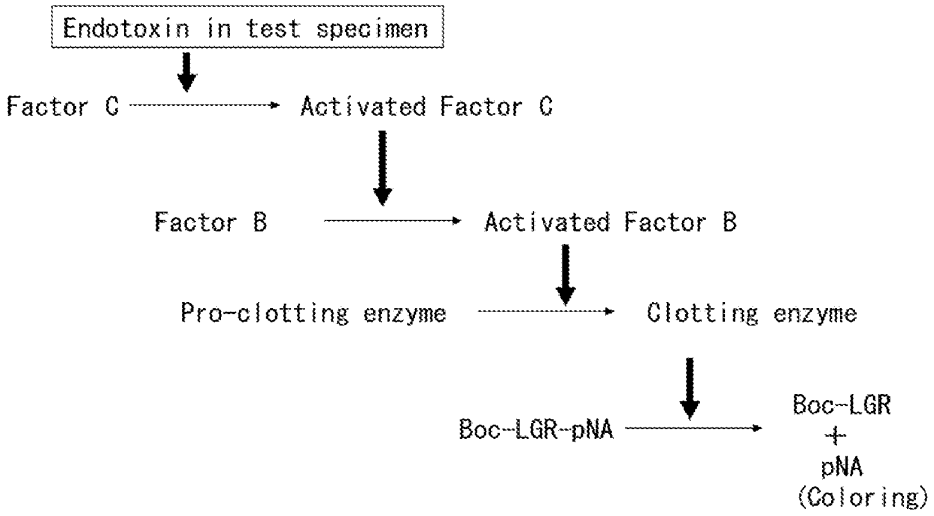
SUGHRUE MION, PLLC
Telephone: 202.293.7060
Facsimile: 202.293.7860

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23373
CUSTOMER NUMBER

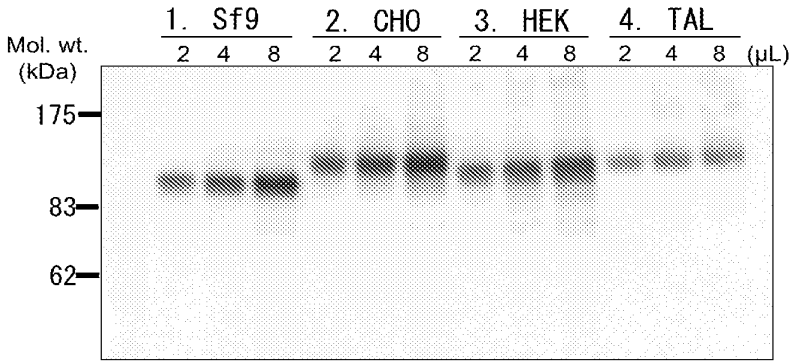
Date: April 30, 2019

Figures

[Fig. 1]

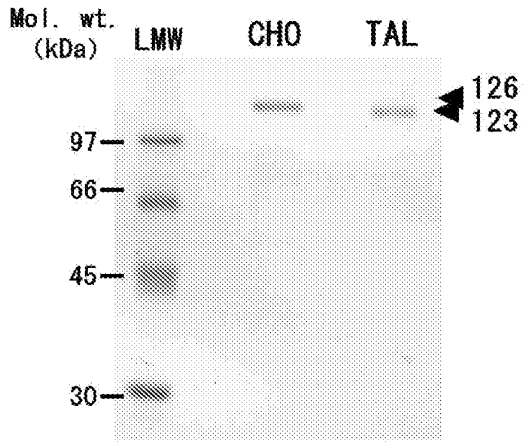


[Fig. 2]



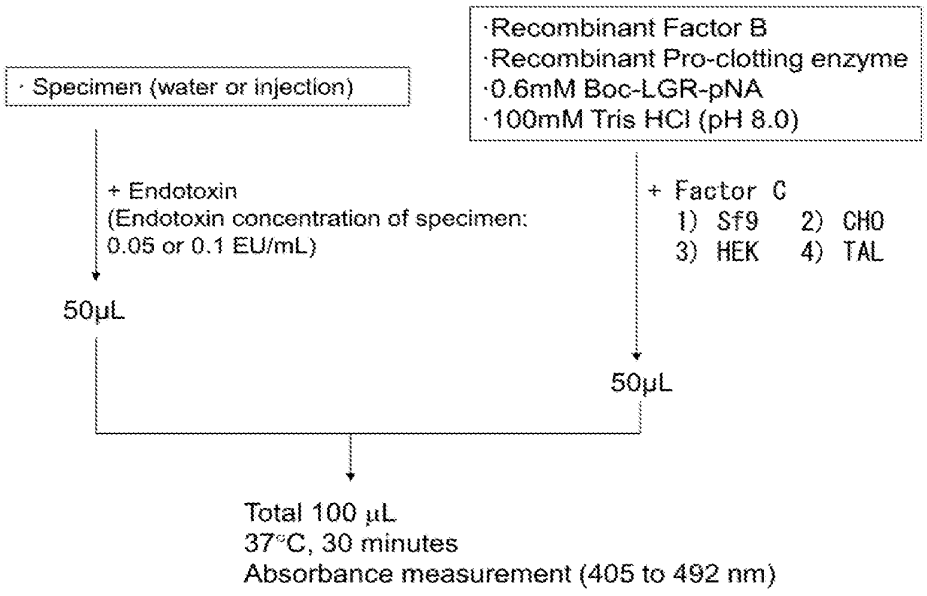
Primary antibody: Anti-factor C monoclonal antibody (2C12)
Electrophoresis conditions: non-reducing

[Fig. 3]

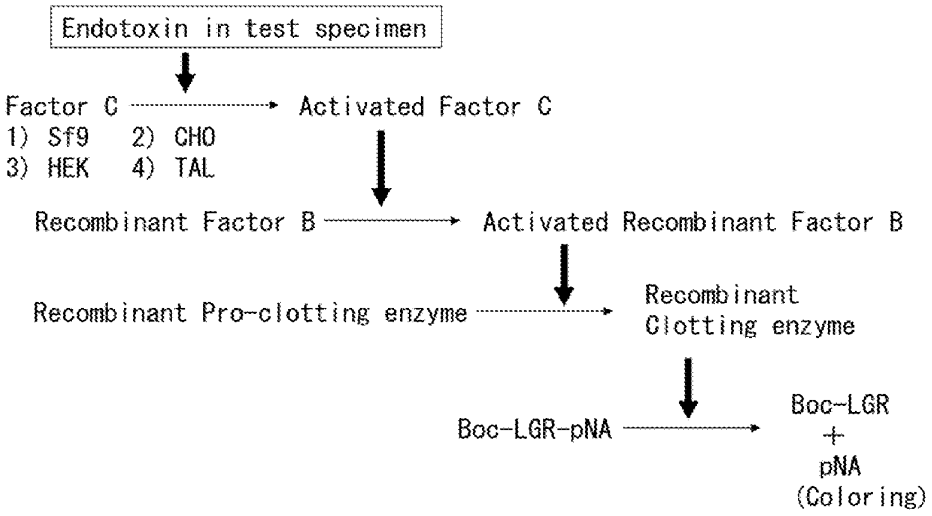


15% Gel (Non-reducing)
CBB staining

[Fig. 4]



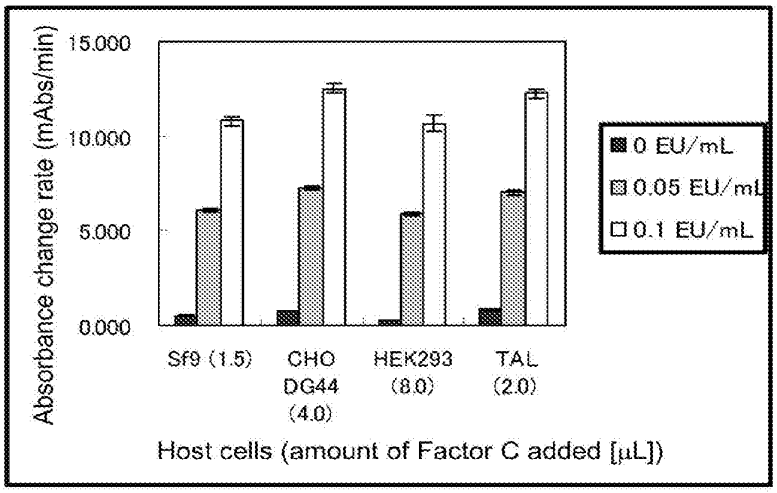
[Fig. 5]


Endotoxin in test specimen
Factor C
Activated Factor C
1) Sf9 2) CHO
3) HEK 4) TAL
Recombinant Factor B
Activated Recombinant Factor B
Recombinant Pro-clotting enzyme
Recombinant
Clotting enzyme
Boc-LGR-pNA
Boc-LGR
+
pNA
(Coloring)

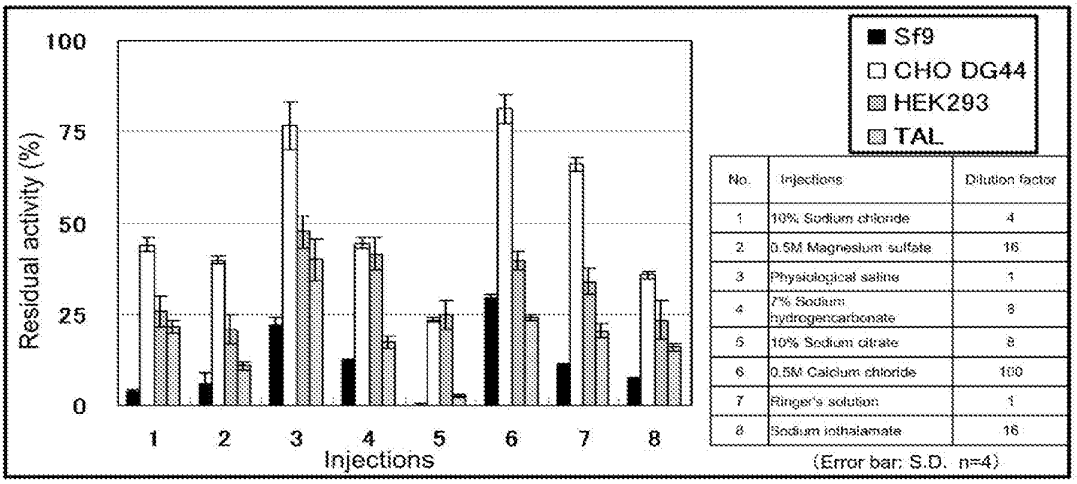
[Fig. 6]

Host cells for Factor C	Amount of Factor C added (μL)	Endotoxin concentration (EU/mL)	Absorbance change rate (av. ± S.D.) (mABs/min)
Sf9	1.5	0	0.520 ± 0.015
		0.05	6.095 ± 0.108
		0.1	10.795 ± 0.246
CHO DG44	4	0	0.770 ± 0.021
		0.05	7.260 ± 0.087
		0.1	12.510 ±0.255
HEK293	8	0	0.300 ± 0.017
		0.05	5.885 ± 0.125
		0.1	10.685 ± 0.417
TAL	2	0	0.840 ± 0.055
		0.05	7.030 ± 0.140
		0.1	12.270 ± 0.255

n=3

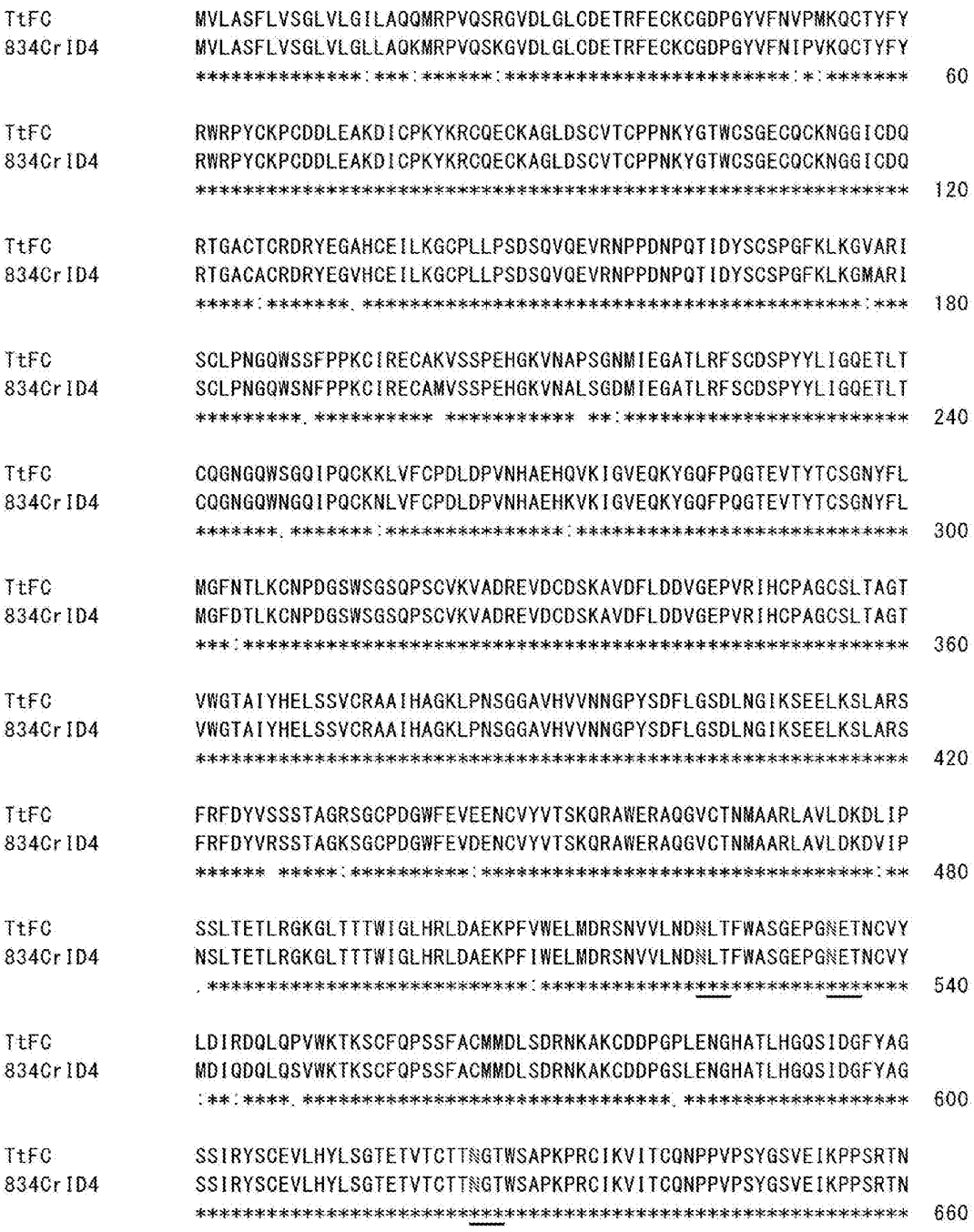


[Fig. 7]

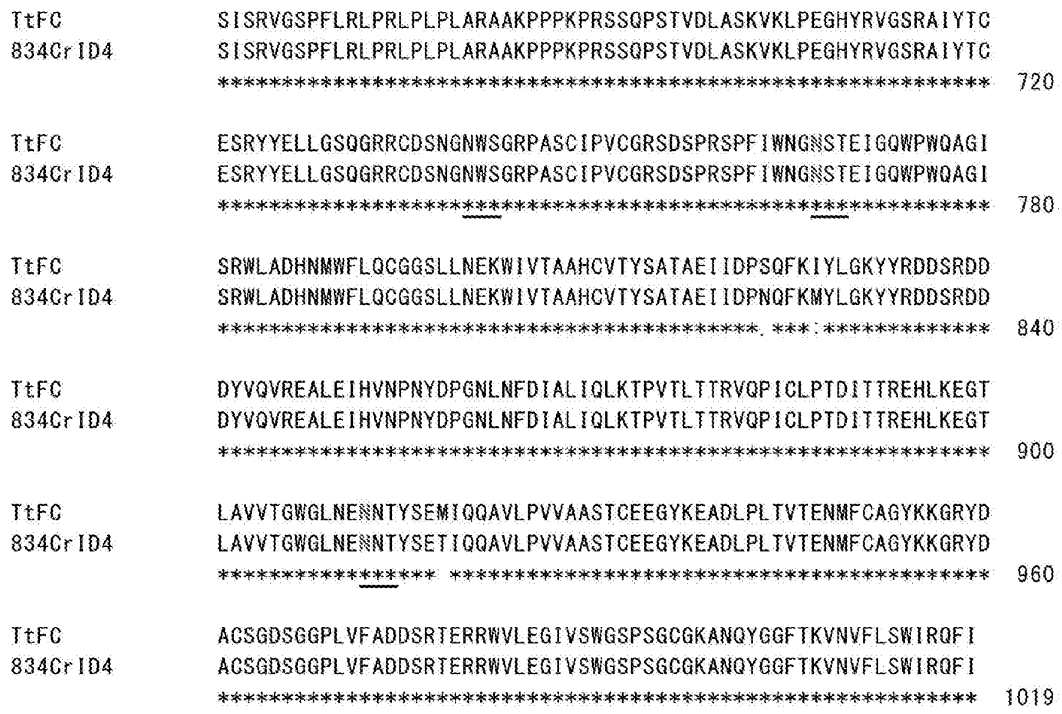


No.	Injections	Dilution factor
1	10% Sodium chloride	4
2	0.5M Magnesium sulfate	16
3	Physiological saline	1
4	7% Sodium hydrogencarbonate	8
5	10% Sodium citrate	8
6	0.5M Calcium chloride	100
7	Ringer's solution	1
8	Sodium isothalamate	16

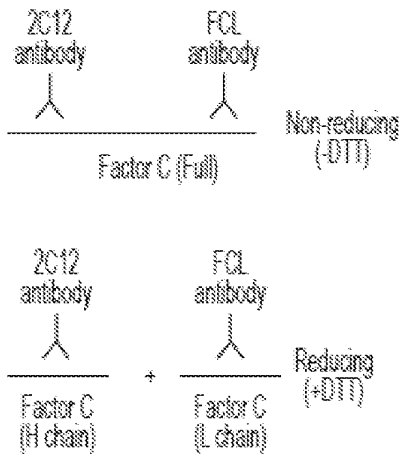
[Fig. 8]


TtFC MVLASFLVSGLVLGILAQQMRPVQSRGVDLGLCDETRFECKCGDPGYVFNVPMKQCTYFY
834CrID4 MVLASFLVSGLVLGLLAQKMRPVQSKGVDLGLCDETRFECKCGDPGYVFNIPVKQCTYFY
60
TtFC RWRPYCKPCDDLEAKDICPKYKRCQECKAGLDSCVTCPPNKYGTWCSGECQCKNGGICDQ
834CrID4 RWRPYCKPCDDLEAKDICPKYKRCQECKAGLDSCVTCPPNKYGTWCSGECQCKNGGICDQ
120
TtFC RTGACTCRDRYEGAHCEILKGCPLLPSDSQVQEVRNPPDNPQTIDYSCSPGFKLKGVARI
834CrID4 RTGACACRDRYEGVHCEILKGCPLLPSDSQVQEVRNPPDNPQTIDYSCSPGFKLKGMARI
180
TtFC SCLPNGQWSSFPPKCIRECAKVSSPEHGKVNAPSGNMIEGATLRFSCDSPYYLIGQETLT
834CrID4 SCLPNGQWSNFPPKCIRECAMVSSPEHGKVNALSGDMIEGATLRFSCDSPYYLIGQETLT
240
TtFC CQGNGQWSGQIPQCKKLVFCPDLDPVNHAEHQVKIGVEQKYGQFPQGTEVTYTCSGNYFL
834CrID4 CQGNGQWNGQIPQCKNLVFCPDLDPVNHAEHKVKIGVEQKYGQFPQGTEVTYTCSGNYFL
300
TtFC MGFNTLKCNPDGSWSGSQPSCVKVADREVDCDSKAVDFLDDVGEPVRIHCPAGCSLTAGT
834CrID4 MGFDTLKCNPDGSWSGSQPSCVKVADREVDCDSKAVDFLDDVGEPVRIHCPAGCSLTAGT
360
TtFC VWGTAIYHELSSVCRAAIHAGKLPNSGGAVHVVNNGPYSDFLGSDLNGIKSEELKSLARS
834CrID4 VWGTAIYHELSSVCRAAIHAGKLPNSGGAVHVVNNGPYSDFLGSDLNGIKSEELKSLARS
420
TtFC FRFDYVSSSTAGRSGCPDGWFEVEENCVYVTSKQRAWERAQGVCTNMAARLAVLDKDLIP
834CrID4 FRFDYVRSSTAGKSGCPDGWFEVDENCVYVTSKQRAWERAQGVCTNMAARLAVLDKDVIP
480
TtFC SSLTETLRGKGLTTTWIGLHRLDAEKPFVWELMDRSNVVLNDNLTFWASGEPGNETNCVY
834CrID4 NSLTETLRGKGLTTTWIGLHRLDAEKPFIWELMDRSNVVLNDNLTFWASGEPGNETNCVY
540
TtFC LDIRDQLQPVWKTKSCFQPSSFACMMDLSDRNKAKCDDPGPLENGHATLHGQSIDGFYAG
834CrID4 MDIQDQLQSVWKTKSCFQPSSFACMMDLSDRNKAKCDDPGSLENGHATLHGQSIDGFYAG
600
TtFC SSIRYSCEVLHYLSGTETVTCTTNGTWSAPKPRCIKVITCQNPPVPSYGSVEIKPPSRTN
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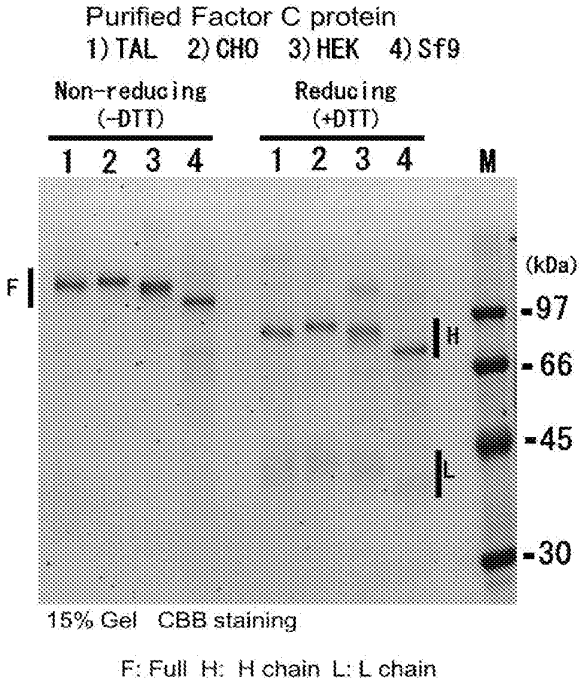
[Fig. 9]


TtFC SISRVGSPFLRLPRLPLPLARAAKPPPKPRSSQPSTVDLASKVKLPEGHYRVGSRAIYTC
834CrID4 SISRVGSPFLRLPRLPLPLARAAKPPPKPRSSQPSTVDLASKVKLPEGHYRVGSRAIYTC
720
TtFC ESRYYELLGSQGRRCDSNGNWSGRPASCIPVCGRSDSPRSPFIWNGNSTEIGQWPWQAGI
834CrID4 ESRYYELLGSQGRRCDSNGNWSGRPASCIPVCGRSDSPRSPFIWNGNSTEIGQWPWQAGI
780
TtFC SRWLADHNMWFLQCGGSLLNEKWIVTAAHCVTYSATAEIIDPSQFKIYLGKYYRDDSRDD
834CrID4 SRWLADHNMWFLQCGGSLLNEKWIVTAAHCVTYSATAEIIDPNQFKMYLGKYYRDDSRDD
840
TtFC DYVQVREALEIHVNPNYDPGNLNFDIALIQLKTPVTLTTRVQPICLPTDITTREHLKEGT
834CrID4 DYVQVREALEIHVNPNYDPGNLNFDIALIQLKTPVTLTTRVQPICLPTDITTREHLKEGT
900
TtFC LAVVTGWGLNENNTYSEMIQQAVLPVVAASTCEEGYKEADLPLTVTENMFCAGYKKGRYD
834CrID4 LAVVTGWGLNENNTYSETIQQAVLPVVAASTCEEGYKEADLPLTVTENMFCAGYKKGRYD
960
TtFC ACSGDSGGPLVFADDSRTERRWVLEGIVSWGSPSGCGKANQYGGFTKVNVFLSWIRQFI
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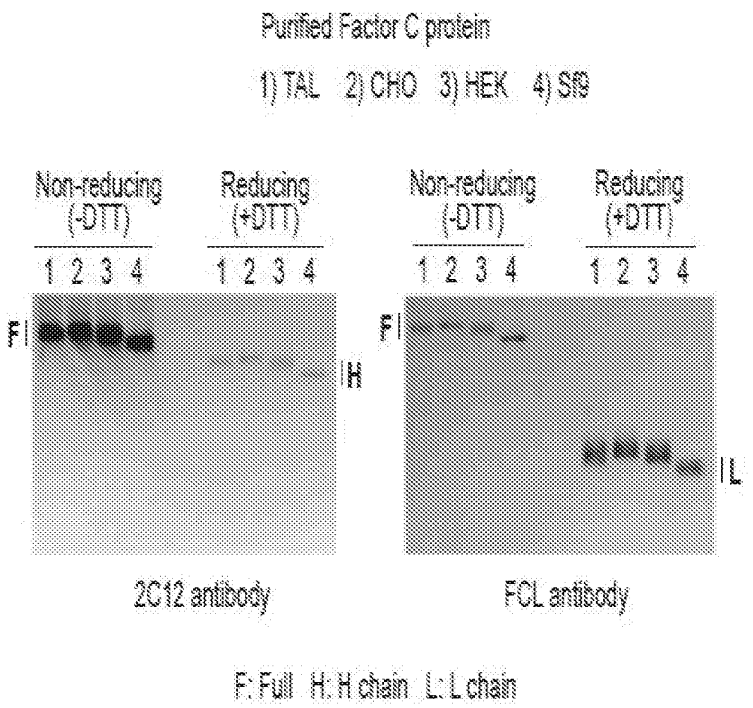
[Fig. 10]


2C12 antibody
FCL antibody
Non-reducing (-DTT)
Factor C (Full)
2C12 antibody
FCL antibody
+
Reducing (+DTT)
Factor C (H chain)
Factor C (L chain)

[Fig. 11]

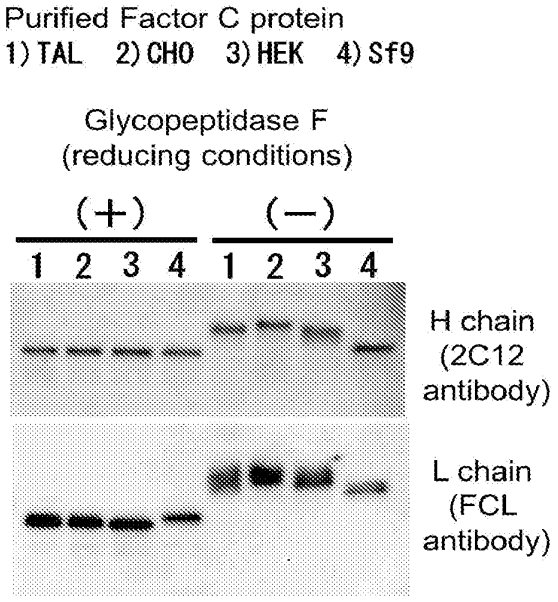

Purified Factor C protein
1) TAL 2) CHO 3) HEK 4) Sf9
Non-reducing (-DTT)
Reducing (+DTT)
1 2 3 4 1 2 3 4 M
(kDa)
F
H
L
-97
-66
-45
-30
15% Gel CBB staining
F: Full H: H chain L: L chain

[Fig. 12]

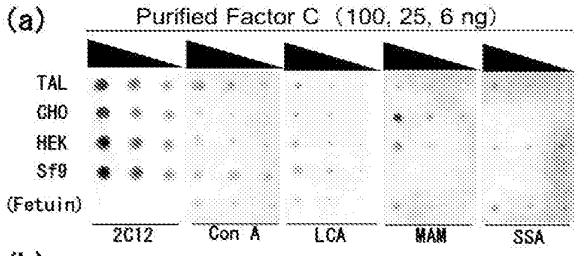


F: Full H: H chain L: L chain

[Fig. 13]



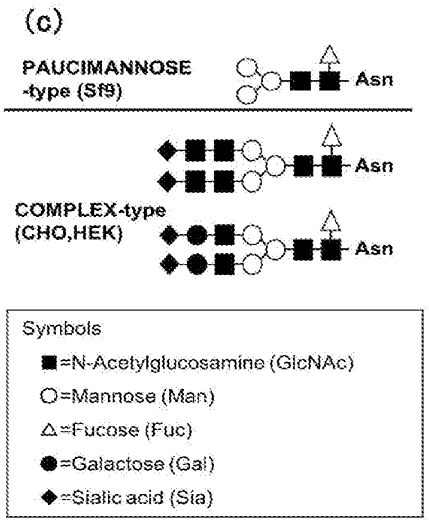
[Fig. 14]



(b)

Binding specificity of Lectin

Name	Origin	Specificity
Con A	*Canavalia ensiformis*	Biantennaly Hybrid type, High mannose type, Complex type
LCA	*Lens culinaris*	Fucose linked α(1,6) to core GlcNAc of N-linked glycopeptide
MAM	*Maackia amurensis*	Sia(α-2,3)Gal(β-1,4)GlcNAc
SSA	*Sambucas sieboldiana*	Sia(α-2,6)Gal



Docket No.:
Only After Sept 16, 2012

ASSIGNMENT WITH DECLARATION FOR PATENT APPLICATION (37 CFR 1.63)

特許出願宣言書付き譲渡書 (37 CFR 1.63)

Japanese Language Assignment with Declaration

下記に署名した発明者であり、以下に譲渡人と称される私（我々）は、下記の出願に記載された改良に関する発明をし、

_____ の_____（譲受人）は、本件出願および本件発明、ならびに当該出願から得られるいかなる米国特許に関する全ての権利、権原、および利益の取得を要求します。

よって、価値ある対価の受領をここに確認し、

上で譲渡人として指名された私（我々）は、米国における本件出願およびその発明開示、全ての分割出願および継続出願、これら出願について許可された米国特許証、全ての再発行特許、ならびに全ての外国出願に関する全ての権利（改正後のパリ条約（1883 年）に基づく優先権、ならびに 35 USC 119 に基づき優先権を主張する権利と過去分の損害賠償請求権を含む）、権原、および利益を、上で指定した譲受人、その承継人ないし権利承継者に売却、譲渡、移転するとともに、私（我々）は米国特許商標庁長官に対し、本件出願に記載の発明に対する特許証を、譲受人、その承継人ないし権利承継者に発行することを要請し、また、私（我々）は、譲受人が本件発明に係る外国特許出願を行うことに同意し、これ以上の対価なしに、譲受人の求めにより、本件米国・外国特許出願に関して譲受人が必要とみなす全ての書類を作成します。

（公証人による認証は不要であるが、これらの書類は、35 USC 261 に基づく推定された証拠となる）

私は，下記に記載の発明者として，以下の通り宣言します。

本宣言付き譲渡書は、以下に関します。

☐ 添付の出願書、または、

☐ _____年__月__日に出願された米国出願あるいは PCT 国際出願番号_____（確認番号_____）

本件出願の名称は、以下の通りです。

_____

_____

_____

Whereas, I/We, the undersigned inventor(s) hereinafter called assignor(s), have invented certain improvements described in the application identified below; and

Whereas, SEIKAGAKU CORPORATION of 6-1, Marunouchi 1-chome, Chiyoda-ku, Tokyo 100-0005 Japan, (assignee), desires to acquire the entire right, title, and interest in the application and invention, and to any United States patents to be obtained therefor;

Now therefore, for valuable consideration, receipt whereof is hereby acknowledged,

I/We, the above named assignor(s), hereby sell, assign and transfer to the above named assignee, its successors and assigns, the entire right, title and interest in the application and the invention disclosed therein for the United States of America, including all divisions, and continuations thereof, and all Letters Patent of the United States that may be granted thereon, and all reissues thereof, and all countries foreign thereto, including rights of priority under the International Convention of Paris (1883) as amended, including the right to claim priority under 35 USC 119 and the right to sue for past damages, and I/we request the Director of the U.S. Patent and Trademark Office to issue any Letters Patent granted upon the invention set forth in the application to the assignee, its successors and assigns; and I/we hereby agree that the assignee may apply for foreign Letters Patent on the invention and I/we will execute without further consideration all papers deemed necessary by the assignee in connection with the United States and foreign applications when called upon to do so by the assignee.

(Legalization not required for recording but is prima facie evidence of execution under 35 USC 261)

As the below named inventor, I hereby declare that:

This assignment with declaration is directed to:

☐ The attached application, or

☒ United States Application or PCT International Application Number PCT/JP2013/083082 filed on December 10, 2013 (Confirmation No. _____).

The application is entitled:

Novel Recombinant Factor C and Method for Producing the Same, and Method for Measuring Endotoxin

Japanese Language Assignment with Declaration

上記に明示された出願は，私が作成した、または作成を許可したものです。

私は、本件出願において請求項に記載された発明の本来の発明者、または、本来の共同発明者であると信じています。

私は、本宣言付き譲渡書の提出に関わる本件出願の内容を検討し、理解しています。

私は、37 CFR 1.56 に定義されるように、自身が知る全ての特許性に関する重要な情報を、米国特許商標庁に開示する義務があることを認識しています。

私は、本宣言付き譲渡書において故意に虚偽の供述を行った場合、18 USC 1001 に基づき、罰金あるいは 5 年以下の懲役、または両方による処罰の対象となることを認識しています。

The above identified application was made or was 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.

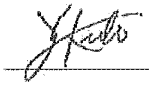
I have reviewed and understand the contents of the application for which this assignment with declaration is being submitted.

I am aware of the duty to disclose to the Office all information known to me to be material to patentability as defined in 37 CFR 1.56.

I hereby acknowledge that any willful false statement made in this assignment with declaration is punishable under 18 USC 1001 by fine or imprisonment of not more than five (5) years, or both.

STATEMENT OF ACCURATE TRANSLATION IN ACCORDANCE WITH 37 CFR 1.69(b):

The assignment with declaration is an accurate translation of the corresponding English language assignment with declaration.

Signature 

Date March 11, 2014

NAME OF SOLE OR FIRST INVENTOR: 唯一あるいは第一の発明者名	
Given Name (first and middle [if any]) 名（名およびミドルネーム[該当する場合]） Hikaru	Family Name or Surname 姓 MIZUMURA
Inventor's signature 発明者の署名 Hikaru Mizumura	Date 日付 May. 22. 2015
Residence: 住所： Tokyo, Japan	
Mailing Address: 郵送先： c/o SEIKAGAKU CORPORATION Central Research Laboratories, 1253, Tateno 3-chome, Higashiyamato-shi, Tokyo 207-0021 Japan	
NAME OF SECOND INVENTOR: 第二の発明者名：	
Given Name (first and middle [if any]) 名（名およびミドルネーム[該当する場合]） Toshio	Family Name or Surname 姓 ODA
Inventor's signature 発明者の署名 Toshio Oda	Date 日付 May, 22, 2015
Residence: 住所： Tokyo, Japan	
Mailing Address: 郵送先： c/o SEIKAGAKU CORPORATION Central Research Laboratories, 1253, Tateno 3-chome, Higashiyamato-shi, Tokyo 207-0021 Japan	
NAME OF THIRD INVENTOR: 第三の発明者名：	
Given Name (first and middle [if any]) 名（名およびミドルネーム[該当する場合]） Shun-ichiro	Family Name or Surname 姓 KAWABATA
Inventor's signature 発明者の署名 Shun-ichiro Kawabata	Date 日付 May. 27, 2015
Residence: 住所： Fukuoka, Japan	
Mailing Address: 郵送先： c/o KYUSHU UNIVERSITY, 6-10-1 Hakozaki, Higashi-ku, Fukuoka-shi, Fukuoka 812-8581 Japan	

Electronic Acknowledgement Receipt

EFS ID:	35879555
Application Number:	16399386
International Application Number:	
Confirmation Number:	9293
Title of Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
First Named Inventor/Applicant Name:	Hikaru MIZUMURA
Customer Number:	23373
Filer:	Arun Shome/Veronica Gardner
Filer Authorized By:	Arun Shome
Attorney Docket Number:	Q246419
Receipt Date:	30-APR-2019
Filing Date:	
Time Stamp:	16:53:10
Application Type:	Utility under 35 USC 111(a)

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.)
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	Claims		100	104	
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6	Drawings-only black and white line drawings	Q246419Figs114asfiled.pdf	2182960 caeae4842bd8660f5b4a3702c7f6418c8c4ee985	no	10
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Information:					
		Total Files Size (in bytes):	4173712		

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.

REQUEST FOR TRANSFER OF A COMPUTER READABLE FORM UNDER 37 CFR 1.821(e)

Application No.:	Not Yet Assigned	First Named Inventor:	Hikaru MIZUMURA
Filing Date:	April 30, 2019	Attorney Docket No.:	Q246419
Title of the Invention:	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN		

The sequence information in the paper copy or PDF file of the Sequence Listing submitted in this application in accordance with 37 CFR 1.821(c),

- ☐ as part of the originally-filed specification of this application
- ☑ as a separate amendment of the specification filed
  - ☐ herewith
  - ☐ on _____

is identical to the sequence information in the

- ☐ only computer readable form
- ☑ last filed computer readable form
- ☐ other computer readable form, which was filed on May 18, 2018 (Request for Transfer from parent)

in application number 15/983,725 **, which was filed on** May 18, 2018. The original computer readable form was filed June 9, 2015 in grandparent application no. 14/650,767. **.**

This computer readable form was compliant with 37 CFR 1.821-1.825, and applicant hereby requests that it be used as the computer readable form for the present application, in accordance with 37 CFR 1.821(e).

The above referenced paper copy or PDF file of the Sequence Listing contains no new matter.

A sequence listing text file submitted via EFS-Web that complies with the requirements of 37 CFR 1.824(a) (2)-(6) and (b) (i.e., is a compliant sequence listing ASCII text file), serves as both the paper copy required by 37 CFR 1.821(c) and the CRF required by 37 CRF 1.821(e). If a user submits a compliant sequence listing ASCII text file via EFS-Web, the U.S. Patent and Trademark Office will not carry out a request to use a compliant computer readable "Sequence Listing" that is already on file for another application pursuant to 37 CFR 1.821(e) but will use the sequence listing submitted with the application as originally filed via EFS-Web.

It is understood that upon the transfer of a copy of the computer readable form to this application, the U.S. Patent and Trademark Office will update the copy of the computer readable form to reflect the application number and filing date for this application.

Signature /markboland/	Date April 30, 2019
Name (Print/Typed) Mark Boland	Registration Number 32,197

***Note**: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required.**

☐ *Total of ___1___ forms are submitted.

SEQUENCE LISTING

<110> SEIKAGAKU CORPORATION

<120> Novel Recombinant Factor C and Method for Producing the Same, and Method for Measuring Endotoxin

<130> Q220395

<150> PCT/JP2013/083082
<151> 2013-12-10

<150> JP2012-269840
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Ala Gln Gln Met Arg Pro Val Gln Ser Arg Gly Val Asp Leu Gly Leu
            20                  25                  30

tgt gat gaa acg agg ttc gag tgt aag tgt gga gat cca ggc tat gtg         144
Cys Asp Glu Thr Arg Phe Glu Cys Lys Cys Gly Asp Pro Gly Tyr Val
        35                  40                  45

ttc aac gtc cct atg aaa caa tgc acg tac ttc tat cga tgg agg cct         192
Phe Asn Val Pro Met Lys Gln Cys Thr Tyr Phe Tyr Arg Trp Arg Pro
    50                  55                  60

tat tgt aaa cca tgt gat gac ctg gag gct aag gac att tgt cca aag         240
Tyr Cys Lys Pro Cys Asp Asp Leu Glu Ala Lys Asp Ile Cys Pro Lys
65                  70                  75                  80

tac aaa cga tgt caa gag tgt aag gct ggt ctt gat agt tgt gtt act         288
Tyr Lys Arg Cys Gln Glu Cys Lys Ala Gly Leu Asp Ser Cys Val Thr
                85                  90                  95

tgt cca cct aac aaa tat ggt act tgg tgt agc ggt gaa tgt caa tgt         336
Cys Pro Pro Asn Lys Tyr Gly Thr Trp Cys Ser Gly Glu Cys Gln Cys
            100                 105                 110

aag aat gga ggt atc tgt gac cag agg aca gga gct tgt acc tgt cgt         384
Lys Asn Gly Gly Ile Cys Asp Gln Arg Thr Gly Ala Cys Thr Cys Arg
        115                 120                 125

gac aga tat gaa gga gcg cac tgt gaa att ctc aaa ggt tgt cct ctt         432
Asp Arg Tyr Glu Gly Ala His Cys Glu Ile Leu Lys Gly Cys Pro Leu
                                (1)
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    130                 135                 140

ctt cca tcg gat tct caa gtt cag gaa gtc aga aac cca cca gat aat        480
Leu Pro Ser Asp Ser Gln Val Gln Glu Val Arg Asn Pro Pro Asp Asn
145                 150                 155                 160

ccc caa act att gac tac agc tgt tca cca ggg ttc aag ctt aaa ggc        528
Pro Gln Thr Ile Asp Tyr Ser Cys Ser Pro Gly Phe Lys Leu Lys Gly
                165                 170                 175

gtg gca cga att agc tgt ctc cca aat gga cag tgg agt agc ttt cca        576
Val Ala Arg Ile Ser Cys Leu Pro Asn Gly Gln Trp Ser Ser Phe Pro
            180                 185                 190

ccc aaa tgt att cga gaa tgt gcc aag gtt tca tct cca gaa cac ggg        624
Pro Lys Cys Ile Arg Glu Cys Ala Lys Val Ser Ser Pro Glu His Gly
        195                 200                 205

aaa gtg aat gct cct agt ggc aat atg ata gaa ggg gct act tta cgg        672
Lys Val Asn Ala Pro Ser Gly Asn Met Ile Glu Gly Ala Thr Leu Arg
    210                 215                 220

ttc tca tgt gat agt ccc tac tac ttg att ggt caa gaa aca tta acc        720
Phe Ser Cys Asp Ser Pro Tyr Tyr Leu Ile Gly Gln Glu Thr Leu Thr
225                 230                 235                 240

tgc cag ggt aat ggt cag tgg agt gga caa ata cca caa tgt aag aag        768
Cys Gln Gly Asn Gly Gln Trp Ser Gly Gln Ile Pro Gln Cys Lys Lys
                245                 250                 255

ttg gtc ttc tgt cct gac ctt gat cct gta aac cat gct gaa cac cag        816
Leu Val Phe Cys Pro Asp Leu Asp Pro Val Asn His Ala Glu His Gln
            260                 265                 270

gtt aaa att ggt gtg gaa caa aaa tat ggt cag ttt cct caa ggc act        864
Val Lys Ile Gly Val Glu Gln Lys Tyr Gly Gln Phe Pro Gln Gly Thr
        275                 280                 285

gaa gtg acc tat acg tgt tcg ggt aac tac ttc ttg atg ggt ttt aac        912
Glu Val Thr Tyr Thr Cys Ser Gly Asn Tyr Phe Leu Met Gly Phe Asn
    290                 295                 300

acc tta aaa tgt aac cct gat ggg tcc tgg tca gga tca cag cca tcc        960
Thr Leu Lys Cys Asn Pro Asp Gly Ser Trp Ser Gly Ser Gln Pro Ser
305                 310                 315                 320

tgt gtt aaa gtg gca gac aga gag gtc gac tgt gac agt aaa gct gta       1008
Cys Val Lys Val Ala Asp Arg Glu Val Asp Cys Asp Ser Lys Ala Val
                325                 330                 335

gac ttc ttg gat gat gtt ggt gaa cct gtc agg atc cac tgt cct gct       1056
Asp Phe Leu Asp Asp Val Gly Glu Pro Val Arg Ile His Cys Pro Ala
            340                 345                 350

ggc tgt tct ttg aca gct ggt act gtg tgg ggt aca gcc ata tac cac       1104
Gly Cys Ser Leu Thr Ala Gly Thr Val Trp Gly Thr Ala Ile Tyr His
        355                 360                 365

gaa ctt tcc tca gtg tgt cgt gca gcc atc cat gct ggc aag ctt cca       1152
Glu Leu Ser Ser Val Cys Arg Ala Ala Ile His Ala Gly Lys Leu Pro
    370                 375                 380

aac tct gga ggg gcg gtg cat gta gtg aac aat ggc ccc tac tcg gac       1200
Asn Ser Gly Gly Ala Val His Val Val Asn Asn Gly Pro Tyr Ser Asp
                                    (2)
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```
385                 390                 395                 400

ttt ctg ggt agt gac ctg aat ggg ata aaa tcg gaa gag ttg aag tct      1248
Phe Leu Gly Ser Asp Leu Asn Gly Ile Lys Ser Glu Glu Leu Lys Ser
                405                 410                 415

ctt gcc cgc agt ttt cga ttt gat tat gtc agt tca tcc aca gca ggt      1296
Leu Ala Arg Ser Phe Arg Phe Asp Tyr Val Ser Ser Ser Thr Ala Gly
            420                 425                 430

aga tca gga tgt cct gat gga tgg ttt gag gta gaa gag aac tgt gtg      1344
Arg Ser Gly Cys Pro Asp Gly Trp Phe Glu Val Glu Glu Asn Cys Val
        435                 440                 445

tac gtt aca tca aaa cag aga gcc tgg gaa aga gct caa ggt gtg tgt      1392
Tyr Val Thr Ser Lys Gln Arg Ala Trp Glu Arg Ala Gln Gly Val Cys
    450                 455                 460

acc aat atg gct gct cgt ctt gct gtg cta gac aaa gat cta att ccg      1440
Thr Asn Met Ala Ala Arg Leu Ala Val Leu Asp Lys Asp Leu Ile Pro
465                 470                 475                 480

agt tcc ttg act gag act cta cga ggg aaa ggg tta aca acc aca tgg      1488
Ser Ser Leu Thr Glu Thr Leu Arg Gly Lys Gly Leu Thr Thr Thr Trp
                485                 490                 495

ata gga ttg cac aga cta gat gct gag aag ccc ttt gtt tgg gag cta      1536
Ile Gly Leu His Arg Leu Asp Ala Glu Lys Pro Phe Val Trp Glu Leu
            500                 505                 510

atg gat cgt agt aat gtg gtt ctg aat gat aac cta aca ttc tgg gcc      1584
Met Asp Arg Ser Asn Val Val Leu Asn Asp Asn Leu Thr Phe Trp Ala
        515                 520                 525

tct ggc gaa cct gga aat gaa act aac tgt gta tat ctg gac atc cga      1632
Ser Gly Glu Pro Gly Asn Glu Thr Asn Cys Val Tyr Leu Asp Ile Arg
    530                 535                 540

gat cag ctg cag cct gtg tgg aaa acc aag tca tgt ttt cag ccc tca      1680
Asp Gln Leu Gln Pro Val Trp Lys Thr Lys Ser Cys Phe Gln Pro Ser
545                 550                 555                 560

agc ttt gct tgc atg atg gat ttg tca gac aga aat aaa gcc aaa tgc      1728
Ser Phe Ala Cys Met Met Asp Leu Ser Asp Arg Asn Lys Ala Lys Cys
                565                 570                 575

gat gac cct gga cca ctg gaa aat gga cac gcc aca ctt cat gga caa      1776
Asp Asp Pro Gly Pro Leu Glu Asn Gly His Ala Thr Leu His Gly Gln
            580                 585                 590

agt att gat ggg ttc tat gct ggt tct tct ata agg tac agc tgt gag      1824
Ser Ile Asp Gly Phe Tyr Ala Gly Ser Ser Ile Arg Tyr Ser Cys Glu
        595                 600                 605

gtt ctc cac tac ctc agt gga act gag acc gta act tgt aca aca aat      1872
Val Leu His Tyr Leu Ser Gly Thr Glu Thr Val Thr Cys Thr Thr Asn
    610                 615                 620

ggc aca tgg agt gct cct aaa cct cga tgt atc aaa gtc atc acc tgc      1920
Gly Thr Trp Ser Ala Pro Lys Pro Arg Cys Ile Lys Val Ile Thr Cys
625                 630                 635                 640

caa aac cct cct gta cca tca tat ggt tct gtg gaa atc aaa ccc cca      1968
Gln Asn Pro Pro Val Pro Ser Tyr Gly Ser Val Glu Ile Lys Pro Pro
                                    (3)
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                645                 650                 655

agt cgg aca aac tcg atc agt cgt gtt ggg tca cct ttc ttg agg ttg       2016
Ser Arg Thr Asn Ser Ile Ser Arg Val Gly Ser Pro Phe Leu Arg Leu
            660                 665                 670

cca cgg tta ccc ctc cca tta gcc aga gca gcc aaa cct cct cca aaa       2064
Pro Arg Leu Pro Leu Pro Leu Ala Arg Ala Ala Lys Pro Pro Pro Lys
        675                 680                 685

cct aga tcc tca caa ccc tct act gtg gac ttg gct tct aaa gtt aaa       2112
Pro Arg Ser Ser Gln Pro Ser Thr Val Asp Leu Ala Ser Lys Val Lys
    690                 695                 700

cta cct gaa ggt cat tac cgg gta ggg tct cga gcc att tac acg tgc       2160
Leu Pro Glu Gly His Tyr Arg Val Gly Ser Arg Ala Ile Tyr Thr Cys
705                 710                 715                 720

gag tcg aga tac tac gaa cta ctt gga tct caa ggc aga aga tgt gac       2208
Glu Ser Arg Tyr Tyr Glu Leu Leu Gly Ser Gln Gly Arg Arg Cys Asp
                725                 730                 735

tct aat gga aac tgg agt ggt cgg ccc gct agc tgt att cca gtt tgt       2256
Ser Asn Gly Asn Trp Ser Gly Arg Pro Ala Ser Cys Ile Pro Val Cys
            740                 745                 750

gga cgg tca gac tct cct cgt tct cct ttc atc tgg aat ggg aat tct       2304
Gly Arg Ser Asp Ser Pro Arg Ser Pro Phe Ile Trp Asn Gly Asn Ser
        755                 760                 765

aca gaa ata ggt cag tgg ccg tgg cag gca gga atc tct cga tgg ctt       2352
Thr Glu Ile Gly Gln Trp Pro Trp Gln Ala Gly Ile Ser Arg Trp Leu
    770                 775                 780

gca gac cac aat atg tgg ttt ctc cag tgt gga gga tcc cta ttg aat       2400
Ala Asp His Asn Met Trp Phe Leu Gln Cys Gly Gly Ser Leu Leu Asn
785                 790                 795                 800

gag aaa tgg atc gtc act gct gcc cac tgt gtc acc tac tct gct act       2448
Glu Lys Trp Ile Val Thr Ala Ala His Cys Val Thr Tyr Ser Ala Thr
                805                 810                 815

gct gag ata att gat ccc agt cag ttt aaa atc tat ctg ggc aag tac       2496
Ala Glu Ile Ile Asp Pro Ser Gln Phe Lys Ile Tyr Leu Gly Lys Tyr
            820                 825                 830

tac cgt gat gac agt aga gac gat gac tac gta caa gta aga gag gct       2544
Tyr Arg Asp Asp Ser Arg Asp Asp Asp Tyr Val Gln Val Arg Glu Ala
        835                 840                 845

ctc gag atc cac gta aat cct aac tac gac ccc ggc aat ctc aac ttt       2592
Leu Glu Ile His Val Asn Pro Asn Tyr Asp Pro Gly Asn Leu Asn Phe
    850                 855                 860

gac ata gcc cta att caa ctg aaa act cct gtt act ttg aca aca cga       2640
Asp Ile Ala Leu Ile Gln Leu Lys Thr Pro Val Thr Leu Thr Thr Arg
865                 870                 875                 880

gtc caa cca atc tgt ctg cct act gac atc aca aca aga gaa cac ttg       2688
Val Gln Pro Ile Cys Leu Pro Thr Asp Ile Thr Thr Arg Glu His Leu
                885                 890                 895

aag gag gga aca tta gca gtg gtg aca ggt tgg ggt ttg aat gaa aac       2736
Lys Glu Gly Thr Leu Ala Val Val Thr Gly Trp Gly Leu Asn Glu Asn
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(4)

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            900                 905                 910

aac aca tat tca gag atg att caa caa gct gtg cta cct gtt gtt gca       2784
Asn Thr Tyr Ser Glu Met Ile Gln Gln Ala Val Leu Pro Val Val Ala
        915                 920                 925

gca agc acc tgt gaa gag ggg tac aag gaa gca gac tta cca ctg aca       2832
Ala Ser Thr Cys Glu Glu Gly Tyr Lys Glu Ala Asp Leu Pro Leu Thr
    930                 935                 940

gta aca gag aac atg ttc tgt gca ggt tac aag aag gga cgt tat gat       2880
Val Thr Glu Asn Met Phe Cys Ala Gly Tyr Lys Lys Gly Arg Tyr Asp
945                 950                 955                 960

gcc tgc agt ggg gac agt gga gga cca tta gtg ttt gct gat gat tcc       2928
Ala Cys Ser Gly Asp Ser Gly Gly Pro Leu Val Phe Ala Asp Asp Ser
                965                 970                 975

cgt acc gaa agg cgg tgg gtc ttg gaa ggg att gtc agc tgg ggc agt       2976
Arg Thr Glu Arg Arg Trp Val Leu Glu Gly Ile Val Ser Trp Gly Ser
            980                 985                 990

ccc agt gga tgt ggc aag gct aac  cag tat ggg ggc ttc  act aaa gtt      3024
Pro Ser Gly Cys Gly Lys Ala Asn  Gln Tyr Gly Gly Phe  Thr Lys Val
        995                 1000                1005

aac gtt  ttt cta tca tgg att  agg cag ttc att tga                      3060
Asn Val  Phe Leu Ser Trp Ile  Arg Gln Phe Ile
    1010                1015
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<210> 2
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<212> PRT
<213> Tachypleus tridentatus

<400> 2

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Met Val Leu Ala Ser Phe Leu Val Ser Gly Leu Val Leu Gly Ile Leu
1               5                   10                  15

Ala Gln Gln Met Arg Pro Val Gln Ser Arg Gly Val Asp Leu Gly Leu
            20                  25                  30

Cys Asp Glu Thr Arg Phe Glu Cys Lys Cys Gly Asp Pro Gly Tyr Val
        35                  40                  45

Phe Asn Val Pro Met Lys Gln Cys Thr Tyr Phe Tyr Arg Trp Arg Pro
    50                  55                  60

Tyr Cys Lys Pro Cys Asp Asp Leu Glu Ala Lys Asp Ile Cys Pro Lys
65                  70                  75                  80

Tyr Lys Arg Cys Gln Glu Cys Lys Ala Gly Leu Asp Ser Cys Val Thr
                85                  90                  95

Cys Pro Pro Asn Lys Tyr Gly Thr Trp Cys Ser Gly Glu Cys Gln Cys
            100                 105                 110
```

```
Lys Asn Gly Gly Ile Cys Asp Gln Arg Thr Gly Ala Cys Thr Cys Arg
        115                 120                 125

Asp Arg Tyr Glu Gly Ala His Cys Glu Ile Leu Lys Gly Cys Pro Leu
    130                 135                 140

Leu Pro Ser Asp Ser Gln Val Gln Glu Val Arg Asn Pro Pro Asp Asn
145                 150                 155                 160

Pro Gln Thr Ile Asp Tyr Ser Cys Ser Pro Gly Phe Lys Leu Lys Gly
                165                 170                 175

Val Ala Arg Ile Ser Cys Leu Pro Asn Gly Gln Trp Ser Ser Phe Pro
            180                 185                 190

Pro Lys Cys Ile Arg Glu Cys Ala Lys Val Ser Ser Pro Glu His Gly
        195                 200                 205

Lys Val Asn Ala Pro Ser Gly Asn Met Ile Glu Gly Ala Thr Leu Arg
    210                 215                 220

Phe Ser Cys Asp Ser Pro Tyr Tyr Leu Ile Gly Gln Glu Thr Leu Thr
225                 230                 235                 240

Cys Gln Gly Asn Gly Gln Trp Ser Gly Gln Ile Pro Gln Cys Lys Lys
                245                 250                 255

Leu Val Phe Cys Pro Asp Leu Asp Pro Val Asn His Ala Glu His Gln
            260                 265                 270

Val Lys Ile Gly Val Glu Gln Lys Tyr Gly Gln Phe Pro Gln Gly Thr
        275                 280                 285

Glu Val Thr Tyr Thr Cys Ser Gly Asn Tyr Phe Leu Met Gly Phe Asn
    290                 295                 300

Thr Leu Lys Cys Asn Pro Asp Gly Ser Trp Ser Gly Ser Gln Pro Ser
305                 310                 315                 320

Cys Val Lys Val Ala Asp Arg Glu Val Asp Cys Asp Ser Lys Ala Val
                325                 330                 335

Asp Phe Leu Asp Asp Val Gly Glu Pro Val Arg Ile His Cys Pro Ala
            340                 345                 350

Gly Cys Ser Leu Thr Ala Gly Thr Val Trp Gly Thr Ala Ile Tyr His
        355                 360                 365
```

```
Glu Leu Ser Ser Val Cys Arg Ala Ala Ile His Ala Gly Lys Leu Pro
    370                 375                 380

Asn Ser Gly Gly Ala Val His Val Val Asn Asn Gly Pro Tyr Ser Asp
385                 390                 395                 400

Phe Leu Gly Ser Asp Leu Asn Gly Ile Lys Ser Glu Glu Leu Lys Ser
                405                 410                 415

Leu Ala Arg Ser Phe Arg Phe Asp Tyr Val Ser Ser Ser Thr Ala Gly
            420                 425                 430

Arg Ser Gly Cys Pro Asp Gly Trp Phe Glu Val Glu Glu Asn Cys Val
        435                 440                 445

Tyr Val Thr Ser Lys Gln Arg Ala Trp Glu Arg Ala Gln Gly Val Cys
    450                 455                 460

Thr Asn Met Ala Ala Arg Leu Ala Val Leu Asp Lys Asp Leu Ile Pro
465                 470                 475                 480

Ser Ser Leu Thr Glu Thr Leu Arg Gly Lys Gly Leu Thr Thr Thr Trp
                485                 490                 495

Ile Gly Leu His Arg Leu Asp Ala Glu Lys Pro Phe Val Trp Glu Leu
            500                 505                 510

Met Asp Arg Ser Asn Val Val Leu Asn Asp Asn Leu Thr Phe Trp Ala
        515                 520                 525

Ser Gly Glu Pro Gly Asn Glu Thr Asn Cys Val Tyr Leu Asp Ile Arg
    530                 535                 540

Asp Gln Leu Gln Pro Val Trp Lys Thr Lys Ser Cys Phe Gln Pro Ser
545                 550                 555                 560

Ser Phe Ala Cys Met Met Asp Leu Ser Asp Arg Asn Lys Ala Lys Cys
                565                 570                 575

Asp Asp Pro Gly Pro Leu Glu Asn Gly His Ala Thr Leu His Gly Gln
            580                 585                 590

Ser Ile Asp Gly Phe Tyr Ala Gly Ser Ser Ile Arg Tyr Ser Cys Glu
        595                 600                 605

Val Leu His Tyr Leu Ser Gly Thr Glu Thr Val Thr Cys Thr Thr Asn
    610                 615                 620
```

(7)

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Gly Thr Trp Ser Ala Pro Lys Pro Arg Cys Ile Lys Val Ile Thr Cys
625                 630                 635                 640

Gln Asn Pro Pro Val Pro Ser Tyr Gly Ser Val Glu Ile Lys Pro Pro
                645                 650                 655

Ser Arg Thr Asn Ser Ile Ser Arg Val Gly Ser Pro Phe Leu Arg Leu
            660                 665                 670

Pro Arg Leu Pro Leu Pro Leu Ala Arg Ala Ala Lys Pro Pro Pro Lys
        675                 680                 685

Pro Arg Ser Ser Gln Pro Ser Thr Val Asp Leu Ala Ser Lys Val Lys
    690                 695                 700

Leu Pro Glu Gly His Tyr Arg Val Gly Ser Arg Ala Ile Tyr Thr Cys
705                 710                 715                 720

Glu Ser Arg Tyr Tyr Glu Leu Leu Gly Ser Gln Gly Arg Arg Cys Asp
                725                 730                 735

Ser Asn Gly Asn Trp Ser Gly Arg Pro Ala Ser Cys Ile Pro Val Cys
            740                 745                 750

Gly Arg Ser Asp Ser Pro Arg Ser Pro Phe Ile Trp Asn Gly Asn Ser
        755                 760                 765

Thr Glu Ile Gly Gln Trp Pro Trp Gln Ala Gly Ile Ser Arg Trp Leu
    770                 775                 780

Ala Asp His Asn Met Trp Phe Leu Gln Cys Gly Gly Ser Leu Leu Asn
785                 790                 795                 800

Glu Lys Trp Ile Val Thr Ala Ala His Cys Val Thr Tyr Ser Ala Thr
                805                 810                 815

Ala Glu Ile Ile Asp Pro Ser Gln Phe Lys Ile Tyr Leu Gly Lys Tyr
            820                 825                 830

Tyr Arg Asp Asp Ser Arg Asp Asp Asp Tyr Val Gln Val Arg Glu Ala
        835                 840                 845

Leu Glu Ile His Val Asn Pro Asn Tyr Asp Pro Gly Asn Leu Asn Phe
    850                 855                 860

Asp Ile Ala Leu Ile Gln Leu Lys Thr Pro Val Thr Leu Thr Thr Arg
865                 870                 875                 880
                                        (8)
```

```
Val Gln Pro Ile Cys Leu Pro Thr Asp Ile Thr Thr Arg Glu His Leu
                885                 890                 895

Lys Glu Gly Thr Leu Ala Val Val Thr Gly Trp Gly Leu Asn Glu Asn
            900                 905                 910

Asn Thr Tyr Ser Glu Met Ile Gln Gln Ala Val Leu Pro Val Val Ala
        915                 920                 925

Ala Ser Thr Cys Glu Glu Gly Tyr Lys Glu Ala Asp Leu Pro Leu Thr
    930                 935                 940

Val Thr Glu Asn Met Phe Cys Ala Gly Tyr Lys Lys Gly Arg Tyr Asp
945                 950                 955                 960

Ala Cys Ser Gly Asp Ser Gly Gly Pro Leu Val Phe Ala Asp Asp Ser
                965                 970                 975

Arg Thr Glu Arg Arg Trp Val Leu Glu Gly Ile Val Ser Trp Gly Ser
            980                 985                 990

Pro Ser Gly Cys Gly Lys Ala Asn  Gln Tyr Gly Gly Phe  Thr Lys Val
        995                 1000                1005

Asn Val  Phe Leu Ser Trp Ile  Arg Gln Phe Ile
    1010                1015
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<210> 3
<211> 3060
<212> DNA
<213> Carcinoscorpius rotundicauda

<220>
<221> CDS
<222> (1)..(3060)

<400> 3

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atg gtc tta gcg tcg ttt ttg gtg tct ggt tta gtt cta ggg cta cta          48
Met Val Leu Ala Ser Phe Leu Val Ser Gly Leu Val Leu Gly Leu Leu
1               5                   10                  15

gcc caa aaa atg cgc cca gtt cag tcc aaa gga gta gat cta ggc ttg          96
Ala Gln Lys Met Arg Pro Val Gln Ser Lys Gly Val Asp Leu Gly Leu
            20                  25                  30

tgt gat gaa acg agg ttc gag tgt aag tgt ggc gat cca ggc tat gtg         144
Cys Asp Glu Thr Arg Phe Glu Cys Lys Cys Gly Asp Pro Gly Tyr Val
        35                  40                  45

ttc aac att cca gtg aaa caa tgt aca tac ttt tat cga tgg agg ccg         192
Phe Asn Ile Pro Val Lys Gln Cys Thr Tyr Phe Tyr Arg Trp Arg Pro
    50                  55                  60
```

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tat tgt aaa cca tgt gat gac ctg gag gct aag gat att tgt cca aag        240
Tyr Cys Lys Pro Cys Asp Asp Leu Glu Ala Lys Asp Ile Cys Pro Lys
65                  70                  75                  80

tac aaa cga tgt caa gag tgt aag gct ggt ctt gat agt tgt gtt act        288
Tyr Lys Arg Cys Gln Glu Cys Lys Ala Gly Leu Asp Ser Cys Val Thr
                85                  90                  95

tgt cca cct aac aaa tat ggt act tgg tgt agc ggt gaa tgt cag tgt        336
Cys Pro Pro Asn Lys Tyr Gly Thr Trp Cys Ser Gly Glu Cys Gln Cys
            100                 105                 110

aag aat gga ggt atc tgt gac cag agg aca gga gct tgt gca tgt cgt        384
Lys Asn Gly Gly Ile Cys Asp Gln Arg Thr Gly Ala Cys Ala Cys Arg
        115                 120                 125

gac aga tat gaa ggg gtg cac tgt gaa att ctc aaa ggt tgt cct ctt        432
Asp Arg Tyr Glu Gly Val His Cys Glu Ile Leu Lys Gly Cys Pro Leu
    130                 135                 140

ctt cca tcg gat tct cag gtt cag gaa gtc aga aat cca cca gat aat        480
Leu Pro Ser Asp Ser Gln Val Gln Glu Val Arg Asn Pro Pro Asp Asn
145                 150                 155                 160

ccc caa act att gac tac agc tgt tca cca ggg ttc aag ctt aag ggt        528
Pro Gln Thr Ile Asp Tyr Ser Cys Ser Pro Gly Phe Lys Leu Lys Gly
                165                 170                 175

atg gca cga att agc tgt ctc cca aat gga cag tgg agt aac ttt cca        576
Met Ala Arg Ile Ser Cys Leu Pro Asn Gly Gln Trp Ser Asn Phe Pro
            180                 185                 190

ccc aaa tgt att cga gaa tgt gcc atg gtt tca tct cca gaa cat ggg        624
Pro Lys Cys Ile Arg Glu Cys Ala Met Val Ser Ser Pro Glu His Gly
        195                 200                 205

aaa gtg aat gct ctt agt ggt gat atg ata gaa ggg gct act tta cgg        672
Lys Val Asn Ala Leu Ser Gly Asp Met Ile Glu Gly Ala Thr Leu Arg
    210                 215                 220

ttc tca tgt gat agt ccc tac tac ttg att ggt caa gaa aca tta acc        720
Phe Ser Cys Asp Ser Pro Tyr Tyr Leu Ile Gly Gln Glu Thr Leu Thr
225                 230                 235                 240

tgt cag ggt aat ggt cag tgg aat gga cag ata cca caa tgt aag aac        768
Cys Gln Gly Asn Gly Gln Trp Asn Gly Gln Ile Pro Gln Cys Lys Asn
                245                 250                 255

ttg gtc ttc tgt cct gac ctg gat cct gta aac cat gct gaa cac aag        816
Leu Val Phe Cys Pro Asp Leu Asp Pro Val Asn His Ala Glu His Lys
            260                 265                 270

gtt aaa att ggt gtg gaa caa aaa tat ggt cag ttt cct caa ggc act        864
Val Lys Ile Gly Val Glu Gln Lys Tyr Gly Gln Phe Pro Gln Gly Thr
        275                 280                 285

gaa gtg acc tat acg tgt tcg ggt aac tac ttc ttg atg ggt ttt gac        912
Glu Val Thr Tyr Thr Cys Ser Gly Asn Tyr Phe Leu Met Gly Phe Asp
    290                 295                 300

acc tta aaa tgt aac cct gat ggg tct tgg tca gga tca cag cca tcc        960
Thr Leu Lys Cys Asn Pro Asp Gly Ser Trp Ser Gly Ser Gln Pro Ser
305                 310                 315                 320
                                        (10)
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tgt gtt aaa gtg gca gac aga gag gtc gac tgt gac agt aaa gct gta       1008
Cys Val Lys Val Ala Asp Arg Glu Val Asp Cys Asp Ser Lys Ala Val
                325                 330                 335

gac ttc ttg gat gat gtt ggt gaa cct gtc agg atc cac tgt cct gct       1056
Asp Phe Leu Asp Asp Val Gly Glu Pro Val Arg Ile His Cys Pro Ala
            340                 345                 350

ggc tgt tct ttg aca gct ggt act gtg tgg ggt aca gcc ata tac cat       1104
Gly Cys Ser Leu Thr Ala Gly Thr Val Trp Gly Thr Ala Ile Tyr His
        355                 360                 365

gaa ctt tcc tca gtg tgt cgt gca gcc atc cat gct ggc aag ctt cca       1152
Glu Leu Ser Ser Val Cys Arg Ala Ala Ile His Ala Gly Lys Leu Pro
    370                 375                 380

aac tct gga gga gcg gtg cat gtt gtg aac aat ggc ccc tac tcg gac       1200
Asn Ser Gly Gly Ala Val His Val Val Asn Asn Gly Pro Tyr Ser Asp
385                 390                 395                 400

ttt ctg ggt agt gac ctg aat ggg ata aaa tcg gaa gag ttg aag tct       1248
Phe Leu Gly Ser Asp Leu Asn Gly Ile Lys Ser Glu Glu Leu Lys Ser
                405                 410                 415

ctt gcc cgg agt ttc cga ttc gat tat gtc cgt tcc tcc aca gca ggt       1296
Leu Ala Arg Ser Phe Arg Phe Asp Tyr Val Arg Ser Ser Thr Ala Gly
            420                 425                 430

aaa tca gga tgt cct gat gga tgg ttt gag gta gac gag aac tgt gtg       1344
Lys Ser Gly Cys Pro Asp Gly Trp Phe Glu Val Asp Glu Asn Cys Val
        435                 440                 445

tac gtt aca tca aaa cag aga gcc tgg gaa aga gct caa ggt gtg tgt       1392
Tyr Val Thr Ser Lys Gln Arg Ala Trp Glu Arg Ala Gln Gly Val Cys
    450                 455                 460

acc aat atg gct gct cgt ctt gct gtg ctg gac aaa gat gta att cca       1440
Thr Asn Met Ala Ala Arg Leu Ala Val Leu Asp Lys Asp Val Ile Pro
465                 470                 475                 480

aat tcg ttg act gag act cta cga ggg aaa ggg tta aca acc acg tgg       1488
Asn Ser Leu Thr Glu Thr Leu Arg Gly Lys Gly Leu Thr Thr Thr Trp
                485                 490                 495

ata gga ttg cac aga cta gat gct gag aag ccc ttt att tgg gag tta       1536
Ile Gly Leu His Arg Leu Asp Ala Glu Lys Pro Phe Ile Trp Glu Leu
            500                 505                 510

atg gat cgt agt aat gtg gtt ctg aat gat aac cta aca ttc tgg gcc       1584
Met Asp Arg Ser Asn Val Val Leu Asn Asp Asn Leu Thr Phe Trp Ala
        515                 520                 525

tct ggc gaa cct gga aat gaa act aac tgt gta tat atg gac atc caa       1632
Ser Gly Glu Pro Gly Asn Glu Thr Asn Cys Val Tyr Met Asp Ile Gln
    530                 535                 540

gat cag ttg cag tct gtg tgg aaa acc aag tca tgt ttt cag ccc tca       1680
Asp Gln Leu Gln Ser Val Trp Lys Thr Lys Ser Cys Phe Gln Pro Ser
545                 550                 555                 560

agt ttt gct tgc atg atg gat ctg tca gac aga aat aaa gcc aaa tgc       1728
Ser Phe Ala Cys Met Met Asp Leu Ser Asp Arg Asn Lys Ala Lys Cys
                565                 570                 575
                                    (11)
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gat gat cct gga tca ctg gaa aat gga cac gcc aca ctt cat gga caa       1776
Asp Asp Pro Gly Ser Leu Glu Asn Gly His Ala Thr Leu His Gly Gln
            580                 585                 590

agt att gat ggg ttc tat gct ggt tct tct ata agg tac agc tgt gag       1824
Ser Ile Asp Gly Phe Tyr Ala Gly Ser Ser Ile Arg Tyr Ser Cys Glu
        595                 600                 605

gtt ctc cac tac ctc agt gga act gaa acc gta act tgt aca aca aat       1872
Val Leu His Tyr Leu Ser Gly Thr Glu Thr Val Thr Cys Thr Thr Asn
    610                 615                 620

ggc aca tgg agt gct cct aaa cct cga tgt atc aaa gtc atc acc tgc       1920
Gly Thr Trp Ser Ala Pro Lys Pro Arg Cys Ile Lys Val Ile Thr Cys
625                 630                 635                 640

caa aac ccc cct gta cca tca tat ggt tct gtg gaa atc aaa ccc cca       1968
Gln Asn Pro Pro Val Pro Ser Tyr Gly Ser Val Glu Ile Lys Pro Pro
                645                 650                 655

agt cgg aca aac tcg ata agt cgt gtt ggg tca cct ttc ttg agg ttg       2016
Ser Arg Thr Asn Ser Ile Ser Arg Val Gly Ser Pro Phe Leu Arg Leu
            660                 665                 670

cca cgg tta ccc ctc cca tta gct aga gca gcc aaa cct cct cca aaa       2064
Pro Arg Leu Pro Leu Pro Leu Ala Arg Ala Ala Lys Pro Pro Pro Lys
        675                 680                 685

cct aga tcc tca caa ccc tct act gtg gac ttg gct tct aaa gtt aaa       2112
Pro Arg Ser Ser Gln Pro Ser Thr Val Asp Leu Ala Ser Lys Val Lys
    690                 695                 700

cta cct gaa ggt cat tac cgg gta ggg tct cga gcc atc tac acg tgc       2160
Leu Pro Glu Gly His Tyr Arg Val Gly Ser Arg Ala Ile Tyr Thr Cys
705                 710                 715                 720

gag tcg aga tac tac gaa cta ctt gga tct caa ggc aga aga tgt gac       2208
Glu Ser Arg Tyr Tyr Glu Leu Leu Gly Ser Gln Gly Arg Arg Cys Asp
                725                 730                 735

tct aat gga aac tgg agt ggt cgg cca gcg agc tgt att cca gtt tgt       2256
Ser Asn Gly Asn Trp Ser Gly Arg Pro Ala Ser Cys Ile Pro Val Cys
            740                 745                 750

gga cgg tca gac tct cct cgt tct cct ttt atc tgg aat ggg aat tct       2304
Gly Arg Ser Asp Ser Pro Arg Ser Pro Phe Ile Trp Asn Gly Asn Ser
        755                 760                 765

aca gaa ata ggt cag tgg ccg tgg cag gca gga atc tct aga tgg ctt       2352
Thr Glu Ile Gly Gln Trp Pro Trp Gln Ala Gly Ile Ser Arg Trp Leu
    770                 775                 780

gca gac cac aat atg tgg ttt ctc cag tgt gga gga tct cta ttg aat       2400
Ala Asp His Asn Met Trp Phe Leu Gln Cys Gly Gly Ser Leu Leu Asn
785                 790                 795                 800

gag aaa tgg atc gtc act gct gcc cac tgt gtc acc tac tct gct act       2448
Glu Lys Trp Ile Val Thr Ala Ala His Cys Val Thr Tyr Ser Ala Thr
                805                 810                 815

gct gag att att gac ccc aat cag ttt aaa atg tat ctg ggc aag tac       2496
Ala Glu Ile Ile Asp Pro Asn Gln Phe Lys Met Tyr Leu Gly Lys Tyr
            820                 825                 830
```

(12)

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tac cgt gat gac agt aga gac gat gac tat gta caa gta aga gag gct       2544
Tyr Arg Asp Asp Ser Arg Asp Asp Asp Tyr Val Gln Val Arg Glu Ala
        835                 840                 845

ctt gag atc cac gtg aat cct aac tac gac ccc ggc aat ctc aac ttt       2592
Leu Glu Ile His Val Asn Pro Asn Tyr Asp Pro Gly Asn Leu Asn Phe
    850                 855                 860

gac ata gcc cta att caa ctg aaa act cct gtt act ttg aca aca cga       2640
Asp Ile Ala Leu Ile Gln Leu Lys Thr Pro Val Thr Leu Thr Thr Arg
865                 870                 875                 880

gtc caa cca atc tgt ctg cct act gac atc aca aca aga gaa cac ttg       2688
Val Gln Pro Ile Cys Leu Pro Thr Asp Ile Thr Thr Arg Glu His Leu
                885                 890                 895

aag gag gga aca tta gca gtg gtg aca ggt tgg ggt ttg aat gaa aac       2736
Lys Glu Gly Thr Leu Ala Val Val Thr Gly Trp Gly Leu Asn Glu Asn
            900                 905                 910

aac acc tat tca gag acg att caa caa gct gtg cta cct gtt gtt gca       2784
Asn Thr Tyr Ser Glu Thr Ile Gln Gln Ala Val Leu Pro Val Val Ala
        915                 920                 925

gcc agc acc tgt gaa gag ggg tac aag gaa gca gac tta cca ctg aca       2832
Ala Ser Thr Cys Glu Glu Gly Tyr Lys Glu Ala Asp Leu Pro Leu Thr
    930                 935                 940

gta aca gag aac atg ttc tgt gca ggt tac aag aag gga cgt tat gat       2880
Val Thr Glu Asn Met Phe Cys Ala Gly Tyr Lys Lys Gly Arg Tyr Asp
945                 950                 955                 960

gcc tgc agt ggg gac agt gga gga cct tta gtg ttt gct gat gat tcc       2928
Ala Cys Ser Gly Asp Ser Gly Gly Pro Leu Val Phe Ala Asp Asp Ser
                965                 970                 975

cgt acc gaa agg cgg tgg gtc ttg gaa ggg att gtc agc tgg ggc agt       2976
Arg Thr Glu Arg Arg Trp Val Leu Glu Gly Ile Val Ser Trp Gly Ser
            980                 985                 990

ccc agt gga tgt ggc aag gcg aac  cag tac ggg ggc ttc  act aaa gtt     3024
Pro Ser Gly Cys Gly Lys Ala Asn  Gln Tyr Gly Gly Phe  Thr Lys Val
        995                 1000                1005

aac gtt  ttc ctg tca tgg att  agg cag ttc att tga                     3060
Asn Val  Phe Leu Ser Trp Ile  Arg Gln Phe Ile
    1010                1015


<210>  4
<211>  1019
<212>  PRT
<213>  Carcinoscorpius rotundicauda

<400>  4

Met Val Leu Ala Ser Phe Leu Val Ser Gly Leu Val Leu Gly Leu Leu
1               5                   10                  15


Ala Gln Lys Met Arg Pro Val Gln Ser Lys Gly Val Asp Leu Gly Leu
            20                  25                  30
```

```
Cys Asp Glu Thr Arg Phe Glu Cys Lys Cys Gly Asp Pro Gly Tyr Val
        35                  40                  45

Phe Asn Ile Pro Val Lys Gln Cys Thr Tyr Phe Tyr Arg Trp Arg Pro
    50                  55                  60

Tyr Cys Lys Pro Cys Asp Asp Leu Glu Ala Lys Asp Ile Cys Pro Lys
65                  70                  75                  80

Tyr Lys Arg Cys Gln Glu Cys Lys Ala Gly Leu Asp Ser Cys Val Thr
                85                  90                  95

Cys Pro Pro Asn Lys Tyr Gly Thr Trp Cys Ser Gly Glu Cys Gln Cys
            100                 105                 110

Lys Asn Gly Gly Ile Cys Asp Gln Arg Thr Gly Ala Cys Ala Cys Arg
        115                 120                 125

Asp Arg Tyr Glu Gly Val His Cys Glu Ile Leu Lys Gly Cys Pro Leu
    130                 135                 140

Leu Pro Ser Asp Ser Gln Val Gln Glu Val Arg Asn Pro Pro Asp Asn
145                 150                 155                 160

Pro Gln Thr Ile Asp Tyr Ser Cys Ser Pro Gly Phe Lys Leu Lys Gly
                165                 170                 175

Met Ala Arg Ile Ser Cys Leu Pro Asn Gly Gln Trp Ser Asn Phe Pro
            180                 185                 190

Pro Lys Cys Ile Arg Glu Cys Ala Met Val Ser Ser Pro Glu His Gly
        195                 200                 205

Lys Val Asn Ala Leu Ser Gly Asp Met Ile Glu Gly Ala Thr Leu Arg
    210                 215                 220

Phe Ser Cys Asp Ser Pro Tyr Tyr Leu Ile Gly Gln Glu Thr Leu Thr
225                 230                 235                 240

Cys Gln Gly Asn Gly Gln Trp Asn Gly Gln Ile Pro Gln Cys Lys Asn
                245                 250                 255

Leu Val Phe Cys Pro Asp Leu Asp Pro Val Asn His Ala Glu His Lys
            260                 265                 270

Val Lys Ile Gly Val Glu Gln Lys Tyr Gly Gln Phe Pro Gln Gly Thr
        275                 280                 285
```

```
Glu Val Thr Tyr Thr Cys Ser Gly Asn Tyr Phe Leu Met Gly Phe Asp
    290                 295                 300

Thr Leu Lys Cys Asn Pro Asp Gly Ser Trp Ser Gly Ser Gln Pro Ser
305                 310                 315                 320

Cys Val Lys Val Ala Asp Arg Glu Val Asp Cys Asp Ser Lys Ala Val
                325                 330                 335

Asp Phe Leu Asp Asp Val Gly Glu Pro Val Arg Ile His Cys Pro Ala
            340                 345                 350

Gly Cys Ser Leu Thr Ala Gly Thr Val Trp Gly Thr Ala Ile Tyr His
        355                 360                 365

Glu Leu Ser Ser Val Cys Arg Ala Ala Ile His Ala Gly Lys Leu Pro
    370                 375                 380

Asn Ser Gly Gly Ala Val His Val Val Asn Asn Gly Pro Tyr Ser Asp
385                 390                 395                 400

Phe Leu Gly Ser Asp Leu Asn Gly Ile Lys Ser Glu Glu Leu Lys Ser
                405                 410                 415

Leu Ala Arg Ser Phe Arg Phe Asp Tyr Val Arg Ser Ser Thr Ala Gly
            420                 425                 430

Lys Ser Gly Cys Pro Asp Gly Trp Phe Glu Val Asp Glu Asn Cys Val
        435                 440                 445

Tyr Val Thr Ser Lys Gln Arg Ala Trp Glu Arg Ala Gln Gly Val Cys
    450                 455                 460

Thr Asn Met Ala Ala Arg Leu Ala Val Leu Asp Lys Asp Val Ile Pro
465                 470                 475                 480

Asn Ser Leu Thr Glu Thr Leu Arg Gly Lys Gly Leu Thr Thr Thr Trp
                485                 490                 495

Ile Gly Leu His Arg Leu Asp Ala Glu Lys Pro Phe Ile Trp Glu Leu
            500                 505                 510

Met Asp Arg Ser Asn Val Val Leu Asn Asp Asn Leu Thr Phe Trp Ala
        515                 520                 525

Ser Gly Glu Pro Gly Asn Glu Thr Asn Cys Val Tyr Met Asp Ile Gln
    530                 535                 540
```

```
Asp Gln Leu Gln Ser Val Trp Lys Thr Lys Ser Cys Phe Gln Pro Ser
545                 550                 555                 560

Ser Phe Ala Cys Met Met Asp Leu Ser Asp Arg Asn Lys Ala Lys Cys
                565                 570                 575

Asp Asp Pro Gly Ser Leu Glu Asn Gly His Ala Thr Leu His Gly Gln
            580                 585                 590

Ser Ile Asp Gly Phe Tyr Ala Gly Ser Ser Ile Arg Tyr Ser Cys Glu
        595                 600                 605

Val Leu His Tyr Leu Ser Gly Thr Glu Thr Val Thr Cys Thr Thr Asn
    610                 615                 620

Gly Thr Trp Ser Ala Pro Lys Pro Arg Cys Ile Lys Val Ile Thr Cys
625                 630                 635                 640

Gln Asn Pro Pro Val Pro Ser Tyr Gly Ser Val Glu Ile Lys Pro Pro
                645                 650                 655

Ser Arg Thr Asn Ser Ile Ser Arg Val Gly Ser Pro Phe Leu Arg Leu
            660                 665                 670

Pro Arg Leu Pro Leu Pro Leu Ala Arg Ala Ala Lys Pro Pro Pro Lys
        675                 680                 685

Pro Arg Ser Ser Gln Pro Ser Thr Val Asp Leu Ala Ser Lys Val Lys
    690                 695                 700

Leu Pro Glu Gly His Tyr Arg Val Gly Ser Arg Ala Ile Tyr Thr Cys
705                 710                 715                 720

Glu Ser Arg Tyr Tyr Glu Leu Leu Gly Ser Gln Gly Arg Arg Cys Asp
                725                 730                 735

Ser Asn Gly Asn Trp Ser Gly Arg Pro Ala Ser Cys Ile Pro Val Cys
            740                 745                 750

Gly Arg Ser Asp Ser Pro Arg Ser Pro Phe Ile Trp Asn Gly Asn Ser
        755                 760                 765

Thr Glu Ile Gly Gln Trp Pro Trp Gln Ala Gly Ile Ser Arg Trp Leu
    770                 775                 780

Ala Asp His Asn Met Trp Phe Leu Gln Cys Gly Gly Ser Leu Leu Asn
785                 790                 795                 800
```

```
Glu Lys Trp Ile Val Thr Ala Ala His Cys Val Thr Tyr Ser Ala Thr
                805                 810                 815

Ala Glu Ile Ile Asp Pro Asn Gln Phe Lys Met Tyr Leu Gly Lys Tyr
            820                 825                 830

Tyr Arg Asp Asp Ser Arg Asp Asp Asp Tyr Val Gln Val Arg Glu Ala
        835                 840                 845

Leu Glu Ile His Val Asn Pro Asn Tyr Asp Pro Gly Asn Leu Asn Phe
    850                 855                 860

Asp Ile Ala Leu Ile Gln Leu Lys Thr Pro Val Thr Leu Thr Thr Arg
865                 870                 875                 880

Val Gln Pro Ile Cys Leu Pro Thr Asp Ile Thr Thr Arg Glu His Leu
                885                 890                 895

Lys Glu Gly Thr Leu Ala Val Val Thr Gly Trp Gly Leu Asn Glu Asn
            900                 905                 910

Asn Thr Tyr Ser Glu Thr Ile Gln Gln Ala Val Leu Pro Val Val Ala
        915                 920                 925

Ala Ser Thr Cys Glu Glu Gly Tyr Lys Glu Ala Asp Leu Pro Leu Thr
    930                 935                 940

Val Thr Glu Asn Met Phe Cys Ala Gly Tyr Lys Lys Gly Arg Tyr Asp
945                 950                 955                 960

Ala Cys Ser Gly Asp Ser Gly Gly Pro Leu Val Phe Ala Asp Asp Ser
                965                 970                 975

Arg Thr Glu Arg Arg Trp Val Leu Glu Gly Ile Val Ser Trp Gly Ser
            980                 985                 990

Pro Ser Gly Cys Gly Lys Ala Asn  Gln Tyr Gly Gly Phe  Thr Lys Val
        995                 1000                1005

Asn Val  Phe Leu Ser Trp Ile  Arg Gln Phe Ile
    1010                1015
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<210> 5
<211> 1203
<212> DNA
<213> Tachypleus tridentatus

<220>

<221> CDS
<222> (1)..(1203)

<400> 5

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atg acg tgg ata tgt gtg ata acg ttg ttt gct ctg gct tct gct acg          48
Met Thr Trp Ile Cys Val Ile Thr Leu Phe Ala Leu Ala Ser Ala Thr
1               5                  10                  15

ttg ggt aac aaa gtt agt aga gtg ggg gtc ctc ttc ccc aag aca cgg          96
Leu Gly Asn Lys Val Ser Arg Val Gly Val Leu Phe Pro Lys Thr Arg
            20                  25                  30

aac gac aat gag tgt aca gca aga ggg gga ttg aaa gga tcc tgc aaa         144
Asn Asp Asn Glu Cys Thr Ala Arg Gly Gly Leu Lys Gly Ser Cys Lys
        35                  40                  45

tcc ctc ata gac tgt cct agt gtc ttg gct acg ttg aag gac agt ttt         192
Ser Leu Ile Asp Cys Pro Ser Val Leu Ala Thr Leu Lys Asp Ser Phe
    50                  55                  60

cct gtc gtt tgc tct tgg aat ggt cga ttt cag cct att gtc tgc tgt         240
Pro Val Val Cys Ser Trp Asn Gly Arg Phe Gln Pro Ile Val Cys Cys
65                  70                  75                  80

cct gat gca ata gca cca cca cct gta acc aca aca gct gta act gta         288
Pro Asp Ala Ile Ala Pro Pro Pro Val Thr Thr Thr Ala Val Thr Val
                85                  90                  95

ata tct aca aaa gaa cca aag ctt cca aga tta cat ata tca ggt tgt         336
Ile Ser Thr Lys Glu Pro Lys Leu Pro Arg Leu His Ile Ser Gly Cys
            100                 105                 110

gga aaa aga aaa gtc aaa ata gat att aca act gtt gga cgc tct gga         384
Gly Lys Arg Lys Val Lys Ile Asp Ile Thr Thr Val Gly Arg Ser Gly
        115                 120                 125

tca cca ata ctt cct ccg ata tct act cct caa aat tca aca ggt ggg         432
Ser Pro Ile Leu Pro Pro Ile Ser Thr Pro Gln Asn Ser Thr Gly Gly
    130                 135                 140

aga gga att att gct gga ggc gta gaa gcc aaa att ggc gcg tgg cct         480
Arg Gly Ile Ile Ala Gly Gly Val Glu Ala Lys Ile Gly Ala Trp Pro
145                 150                 155                 160

tgg atg gca gct gtt ttt gtg aaa aac ttt ggc att ggc aga ttc cac         528
Trp Met Ala Ala Val Phe Val Lys Asn Phe Gly Ile Gly Arg Phe His
                165                 170                 175

tgt gct ggt agc ata atc agt aac aag tac att ttg tca gct gcc cac         576
Cys Ala Gly Ser Ile Ile Ser Asn Lys Tyr Ile Leu Ser Ala Ala His
            180                 185                 190

gcc ttc ctt atc gga ggt cga aag ttg acc cca act cgc tta gct gtc         624
Ala Phe Leu Ile Gly Gly Arg Lys Leu Thr Pro Thr Arg Leu Ala Val
        195                 200                 205

cgt gtg gga ggc cac tac ata aag agg ggt caa gag tat cca gtg aaa         672
Arg Val Gly Gly His Tyr Ile Lys Arg Gly Gln Glu Tyr Pro Val Lys
    210                 215                 220

gac gtg att atc cat cct cat tat gta gaa aag gag aac tac aat gat         720
Asp Val Ile Ile His Pro His Tyr Val Glu Lys Glu Asn Tyr Asn Asp
225                 230                 235                 240
```

```
ata gcc ata atc gag tta aaa gag gaa ctg aac ttt acg gac ttg gtc        768
Ile Ala Ile Ile Glu Leu Lys Glu Glu Leu Asn Phe Thr Asp Leu Val
                245                 250                 255

aat cct ata tgt ctc cct gat cca gag aca gta acg gat cca tta aaa        816
Asn Pro Ile Cys Leu Pro Asp Pro Glu Thr Val Thr Asp Pro Leu Lys
            260                 265                 270

gac aga att gtg act gca gcg gga tgg ggc gat ctg gat ttc tcc ggt        864
Asp Arg Ile Val Thr Ala Ala Gly Trp Gly Asp Leu Asp Phe Ser Gly
        275                 280                 285

cca cgg agc caa gtt cta cgt gag gta agc atc cca gtt gtt cca gtt        912
Pro Arg Ser Gln Val Leu Arg Glu Val Ser Ile Pro Val Val Pro Val
    290                 295                 300

gat aaa tgt gat caa gcc tat gag aaa ctc aac acc cct tca cta aaa        960
Asp Lys Cys Asp Gln Ala Tyr Glu Lys Leu Asn Thr Pro Ser Leu Lys
305                 310                 315                 320

aat ggg ata acg aat aac ttc ctt tgc gct gga ttg gaa gaa gga ggg       1008
Asn Gly Ile Thr Asn Asn Phe Leu Cys Ala Gly Leu Glu Glu Gly Gly
                325                 330                 335

aaa gac gct tgc caa ggc gat tct ggt gga ccg ttg atg cta gtg aac       1056
Lys Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Met Leu Val Asn
            340                 345                 350

aac act agg tgg ata gta gta gga gtt gtg tcg ttc ggg cac aag tgt       1104
Asn Thr Arg Trp Ile Val Val Gly Val Val Ser Phe Gly His Lys Cys
        355                 360                 365

gcc gag gaa gga tat cct ggt gtg tac tcg cgc gta gcg agt tac cta       1152
Ala Glu Glu Gly Tyr Pro Gly Val Tyr Ser Arg Val Ala Ser Tyr Leu
    370                 375                 380

gac tgg atc gcg aaa gtt acg aac tcg tta gat cat gcc gtc act aac       1200
Asp Trp Ile Ala Lys Val Thr Asn Ser Leu Asp His Ala Val Thr Asn
385                 390                 395                 400

taa                                                                  1203
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<210> 6
<211> 400
<212> PRT
<213> Tachypleus tridentatus

<400> 6

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Met Thr Trp Ile Cys Val Ile Thr Leu Phe Ala Leu Ala Ser Ala Thr
1               5                   10                  15

Leu Gly Asn Lys Val Ser Arg Val Gly Val Leu Phe Pro Lys Thr Arg
            20                  25                  30

Asn Asp Asn Glu Cys Thr Ala Arg Gly Gly Leu Lys Gly Ser Cys Lys
        35                  40                  45

Ser Leu Ile Asp Cys Pro Ser Val Leu Ala Thr Leu Lys Asp Ser Phe
    50                  55                  60
```

```
Pro Val Val Cys Ser Trp Asn Gly Arg Phe Gln Pro Ile Val Cys Cys
65                  70                  75                  80

Pro Asp Ala Ile Ala Pro Pro Pro Val Thr Thr Thr Ala Val Thr Val
                85                  90                  95

Ile Ser Thr Lys Glu Pro Lys Leu Pro Arg Leu His Ile Ser Gly Cys
            100                 105                 110

Gly Lys Arg Lys Val Lys Ile Asp Ile Thr Thr Val Gly Arg Ser Gly
        115                 120                 125

Ser Pro Ile Leu Pro Pro Ile Ser Thr Pro Gln Asn Ser Thr Gly Gly
    130                 135                 140

Arg Gly Ile Ile Ala Gly Gly Val Glu Ala Lys Ile Gly Ala Trp Pro
145                 150                 155                 160

Trp Met Ala Ala Val Phe Val Lys Asn Phe Gly Ile Gly Arg Phe His
                165                 170                 175

Cys Ala Gly Ser Ile Ile Ser Asn Lys Tyr Ile Leu Ser Ala Ala His
            180                 185                 190

Ala Phe Leu Ile Gly Gly Arg Lys Leu Thr Pro Thr Arg Leu Ala Val
        195                 200                 205

Arg Val Gly Gly His Tyr Ile Lys Arg Gly Gln Glu Tyr Pro Val Lys
    210                 215                 220

Asp Val Ile Ile His Pro His Tyr Val Glu Lys Glu Asn Tyr Asn Asp
225                 230                 235                 240

Ile Ala Ile Ile Glu Leu Lys Glu Glu Leu Asn Phe Thr Asp Leu Val
                245                 250                 255

Asn Pro Ile Cys Leu Pro Asp Pro Glu Thr Val Thr Asp Pro Leu Lys
            260                 265                 270

Asp Arg Ile Val Thr Ala Ala Gly Trp Gly Asp Leu Asp Phe Ser Gly
        275                 280                 285

Pro Arg Ser Gln Val Leu Arg Glu Val Ser Ile Pro Val Val Pro Val
    290                 295                 300

Asp Lys Cys Asp Gln Ala Tyr Glu Lys Leu Asn Thr Pro Ser Leu Lys
305                 310                 315                 320
                                        (20)
```

```
Asn Gly Ile Thr Asn Asn Phe Leu Cys Ala Gly Leu Glu Glu Gly Gly
                325                 330                 335

Lys Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Met Leu Val Asn
            340                 345                 350

Asn Thr Arg Trp Ile Val Val Gly Val Val Ser Phe Gly His Lys Cys
        355                 360                 365

Ala Glu Glu Gly Tyr Pro Gly Val Tyr Ser Arg Val Ala Ser Tyr Leu
    370                 375                 380

Asp Trp Ile Ala Lys Val Thr Asn Ser Leu Asp His Ala Val Thr Asn
385                 390                 395                 400

<210>  7
<211>  1128
<212>  DNA
<213>  Tachypleus tridentatus

<220>
<221>  CDS
<222>  (1)..(1128)

<400>  7
atg ttg gtg aat aac gtg ttt tca cta ctg tgt ttc cca ctc ttg atg         48
Met Leu Val Asn Asn Val Phe Ser Leu Leu Cys Phe Pro Leu Leu Met
1               5                   10                  15

tct gtg gtt aga tgc agt act ctc agc aga cag cgt aga cag ttt gtt         96
Ser Val Val Arg Cys Ser Thr Leu Ser Arg Gln Arg Arg Gln Phe Val
            20                  25                  30

ttc cct gac gag gaa gaa ctt tgc tca aac cga ttt act gaa gaa gga        144
Phe Pro Asp Glu Glu Glu Leu Cys Ser Asn Arg Phe Thr Glu Glu Gly
        35                  40                  45

aca tgc aaa aat gtc ttg gat tgt aga ata ctt tta caa aaa aat gat        192
Thr Cys Lys Asn Val Leu Asp Cys Arg Ile Leu Leu Gln Lys Asn Asp
    50                  55                  60

tat aat tta ctc aaa gaa tca ata tgc ggc ttt gaa ggc ata aca ccc        240
Tyr Asn Leu Leu Lys Glu Ser Ile Cys Gly Phe Glu Gly Ile Thr Pro
65                  70                  75                  80

aaa gtt tgt tgt ccg aaa tca agc cat gta att tca agt aca cag gca        288
Lys Val Cys Cys Pro Lys Ser Ser His Val Ile Ser Ser Thr Gln Ala
                85                  90                  95

cct cca gaa acc act acg act gaa cgc cca cca aaa cag ata cca ccc        336
Pro Pro Glu Thr Thr Thr Thr Glu Arg Pro Pro Lys Gln Ile Pro Pro
            100                 105                 110

aat ctt cat gaa gtg tgt gga att cac aat act aca act acc agg att        384
Asn Leu His Glu Val Cys Gly Ile His Asn Thr Thr Thr Thr Arg Ile
        115                 120                 125
```

(21)

```
att gga ggt cgg gaa gca cct att gga gcc tgg ccg tgg atg act gct        432
Ile Gly Gly Arg Glu Ala Pro Ile Gly Ala Trp Pro Trp Met Thr Ala
    130                 135                 140

gtc tac ata aaa caa gga gga atc aga agt gtt cag tgt ggt ggc gca        480
Val Tyr Ile Lys Gln Gly Gly Ile Arg Ser Val Gln Cys Gly Gly Ala
145                 150                 155                 160

ctt gtc act aac agg cac gtg att aca gct tcg cac tgt gtt gta aac        528
Leu Val Thr Asn Arg His Val Ile Thr Ala Ser His Cys Val Val Asn
                165                 170                 175

agt gca gga aca gat gtg atg cca gct gat gta ttc tcg gtt cgt ctg        576
Ser Ala Gly Thr Asp Val Met Pro Ala Asp Val Phe Ser Val Arg Leu
            180                 185                 190

ggt gaa cac aat tta tac agt acc gat gac gat tcg aat cca ata gat        624
Gly Glu His Asn Leu Tyr Ser Thr Asp Asp Asp Ser Asn Pro Ile Asp
        195                 200                 205

ttt gca gtt acg tcg gtg aaa cat cac gaa cac ttt gta ctc gcg acg        672
Phe Ala Val Thr Ser Val Lys His His Glu His Phe Val Leu Ala Thr
    210                 215                 220

tat ttg aat gac atc gca att cta acg tta aat gac aca gtt acg ttt        720
Tyr Leu Asn Asp Ile Ala Ile Leu Thr Leu Asn Asp Thr Val Thr Phe
225                 230                 235                 240

aca gac aga att cga ccc att tgt cta cct tat cgt aag ttg aga tac        768
Thr Asp Arg Ile Arg Pro Ile Cys Leu Pro Tyr Arg Lys Leu Arg Tyr
                245                 250                 255

gat gat cta gca atg aga aaa ccg ttt atc act gga tgg gga aca aca        816
Asp Asp Leu Ala Met Arg Lys Pro Phe Ile Thr Gly Trp Gly Thr Thr
            260                 265                 270

gca ttt aac ggc cca tct agt gca gtg ttg aga gaa gta cag tta cca        864
Ala Phe Asn Gly Pro Ser Ser Ala Val Leu Arg Glu Val Gln Leu Pro
        275                 280                 285

ata tgg gaa cac gag gcc tgt aga cag gcc tac gag aag gat tta aat        912
Ile Trp Glu His Glu Ala Cys Arg Gln Ala Tyr Glu Lys Asp Leu Asn
    290                 295                 300

att aca aac gtg tat atg tgt gct ggc ttt gca gat ggc ggg aag gat        960
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gct tgc cag ggt gat tct gga ggt cca atg atg ttg cct gtt aaa acc       1008
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Phe Pro Asp Glu Glu Glu Leu Cys Ser Asn Arg Phe Thr Glu Glu Gly
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Thr Cys Lys Asn Val Leu Asp Cys Arg Ile Leu Leu Gln Lys Asn Asp
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Tyr Asn Leu Leu Lys Glu Ser Ile Cys Gly Phe Glu Gly Ile Thr Pro
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Lys Val Cys Cys Pro Lys Ser Ser His Val Ile Ser Ser Thr Gln Ala
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Pro Pro Glu Thr Thr Thr Thr Glu Arg Pro Pro Lys Gln Ile Pro Pro
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Asn Leu His Glu Val Cys Gly Ile His Asn Thr Thr Thr Thr Arg Ile
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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	Q246419
First Named Inventor	Hikaru MIZUMURA
Title	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN
Priority Mail Express® Label No.	

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: **Commissioner for Patents**
P.O. Box 1450
Alexandria VA 22313-1450

1. ☐ **Fee Transmittal Form** (PTO/SB/17 or equivalent)
2. ☐ **Applicant asserts small entity status.** See 37 CFR 1.27
3. ☐ **Applicant certifies micro entity status.** See 37 CFR 1.29. Applicant must attach form PTO/SB/15A or B or equivalent.
4. ☑ **Specification** [Total Pages 105] Both the claims and abstract must start on a new page. *(See MPEP § 608.01(a) for information on the preferred arrangement)*
5. ☑ **Drawing(s)** (35 U.S.C. 113) [Total Sheets 10]
6. **Inventor's Oath or Declaration** [Total Pages 3] *(including substitute statements under 37 CFR 1.64 and assignments serving as an oath or declaration under 37 CFR 1.63(e))*
   a. ☐ Newly executed (original or copy)
   b. ☑ A copy from a prior application (37 CFR 1.63(d))
7. ☑ **Application Data Sheet.** **See note below.* See 37 CFR 1.76 (PTO/AIA/14 or equivalent)
8. ☐ **CD-ROM or CD-R** in duplicate, large table or Computer Program (*Appendix*)
   ☐ Landscape Table on CD
9. ☐ **Nucleotide and/or Amino Acid Sequence Submission** *(if applications, items a. – c. are required)*
   a. ☐ Computer Readable Form (CRF)
   b. Specification Sequence Listing on:
      i. ☐ CD-ROM or CD-R (2 copies); or
      ii. ☐ Paper
   c. ☐ Statements verifying identity of above copies

ACCOMPANYING APPLICATION PAPERS

10. ☐ **Assignment Papers** (cover sheet & document(s))
    Name of Assignee SEIKAGAKU CORPORATION
11. ☐ **37 CFR 3.73(c) Statement** ☑ **Power of Attorney** *(when there is an assignee)*
12. ☐ **English Translation Document** *(if applicable)*
13. ☑ **Information Disclosure Statement** *(PTO/SB/08 or PTO-1449)*
    ☐ Copies of citations attached
14. ☐ **Preliminary Amendment**
15. ☐ **Return Receipt PostCard** *(MPEP § 503)(Should be specifically itemized)*
16. ☐ **Certified Copy of Priority Document(s)** *(if foreign priority is claimed)*
17. ☐ **Nonpublication Request** Under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
18. ☑ **Priority is claimed from: SEE ATTACHED**
19. ☑ **Other**: PDF of Sequence Listing (25 pgs) and Request for Transfer of Computer Readable Form

Note: (1) Benefit claims under 37 CFR 1.78 and foreign priority claims under 1.55 **must** be included in an Application Data Sheet (ADS).
(2) For applications filed under 35 U.S.C. 111, the application must contain an ADS specifying the applicant if the applicant is an assignee, person to whom the inventor is under an obligation to assign, or person who otherwise shows sufficient proprietary interest in the matter. See 37 CFR 1.46(b).

☑ The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

20. **If a CONTINUING APPLICATION**, *check appropriate box, and supply the requisite information below and in the first sentence of the specification following the title, or in an Application Data Sheet under 37 CFR 1.76*:

☑ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No: **15/983,725**

Prior application information: *Examiner:* **STEADMAN, DAVID** *Art Unit:* **1656**

21. CORRESPONDENCE ADDRESS

☑ The address associated with Customer Number: WASHINGTON OFFICE **23373** CUSTOMER NUMBER OR ☐ Correspondence address below

Signature /markboland/	Date April 30, 2019
Name Mark Boland	Registration No. 32,197

UTILITY PATENT APPLICATION TRANSMITTAL
ADDITIONAL PAGE

Priority is claimed from:

Country	Application No	Filing Date
US	15/983,725	2018-05-18
US	14/650,767	2015-06-09
PCT	PCT/JP2013/083082	2013-12-10
JP	2012-269840	2012-12-10

	Attorney Docket No.	Q246419
CORRESPONDENCE ADDRESS		
☑ The address associated with Customer Number:	WASHINGTON OFFICE 23373 CUSTOMER NUMBER	OR ☐ Correspondence address below

Abstract

A horseshoe crab Factor C protein having activity of Factor C, wherein the horseshoe crab is selected from *Tachypleus tridentatus, Limulus polyphemus, and Carcinoscorpius rotundicauda*, and wherein the horseshoe crab Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or HEK cell.

What is claimed is:

1. A horseshoe crab Factor C protein having activity of Factor C,

wherein the horseshoe crab is selected from the group consisting of *Tachypleus tridentatus, Limulus polyphemus, and Carcinoscorpius rotundicauda*, and wherein the horseshoe crab Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or HEK cell.

2. A Factor C protein having activity of Factor C,

wherein the Factor C protein comprises an amino acid sequence selected from the group consisting of:

(A) the amino acid sequence of SEQ ID NO: 2;

(B) an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:2, and having activity of Factor C;

(C) the amino acid sequence of SEQ ID NO: 4; and

(D) an amino acid sequence having 90% or higher sequence identity to the amino acid sequence of SEQ ID NO:4, and having activity of Factor C,

wherein the Factor C protein is produced through being recombinantly expressed from a Chinese Hamster Ovary (CHO) DG44 cell or HEK cell, and

wherein the Factor C protein exhibits a residual activity of 10% or higher in the presence of 21 mM sodium

citrate.

3. The Factor C protein according to claim 2, which is water soluble.

4. The Factor C protein according to claim 2, which has a molecular weight of 115 kDa to 140 kDa as measured by SDS-PAGE under non-reducing condition.

5. The Factor C protein according to claim 4, which has a molecular weight of 127 kDa±5 kDa.

6. The Factor C protein according to claim 2, which exhibits a residual Factor C activity of 25% or higher in the presence of 52 mM sodium hydrogen carbonate.

7. The Factor C protein according to claim 2, which exhibits a residual Factor C activity of 25% or higher in the presence of 214 mM sodium chloride.

8. The Factor C protein according to claim 2, which exhibits a residual Factor C activity of 15% or higher in the presence of 16 mM magnesium sulfate.

9. The Factor C protein according to claim 2, which exhibits a residual Factor C activity of 35% or higher in the presence of 2.5 mM calcium chloride.

10. The horseshoe crab Factor C protein according to claim 1, which contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a corresponding native horseshoe crab Factor C protein and/or a corresponding horseshoe crab Factor C protein recombinantly expressed using Sf9 as a host cell.

11. The Factor C protein according to claim 2, which contains (α-2,3)-linked terminal sialic acid in a greater amount, as compared with a corresponding native horseshoe crab Factor C protein and/or a corresponding horseshoe crab Factor C protein recombinantly expressed using Sf9 as a host cell.

12. A method for producing the Factor C protein of claim 2, the method comprising culturing a CHO DG44 cell or HEK cell recombinantly expressing said Factor C protein under conditions suitable for the expression of the horseshoe crab

Factor C to produce the Factor C protein.

13. An endotoxin assay kit comprising the Factor C protein according to claim 2 and one or more elements.

14. A method for measuring endotoxin in a test specimen using an endotoxin assay agent comprising the Factor C protein according to claim 2.

15. The method according to claim 14, wherein the test specimen comprises at least one of:
(A) an ion;
(B) a cation;
(C) a metal ion;
(D) an alkali metal ion or an alkaline earth metal ion; and
(E) one or more kinds of ions selected from the group consisting of sodium ion, potassium ion, calcium ion, and magnesium ion.

16. The method according to claim 15, wherein the test specimen comprises a cation and the final concentration of the cation after mixing the test specimen with the assay agent is 1 mM or higher.

17. The method according to claim 14, wherein the test

specimen is an injection.

18. A product which has been subjected to an endotoxin assay using an endotoxin assay agent comprising the Factor C protein according to claim 2,

wherein the product is selected from the group consisting of water for medical use, a pharmaceutical product, an infusion solution, a blood product, a medical apparatus, a medical instrument, a biological specimen, a native protein, a recombinant protein, a nucleic acid, and a saccharide.

Description

Novel Recombinant Factor C and Method for Producing the Same, and Method for Measuring Endotoxin

Cross-Reference To Related Applications

This is a Continuation of U.S. Patent Application 15/983,725 filed May 18, 2018; which is a continuation of U.S. Patent Application 14/650,767 filed June 9,2015, which is National Stage of International Application No. PCT/JP2013/083082 filed December 10, 2013, claiming priority based on Japanese Patent Application No. 2012-269840 filed December 10, 2012, the contents of all of which are incorporated herein by reference in their entirety.

Technical Field

[0001]

The present invention relates to a novel recombinant Factor C, to a method for producing the recombinant Factor C, and to a method for measuring endotoxin.

Background Art

[0002]

Endotoxin is a lipopolysaccharide which is present in the outer membrane of Gram-negative bacteria and is known as a strong pyrogen. It is also known that even a very small

amount of endotoxin causes various pathological conditions via bacterial infection. Such conditions include not only fever but also release of an inflammatory cytokine concomitant with activation of macrophages, induction of endotoxin shock, and the like. Therefore, detection of endotoxin is essential in pharmaceutical products such as an injection, water, medical devices, and the like. From another aspect, endotoxin is a conceivable main cause for a shock involved in an infection with a Gram-negative bacterium. Thus, the presence of infection and a therapeutic effect can be determined through a blood endotoxin analysis.

[0003]

Meanwhile, it has been known that the American horseshoe crab (*Limulus polyphemus*) undergoes blood clotting when it is infected with a Gram-negative bacterium. This phenomenon has been conventionally employed for the detection of endotoxin.

[0004]

Specifically, there is known a method for measuring endotoxin by use of a hematocyte extract of a horseshoe crab (i.e. an amebocyte lysate of a horseshoe crab, hereinafter also referred to simply as a lysate) (see, for example, Non-Patent Document 1). This method is called a "limulus test," which employs a cascade reaction of a variety of proteins present in the lysate, which reaction occurs via contact between endotoxin and the lysate. Fig. 1 shows the scheme of the cascade reaction.

[0005]

When endotoxin comes into contact with the lysate, Factor C, which is a serine protease zymogen present in the lysate, is activated to thereby form activated Factor C. The thus-formed activated Factor C activates Factor B present in the lysate, to thereby form activated Factor B. The thus-formed activated Factor B activates Pro-clotting enzyme present in the lysate, to thereby form a corresponding Clotting enzyme.

[0006]

The Clotting enzyme hydrolyzes a specific site of a coagulogen molecule present in the lysate, thereby coagulin gel is formed, and thus the lysate is coagulated. Thus, endotoxin can be measured through measuring the lysate coagulation reaction.

[0007]

Alternatively, endotoxin may also be measured through coloring reaction between the Clotting enzyme and a synthetic substrate. For example, the Clotting enzyme acts on t-butoxycarbonyl-leucyl-glycyl-arginyl-pNA (Boc-Leu-Gly-Arg-pNA), which is a synthetic substrate, to hydrolyze the amino bonds thereof, and thereby pNA is released. Thus, when the synthetic substrate has been added to the reaction system, endotoxin can be measured through measuring the absorbance (at 405 nm) of the coloring substance (pNA).

[0008]

Furthermore, it is known that a cascade reaction system

can be reconstituted by use of Factor C, Factor B, and Pro-clotting enzyme, which are purified from a lysate of the Japanese horseshoe crab (Non-Patent Document 2).

[0009]

However, for using such a lysate, or Factor C, Factor B, and Pro-clotting enzyme purified from the lysate, horseshoe crabs must be caught and blood must be recovered there from. Hence, from the viewpoint of protection of biological resources, difficulty is encountered in supply of such ingredients in an inexhaustible manner. Under such circumstances, there is demand for a technique that can produce these ingredients by genetic engineering, to thereby reconstitute a cascade reaction system.

[0010]

For example, there is known a case where Factor C, Factor B, and Pro-clotting enzyme were expressed in insect cells as host cells, to thereby reconstitute a cascade reaction system (Patent Documents 1 and 2). However, it has been reported that since the reconstituted system contains sodium chloride, magnesium sulfate, or calcium chloride in the reaction system, the cascade reaction is suppressed (Patent Document 1).

[0011]

Alternatively, there is known a case of using mammalian cells as host cells, wherein Factor C derived from the Singaporean horseshoe crab (*Carcinoscorpius rotundicauda*) was expressed in COS-1, which is a cell line derived from African

green monkey kidney cells, as host cells. However, it has been reported that when COS-1 was used as a host, the expressed Factor C was insoluble (Non-Patent Documents 3 and 4). That is, there has never been known a case where functional Factor C is produced using mammalian cells as host cells.

Prior Art Documents

Patent Documents

[0012]

Patent Document 1: WO 2008/004674, pamphlet

Patent Document 2: WO 2012/118226, pamphlet

Non-Patent Documents

[0013]

Non-Patent Document 1: Iwanaga S., Curr. Opin. Immunol. Feb; 5(1): 74-82 (1993)

Non-Patent Document 2: Nakamura T., *et al.*, J. Biochem. Mar; 99(3): 847-57 (1986)

Non-Patent Document 3: Roopashree S. Dwarakanath, *et al.*, Biotechnology letters 19(4): 357-361 (1997)

Non-Patent Document 4: Jing Wang, Bow Ho and Jeak L. Ding, Biotechnology letters 23: 71-76 (2001)

Summary of the Invention

Problems to be Solved by the Invention

[0014]

An object of the present invention is to provide a

novel recombinant horseshoe crab Factor C, and a method for producing the recombinant Factor C.

[0015]

Meanwhile, injections to be administered in vivo generally contain a variety of salts (ions). In production of such injections, detection of endotoxin is obligated by the pharmacopoeia of the relevant country. Under such circumstances, in an aspect, another object of the present invention is to provide a reconstituted cascade reaction system which is not susceptible to reaction inhibition even in the presence of a salt (ion).

Means for Solving the Problems

[0016]

The present inventors have found that a recombinant horseshoe crab Factor C can be produced by use of human cells or Chinese hamster cells as host cells, and that endotoxin can be measured by use of the thus-produced recombinant Factor C while reaction inhibition in the presence of a salt (ion) is mitigated. The present invention has been accomplished on the basis of these findings.

[0017]

Accordingly, the present invention encompasses the following modes.

[1]

A horseshoe crab Factor C having activity of Factor C.

[1.1.1]

The above-described Factor C, which contains sialic acid.

[1.1.2]

The above-described Factor C, which contains (α-2,3) linked terminal sialic acid.

[1.1.3]

The above-described Factor C, which contains (α-2,3) linked terminal sialic acid in a greater amount, as compared with a native Factor C.

[1.1.4]

The above-described Factor C, which contains (α-2,3) linked terminal sialic acid in a greater amount, as compared with a horseshoe crab Factor C expressed in Sf9 as a host cell.

[1.1.5]

The above-described Factor C, which exhibits a higher reactivity in lectin blotting by use of *Maackia amurensis* agglutinin, as compared with a native Factor C.

[1.1.6]

The above-described Factor C, which exhibits a higher reactivity in lectin blotting by use of *Maackia amurensis* agglutinin, as compared with a horseshoe crab Factor C expressed in Sf9 as a host cell.

[1.2.1]

The above-described Factor C, which exhibits a residual activity of 10% or higher in the presence of 21 mM sodium citrate.

[1.2.2]

The above-described Factor C, which exhibits a residual activity of 20% or higher in the presence of 21 mM sodium citrate.

[1.2.3]

The above-described Factor C, which exhibits a residual activity of 25% or higher in the presence of 52 mM sodium hydrogencarbonate.

[1.2.4]

The above-described Factor C, which exhibits a residual activity of 35% or higher in the presence of 52 mM sodium hydrogencarbonate.

[1.2.5]

The above-described Factor C, which exhibits a residual activity of 25% or higher in the presence of 214 mM sodium chloride.

[1.2.6]

The above-described Factor C, which exhibits a residual activity of 35% or higher in the presence of 214 mM sodium chloride.

[1.2.7]

The above-described Factor C, which exhibits a residual activity of 15% or higher in the presence of 16 mM magnesium sulfate.

[1.2.8]

The above-described Factor C, which exhibits a residual activity of 25% or higher in the presence of 16 mM magnesium

sulfate.

[1.2.9]

The above-described Factor C, which exhibits a residual activity of 35% or higher in the presence of 2.5 mM calcium chloride.

[1.2.10]

The above-described Factor C, which exhibits a residual activity of 45% or higher in the presence of 2.5 mM calcium chloride.

[1.2.11]

The above-described Factor C, which exhibits a higher residual activity in the presence of 21 mM sodium citrate, as compared with a horseshoe crab Factor C expressed in Sf9 as a host cell and/or a native Factor C.

[1.2.12]

The above-described Factor C, which exhibits a higher residual activity in the presence of 52 mM sodium hydrogencarbonate, as compared with a horseshoe crab Factor C expressed in Sf9 as a host cell and/or a native Factor C.

[1.2.13]

The above-described Factor C, which exhibits a higher residual activity in the presence of 214 mM sodium chloride, as compared with a horseshoe crab Factor C expressed in Sf9 as a host cell and/or a native Factor C.

[1.2.14]

The above-described Factor C, which exhibits a higher residual activity in the presence of 16 mM magnesium sulfate,

as compared with a horseshoe crab Factor C expressed in Sf9 as a host cell and/or a native Factor C.

[1.2.15]

The above-described Factor C, which exhibits a higher residual activity in the presence of 2.5 mM calcium chloride, as compared with a horseshoe crab Factor C expressed in Sf9 as a host cell and/or a native Factor C.

[1.3.1]

The Factor C, which is a protein shown in the following (A), (B), (C), or (D):

(A) a protein comprising the amino acid sequence shown in SEQ ID NO: 2;

(B) a protein comprising the amino acid sequence shown in SEQ ID NO:2 but which includes substitution, deletion, insertion, or addition of one or several amino acid residues, and having activity of Factor C;

(C) a protein comprising the amino acid sequence shown in SEQ ID NO: 4;

(D) a protein comprising the amino acid sequence shown in SEQ ID NO:4 but which includes substitution, deletion, insertion, or addition of one or several amino acid residues, and having activity of Factor C.

[1.3.2]

The Factor C, which is a protein shown in the following (A) or (B):

(A) a protein comprising the amino acid sequence shown in SEQ ID NO: 2;

(B) a protein comprising the amino acid sequence shown in SEQ ID NO:2 but which includes substitution, deletion, insertion, or addition of one or several amino acid residues, and having activity of Factor C.

[1.4]

The above-described Factor C, which is a recombinant protein.

[1.5.1]

The above-described Factor C, which has a molecular weight of 115 kDa to 140 kDa, as measured through SDS-PAGE under a non-reducing condition.

[1.5.2]

The above-described Factor C, which has a molecular weight of 115 kDa to 130 kDa, as measured through SDS-PAGE under a non-reducing condition.

[1.5.3]

The above-described Factor C, which has a molecular weight of 120 kDa to 130 kDa, as measured through SDS-PAGE under a non-reducing condition.

[1.5.4]

The above-described Factor C, which has a molecular weight of 120 kDa to 128 kDa, as measured through SDS-PAGE under a non-reducing condition.

[1.5.5]

The above-described Factor C, which has a molecular weight of 127 kDa ± 5 kDa, as measured through SDS-PAGE under a non-reducing condition.

[1.5.6]

The above-described Factor C, which has a molecular weight of 128 kDa ± 2 kDa, as measured through SDS-PAGE under a non-reducing condition.

[1.5.7]

The above-described Factor C, which has a molecular weight of 127 kDa ± 2 kDa, as measured through SDS-PAGE under a non-reducing condition.

[1.5.8]

The above-described Factor C, which has a molecular weight of 126 kDa ± 2 kDa, as measured through SDS-PAGE under a non-reducing condition.

[1.5.9]

The above-described Factor C, which has a molecular weight of 127 kDa ± 1 kDa, as measured through SDS-PAGE under a non-reducing condition.

[1.6]

The above-described Factor C, which is water-soluble.

[1.7]

The above-described Factor C, which is a culture supernatant containing Factor C.

[1.8]

The above-described Factor C, which has the following characteristics (1) to (3):

(1) having activity of Factor C;

(2) having a molecular weight of 115 kDa to 130 kDa, as measured through SDS-PAGE under a non-reducing condition; and

(3) exhibiting a residual activity of 10% or higher in the presence of 21 mM sodium citrate.

[2]

A method for producing a horseshoe crab Factor C, the method comprising expressing the horseshoe crab Factor C in a mammalian cell as a host cell.

[2.1.1]

The above-described method, wherein the mammalian cell is a mammalian cell other than COS-1.

[2.1.2]

The above-described method, wherein the mammalian cell is a cell of a mammal selected from the group consisting of a primate and a rodent.

[2.1.3]

The above-described method, wherein the mammalian cell is a primate cell.

[2.1.4]

The above-described method, wherein the mammalian cell is a rodent cell.

[2.1.5]

The above-described method, wherein the mammalian cell is a cell of a mammal selected from the group consisting of a primate and a rodent, other than monkey.

[2.1.6]

The above-described method, wherein the mammalian cell is a Chinese hamster cell or a human cell.

[2.1.7]

The above-described method, wherein the mammalian cell is a Chinese hamster cell.

[2.1.8]

The above-described method, wherein the mammalian cell is a human cell.

[2.1.9]

The above-described method, wherein the mammalian cell is CHO or HEK.

[2.1.10]

The above-described method, wherein the mammalian cell is CHO.

[2.1.11]

The above-described method, wherein the mammalian cell is HEK.

[2.1.12]

The above-described method, wherein the mammalian cell is CHO DG44 or HEK293.

[2.1.13]

The above-described method, wherein the mammalian cell is CHO DG44.

[2.1.14]

The above-described method, wherein the mammalian cell is HEK293.

[2.2.1]

The above-described method, wherein the Factor C is a protein shown in the following (A), (B), (C), or (D):

(A) a protein comprising the amino acid sequence shown

in SEQ ID NO: 2;

(B) a protein comprising the amino acid sequence shown in SEQ ID NO:2 but which includes substitution, deletion, insertion, or addition of one or several amino acid residues, and having activity of Factor C;

(C) a protein comprising the amino acid sequence shown in SEQ ID NO: 4;

(D) a protein comprising the amino acid sequence shown in SEQ ID NO:4 but which includes substitution, deletion, insertion, or addition of one or several amino acid residues, and having activity of Factor C.

[2.2.2]

The above-described method, wherein the Factor C is a protein shown in the following (A) or (B):

(A) a protein comprising the amino acid sequence shown in SEQ ID NO: 2;

(B) a protein comprising the amino acid sequence shown in SEQ ID NO:2 but which includes substitution, deletion, insertion, or addition of one or several amino acid residues, and having activity of Factor C.

[3]

A horseshoe crab Factor C producible by the above-described method.

[3.1]

The above-described Factor C, which has activity of Factor C.

[3.2.1]

The above-described Factor C, which contains sialic acid.

[3.2.2]

The above-described Factor C, which contains (α-2,3) linked terminal sialic acid.

[3.2.3]

The above-described Factor C, which contains (α-2,3) linked terminal sialic acid in a greater amount, as compared with a native Factor C.

[3.2.4]

The above-described Factor C, which contains (α-2,3) linked terminal sialic acid in a greater amount, as compared with a horseshoe crab Factor C expressed in Sf9 as a host cell.

[3.2.5]

The above-described Factor C, which exhibits a higher reactivity in lectin blotting by use of *Maackia amurensis* agglutinin, as compared with a native Factor C.

[3.2.6]

The above-described Factor C, which exhibits a higher reactivity in lectin blotting by use of *Maackia amurensis* agglutinin, as compared with a horseshoe crab Factor C expressed in Sf9 as a host cell.

[3.3.1]

The above-described Factor C, which exhibits a residual activity of 10% or higher in the presence of 21 mM sodium citrate.

[3.3.2]

The above-described Factor C, which exhibits a residual activity of 20% or higher in the presence of 21 mM sodium citrate.

[3.3.3]

The above-described Factor C, which exhibits a residual activity of 25% or higher in the presence of 52 mM sodium hydrogencarbonate.

[3.3.4]

The above-described Factor C, which exhibits a residual activity of 35% or higher in the presence of 52 mM sodium hydrogencarbonate.

[3.3.5]

The above-described Factor C, which exhibits a residual activity of 25% or higher in the presence of 214 mM sodium chloride.

[3.3.6]

The above-described Factor C, which exhibits a residual activity of 35% or higher in the presence of 214 mM sodium chloride.

[3.3.7]

The above-described Factor C, which exhibits a residual activity of 15% or higher in the presence of 16 mM magnesium sulfate.

[3.3.8]

The above-described Factor C, which exhibits a residual activity of 25% or higher in the presence of 16 mM magnesium

sulfate.

[3.3.9]

The above-described Factor C, which exhibits a residual activity of 35% or higher in the presence of 2.5 mM calcium chloride.

[3.3.10]

The above-described Factor C, which exhibits a residual activity of 45% or higher in the presence of 2.5 mM calcium chloride.

[3.3.11]

The above-described Factor C, which exhibits a higher residual activity in the presence of 21 mM sodium citrate, as compared with a horseshoe crab Factor C expressed in Sf9 as a host cell and/or a native Factor C.

[3.3.12]

The above-described Factor C, which exhibits a higher residual activity in the presence of 52 mM sodium hydrogencarbonate, as compared with a horseshoe crab Factor C expressed in Sf9 as a host cell and/or a native Factor C.

[3.3.13]

The above-described Factor C, which exhibits a higher residual activity in the presence of 214 mM sodium chloride, as compared with a horseshoe crab Factor C expressed in Sf9 as a host cell and/or a native Factor C.

[3.3.14]

The above-described Factor C, which exhibits a higher residual activity in the presence of 16 mM magnesium sulfate,

as compared with a horseshoe crab Factor C expressed in Sf9 as a host cell and/or a native Factor C.

[3.3.15]

The above-described Factor C, which exhibits a higher residual activity in the presence of 2.5 mM calcium chloride, as compared with a horseshoe crab Factor C expressed in Sf9 as a host cell and/or a native Factor C.

[3.4]

The above-described Factor C, which is a recombinant protein.

[3.5.1]

The above-described Factor C, which has a molecular weight of 115 kDa to 140 kDa, as measured through SDS-PAGE under a non-reducing condition.

[3.5.2]

The above-described Factor C, which has a molecular weight of 115 kDa to 130 kDa, as measured through SDS-PAGE under a non-reducing condition.

[3.5.3]

The above-described Factor C, which has a molecular weight of 120 kDa to 130 kDa, as measured through SDS-PAGE under a non-reducing condition.

[3.5.4]

The above-described Factor C, which has a molecular weight of 120 kDa to 128 kDa, as measured through SDS-PAGE under a non-reducing condition.

[3.5.5]

The above-described Factor C, which has a molecular weight of 127 kDa ± 5 kDa, as measured through SDS-PAGE under a non-reducing condition.

[3.5.6]

The above-described Factor C, which has a molecular weight of 128 kDa ± 2 kDa, as measured through SDS-PAGE under a non-reducing condition.

[3.5.7]

The above-described Factor C, which has a molecular weight of 127 kDa ± 2 kDa, as measured through SDS-PAGE under a non-reducing condition.

[3.5.8]

The above-described Factor C, which has a molecular weight of 126 kDa ± 2 kDa, as measured through SDS-PAGE under a non-reducing condition.

[3.5.9]

The above-described Factor C, which has a molecular weight of 127 kDa ± 1 kDa, as measured through SDS-PAGE under a non-reducing condition.

[3.6]

The above-described Factor C, which is water-soluble.

[3.7]

The above-described Factor C, which is a culture supernatant containing Factor C.

[4]

An endotoxin assay agent containing the above-described Factor C.

[4.1]

The above-described assay agent, which further contains a horseshoe crab Factor B and a horseshoe crab Pro-clotting enzyme.

[4.2]

The above-described assay agent, wherein the Factor B is a protein shown in the following (E) or (F) and the Pro-clotting enzyme is a protein shown in the following (G) or (H):

(E) a protein comprising the amino acid sequence shown in SEQ ID NO: 6;

(F) a protein comprising the amino acid sequence shown in SEQ ID NO:6 but which includes substitution, deletion, insertion, or addition of one or several amino acid residues, and having activity of Factor B;

(G) a protein comprising the amino acid sequence shown in SEQ ID NO: 8;

(H) a protein comprising the amino acid sequence shown in SEQ ID NO:8 but which includes substitution, deletion, insertion, or addition of one or several amino acid residues, and having activity of Pro-clotting enzyme.

[5]

An endotoxin assay kit comprising the above-described assay agent.

[6]

A method for measuring endotoxin in a test specimen, the method comprising a step of mixing the above-described

assay agent and the test specimen, and a step of measuring the progress of a cascade reaction.

[6.1.1]

The above-described method, wherein the test specimen contains an ion.

[6.1.2]

The above-described method, wherein the test specimen contains a cation.

[6.1.3]

The above-described method, wherein the test specimen contains a metal ion.

[6.1.4]

The above-described method, wherein the test specimen contains an alkali metal ion or an alkaline earth metal ion.

[6.1.5]

The above-described method, wherein the test specimen contains an alkali metal ion.

[6.1.6]

The above-described method, wherein the test specimen contains an alkaline earth metal ion.

[6.2.1]

The above-described method, wherein the test specimen contains one or more kinds of ions selected from the group consisting of sodium ion, potassium ion, calcium ion, and magnesium ion.

[6.2.2]

The above-described method, wherein the test specimen

contains sodium ion.

[6.2.3]

The above-described method, wherein the test specimen contains potassium ion.

[6.2.4]

The above-described method, wherein the test specimen contains calcium ion.

[6.2.5]

The above-described method, wherein the test specimen contains magnesium ion.

[6.3.1]

The above-described method, wherein the contained amount of the ion in the test specimen is such an amount that the concentration of the cation derived from the test specimen becomes 1 mM or higher, as a final concentration in the reaction system after mixing the test specimen with the assay agent.

[6.3.2]

The above-described method, wherein the contained amount of the ion in the test specimen is such an amount that the concentration of the cation derived from the test specimen becomes 2 mM or higher, as a final concentration in the reaction system after mixing the test specimen with the assay agent.

[6.3.3]

The above-described method, wherein the contained amount of the ion in the test specimen is such an amount that

the concentration of the cation derived from the test specimen becomes 5 mM or higher, as a final concentration in the reaction system after mixing the test specimen with the assay agent.

[6.3.4]

The above-described method, wherein the contained amount of the ion in the test specimen is such an amount that the concentration of the cation derived from the test specimen becomes 10 mM or higher, as a final concentration in the reaction system after mixing the test specimen with the assay agent.

[6.3.5]

The above-described method, wherein the contained amount of the ion in the test specimen is such an amount that the concentration of the cation derived from the test specimen becomes 20 mM or higher, as a final concentration in the reaction system after mixing the test specimen with the assay agent.

[6.3.6]

The above-described method, wherein the contained amount of the ion in the test specimen is such an amount that the concentration of the cation derived from the test specimen becomes 50 mM or higher, as a final concentration in the reaction system after mixing the test specimen with the assay agent.

[6.3.7]

The above-described method, wherein the contained

amount of the ion in the test specimen is such an amount that the concentration of the cation derived from the test specimen becomes 100 mM or higher, as a final concentration in the reaction system after mixing the test specimen with the assay agent.

[6.4]

The above-described method, wherein the test specimen is an injection.

[6.5.1]

The above-described method, wherein the test specimen contains one or more kinds of salts selected from the group consisting of sodium chloride, magnesium sulfate, sodium hydrogencarbonate, sodium citrate, calcium chloride, potassium chloride, sodium iothalamate, calcium disodium edetate, and dihydrogen sodium phosphate.

[6.5.2]

The above-described method, wherein the test specimen contains sodium chloride.

[6.5.3]

The above-described method, wherein the test specimen contains magnesium sulfate.

[6.5.4]

The above-described method, wherein the test specimen contains sodium hydrogencarbonate.

[6.5.5]

The above-described method, wherein the test specimen contains sodium citrate.

[6.5.6]

The above-described method, wherein the test specimen contains calcium chloride.

[6.5.7]

The above-described method, wherein the test specimen contains potassium chloride.

[6.5.8]

The above-described method, wherein the test specimen contains sodium iothalamate.

[6.5.9]

The above-described method, wherein the test specimen contains calcium disodium edetate.

[6.5.10]

The above-described method, wherein the test specimen contains dihydrogen sodium phosphate.

[6.6.1]

The above-described method, wherein the contained amount of the salt in the test specimen is such an amount that the concentration of the salt derived from the test specimen becomes 1 mM or higher, as a final concentration in the reaction system after mixing the test specimen with the assay agent.

[6.6.2]

The above-described method, wherein the contained amount of the salt in the test specimen is such an amount that the concentration of the salt derived from the test specimen becomes 2 mM or higher, as a final concentration in

the reaction system after mixing the test specimen with the assay agent.

[6.6.3]

The above-described method, wherein the contained amount of the salt in the test specimen is such an amount that the concentration of the salt derived from the test specimen becomes 5 mM or higher, as a final concentration in the reaction system after mixing the test specimen with the assay agent.

[6.6.4]

The above-described method, wherein the contained amount of the salt in the test specimen is such an amount that the concentration of the salt derived from the test specimen becomes 10 mM or higher, as a final concentration in the reaction system after mixing the test specimen with the assay agent.

[6.6.5]

The above-described method, wherein the contained amount of the salt in the test specimen is such an amount that the concentration of the salt derived from the test specimen becomes 20 mM or higher, as a final concentration in the reaction system after mixing the test specimen with the assay agent.

[6.6.6]

The above-described method, wherein the contained amount of the salt in the test specimen is such an amount that the concentration of the salt derived from the test

specimen becomes 50 mM or higher, as a final concentration in the reaction system after mixing the test specimen with the assay agent.

[6.6.7]

The above-described method, wherein the contained amount of the salt in the test specimen is such an amount that the concentration of the salt derived from the test specimen becomes 100 mM or higher, as a final concentration in the reaction system after mixing the test specimen with the assay agent.

[6.7]

The above-described method, which further comprises a step of adding, to the reaction system, a substrate for detecting the progress of the cascade reaction.

[6.8]

The above-described method, which further comprises a step of calculating the amount of endotoxin in the test specimen.

Brief Description of the Drawings

[0018]

[Fig. 1] A chart showing a cascade reaction system of the limulus test.

[Fig. 2] A photograph showing the results of western blotting of four types of Factor C (Sf9, CHO, HEK, and TAL) derived from Japanese horseshoe crab (*Tachypleus tridentatus*), under non-reducing conditions.

[Fig. 3] A photograph (SDS-PAGE) showing the molecular weight of each of a native Factor C (TAL) derived from Japanese horseshoe crab (*Tachypleus tridentatus*) and a recombinant Factor C (CHO).

[Fig. 4] A scheme of measuring the activity of Factor C.

[Fig. 5] A chart showing a reconstituted cascade reaction system.

[Fig. 6] A table and a graph showing the activity of the four types of Factor C (Sf9, CHO, HEK, and TAL) derived from Japanese horseshoe crab (*Tachypleus tridentatus*).

[Fig. 7] A table and a graph showing the activity inhibition by injections of the four types of Factor C (Sf9, CHO, HEK, and TAL) derived from Japanese horseshoe crab (*Tachypleus tridentatus*).

[Fig. 8] Alignment of Factor C of *Tachypleus tridentatus* (TtFC; SEQ ID NO: 2) and Factor C of *Carcinoscorpius rotundicauda* (834CrID4; SEQ ID NO: 4) (continued to Fig. 9). Consensus sequences which can undergo N-type sugar chain (N-linked sugar chain) modification are underlined.

[Fig. 9] Alignment of Factor C of *Tachypleus tridentatus* (TtFC; SEQ ID NO: 2) and Factor C of *Carcinoscorpius rotundicauda* (834CrID4; SEQ ID NO: 4) (continued from Fig. 8). Consensus sequences which can undergo N-type sugar chain (N-linked sugar chain) modification are underlined.

[Fig. 10] A schematic representation of reaction sites

between Factor C and specific antibodies under reducing and non-reducing conditions.

[Fig. 11] A photograph (SDS-PAGE) showing the molecular weight of the four types of purified Factor C derived from Japanese horseshoe crab (*Tachypleus tridentatus*).

[Fig. 12] A photograph (western blotting) showing the molecular weight of the four types of purified Factor C derived from Japanese horseshoe crab (*Tachypleus tridentatus*).

[Fig. 13] A photograph (western blotting) showing the results of removal of N-type sugar chain, by glycopeptidase F, from the four types of purified Factor C derived from Japanese horseshoe crab (*Tachypleus tridentatus*).

[Fig. 14] (a) A photograph (lectin blotting) showing the results of detection of N-type sugar chain of the four types of purified Factor C derived from Japanese horseshoe crab (*Tachypleus tridentatus*); (b) a table showing the biding specificity of lectin; and (c) a schematic representation of N-type sugar chain structures of a recombinant Factor C, assumed by the experimental results and reports by documents.

Modes for Carrying Out the Invention

In the present invention, a series of reactions wherein endotoxin activates Factor C to generate active-type Factor C; the active-type Factor C activates Factor B to generate active-type Factor B; and the active-type Factor B activates Pro-clotting enzyme to generate a corresponding Clotting enzyme; may be referred to as “cascade reaction”. In the

present invention, Factor C, Factor B, and Pro-clotting enzyme may be collectively referred to as “factor”. In the present invention, a cascade reaction system constituted by combining the factors may be referred to as “reconstituted system”. In such a reconstituted system, an effect of a contaminant contained in an amebocyte lysate of a horseshoe crab, e.g. Factor G, on the cascade reaction can be eliminated.

[0019]

(1) Factor C of the present invention

The Factor C of the present invention is a Factor C derived from a horseshoe crab. The Factor C of the present invention is a recombinant protein. The term "recombinant protein" refers to a protein obtained through introduction of a gene encoding the protein into a host cell and heterologous expression of the gene. The Factor C of the present invention has activity of Factor C.

[0020]

Examples of the horseshoe crab include *Tachypleus tridentatus* (Japanese horseshoe crab), *Limulus polyphemus* (American horseshoe crab), *Carcinoscorpius rotundicauda* (Southeast Asian (Singaporean) horseshoe crab), and *Tachypleus gigas* (Southeast Asian horseshoe crab). Among them, *Tachypleus tridentatus* (Japanese horseshoe crab) and *Carcinoscorpius rotundicauda* (Southeast Asian (Singaporean) horseshoe crab) are preferred as a Factor C source. Furthermore, among them, *Tachypleus tridentatus* (Japanese

horseshoe crab) is more preferred as a Factor C source. The amino acid sequence of the Factor C derived from *Tachypleus tridentatus* is shown in SEQ ID NO: 2. The nucleotide sequence of a gene encoding the Factor C derived from *Tachypleus tridentatus* is shown in SEQ ID NO: 1. The amino acid sequence of the Factor C derived from *Carcinoscorpius rotundicauda* is shown in SEQ ID NO: 4. The nucleotide sequence of a gene encoding the Factor C derived from *Carcinoscorpius rotundicauda* is shown in SEQ ID NO: 3.

[0021]

The Factor C of the present invention may undergo N-type sugar chain (N-linked sugar chain) modification. The term "N-type sugar chain" refers to a sugar chain which is linked to an asparagine residue of a protein. In the Factor C derived from *Tachypleus tridentatus* and the Factor C derived from *Carcinoscorpius rotundicauda*, the amino acid residues which can undergo N-type sugar chain modification are conserved. Figs. 8 and 9 show alignments and consensus sequences (Asn-Xaa-Ser/Thr, wherein Xaa represents any amino acid residue) which can undergo N-type sugar chain modification of both types of Factor C. Thus, in particular, the Factor C derived from *Carcinoscorpius rotundicauda* is thought to be a suitable Factor C as with the Factor C derived from *Tachypleus tridentatus*.

[0022]

The Factor C of the present invention may contain a sialic acid. Examples of the sialic acid include N-

acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). The expression "containing a sialic acid" refers to the state that a sialic acid is linked to the Factor C of the present invention. The sialic acid may be linked to Factor C directly or indirectly. In one case, a sugar chain containing a sialic acid may be linked to Factor C, whereby the sialic acid may be indirectly linked to Factor C. The sialic acid may be, for example, an (α-2,3)-linked sialic acid, more specifically, an (α-2,3)-linked terminal sialic acid. The term "(α-2,3)-linked sialic acid" refers to a sialic acid linked to a sugar via (α-2,3) glycoside linkage. The term "(α-2,3)-linked terminal sialic acid" refers to a sialic acid which is linked to a sugar via (α-2,3) glycoside linkage and which is located at a terminus of the sugar chain. An example of the sugar chain is an N-type sugar chain (N-linked sugar chain). The sialic acid may be added to Factor C through, for example, expression of Factor C in a host cell which can add a sialic-acid-containing sugar chain to a protein through modification after translation. Examples of such a host cell include mammalian cells. Sialic acid may be detected through, for example, sugar chain analysis. Sugar chain analysis may be carried out through a known technique. For example, sugar chain analysis can be carried out by using lectin or a sugar-chain-recognizing antibody. Specifically, for example, an (α-2,3)-linked terminal sialic acid can be specifically detected through lectin blotting using *Maackia amurensis* agglutinin (MAM).

[0023]

The Factor C of the present invention may contain sialic acid in a greater amount, as compared with a control Factor C. The expression "containing sialic acid in a greater amount, as compared with a control Factor C" refers to the state that the amount of sialic acid linked to the Factor C of the present invention is greater than the amount of sialic acid linked to the control Factor C, in terms of the amount by mole of sialic acid per molecule of Factor C. For example, the amount of sialic acid linked to the Factor C of the present invention may be 1.1 times or greater, 1.2 times or greater, 1.3 times or greater, 1.5 times or greater, 2.0 times or greater, 2.5 times or greater, or 3.0 times or greater than the amount of sialic acid linked to the control Factor C, in terms of the amount by mole of sialic acid per molecule of Factor C.

[0024]

Examples of the control Factor C include a recombinant Factor C expressed in insect cell Sf9, and a native Factor C. The expression "Factor C expressed in Sf9 as a host cell" refers to Factor C obtainable through expression of a gene encoding Factor C consisting of the same amino acid sequence as that of the Factor C of the present invention, in SF9 as a host cell. The term "native Factor C" refers to Factor C isolated and purified from a horseshoe crab lysate. The native Factor C may be Factor C isolated and purified from a lysate of a horseshoe crab from which the Factor C of the

present invention is derived. That is, specifically, for example, when the Factor C of the present invention is a recombinant Factor C of *Tachypleus tridentatus* or a variant thereof, the native Factor C may be Factor C isolated and purified from a lysate of *Tachypleus tridentatus*.

[0025]

Whether or not "the Factor C of the present invention contains sialic acid in a greater amount, as compared with a control Factor C" may be determined through comparison between the sialic acid amount of the Factor C of the present invention and that of the control Factor C. The amount of sialic acid may be determined through, for example, sugar chain analysis. Sugar chain analysis may be carried out through a known technique. For example, sugar chain analysis can be carried out by using lectin or a sugar-chain-recognizing antibody. Specifically, for example, an (α-2,3)-linked terminal sialic acid can be specifically quantitated through lectin blotting using *Maackia amurensis* agglutinin (MAM). It can be confirmed that the higher the reactivity in lectin blotting using MAM, the greater the amount of (α-2,3)-linked terminal sialic acid, and that is, the greater amount of (α-2,3)-linked terminal sialic acid the Factor C contains. In other words, the Factor C of the present invention may exhibit higher reactivity, as compared with the control Factor C, in lectin blotting using MAM. The expression "exhibit higher reactivity, as compared with the control Factor C, in lectin blotting using MAM" refers to the

state that, when the equiamount of the Factor C of the present invention and the control Factor C are subjected to lectin blotting using MAM, the detection intensity (such as the extent of coloring) of the spot attributed to the Factor C of the present invention is higher than that of the spot attributed to the control Factor C.

[0026]

The Factor C of the present invention may have no His-tag added to the C-terminus. Also, the Factor C of the present invention may have no V5-tag added to the C-terminus. Also, the Factor C of the present invention may have no peptide added to the C-terminus. Also, the Factor C of the present invention may have no peptide added to the N-terminus. Also, the Factor C of the present invention may have no peptide added to any of the N-terminus and the C-terminus.

[0027]

So long as the Factor C of the present invention has activity of Factor C, the Factor C may be a variant of any of the aforementioned horseshoe crab Factor C proteins (e.g., a protein having the amino acid sequence shown in SEQ ID NO: 2 or 4). Such a variant includes, for example, a homologue and an artificially modified product of the aforementioned Factor C. Notably, the amino acid sequence of the variant is not necessarily found in an actual horseshoe crab. That is, the Factor C "of a horseshoe crab or derived from a horseshoe crab" includes a variant of Factor C found in a horseshoe crab and having an amino acid sequence which is not found in

any horseshoe crab.

[0028]

The activity of Factor C refers to such an activity that the Factor C is activated in the presence of endotoxin to convert Factor B into activated Factor B. Whether "the Factor C of the present invention has activity of Factor C" can be confirmed, for example, by combining the Factor C of the present invention with a suitable Factor B and a suitable Pro-clotting enzyme in the presence of endotoxin, and detecting the progress of the cascade reaction. Specifically, the protein of SEQ ID NO: 6 and the protein of SEQ ID NO: 8 may be used as a suitable Factor B and a suitable Pro-clotting enzyme, respectively. The progress of the cascade reaction may be determined by use of the below-mentioned substrate for detection.

[0029]

So long as the Factor C of the present invention has activity of Factor C, the Factor C of the present invention may be a protein having the amino acid sequence of the above-mentioned Factor C such as the amino acid sequence shown in SEQ ID NO:2 or 4, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues. Although the meaning of the term "one or several" varies depending on the positions of the amino acid residues in the three-dimensional structure of the protein and the types of the amino acid residues, the term specifically refers to preferably 1 to 20, more preferably 1 to 10, still more

preferably 1 to 5, particularly preferably 1 to 3. The substitution, deletion, insertion, or addition of one or several amino acid residues is a conservative variation where the function of the protein is appropriately maintained. A typical conservative variation is conservative substitution. Conservative substitution is a variation among Phe, Trp, and Tyr, in the case where the substitution occurs at an aromatic amino acid residue; a variation among Leu, Ile, and Val, in the case where the substitution occurs at a hydrophobic amino acid residue; a variation between Gln and Asn, in the case where the substitution occurs at a polar amino acid residue; a variation among Lys, Arg, and His, in the case where the substitution occurs at a basic amino acid residue; a variation between Asp and Glu, in the case where the substitution occurs at an acidic amino acid residue; or a variation between Ser and Thr, in the case where the substitution occurs at an amino acid residue having a hydroxyl group. Specific examples of the substitution which can be recognized as conservative substitution include a substitution of Ala by Ser or Thr; a substitution of Arg by Gln, His, or Lys; a substitution of Asn by Glu, Gln, Lys, His, or Asp; a substitution of Asp by Asn, Glu, or Gln; a substitution of Cys by Ser or Ala; a substitution of Gln by Asn, Glu, Lys, His, Asp, or Arg; a substitution of Glu by Gly, Asn, Gln, Lys, or Asp; a substitution of Gly by Pro; a substitution of His by Asn, Lys, Gln, Arg, or Tyr; a substitution of Ile by Leu, Met, Val, or Phe; a substitution

of Leu by Ile, Met, Val, or Phe; a substitution of Lys by Asn, Glu, Gln, His, or Arg; a substitution of Met by Ile, Leu, Val, or Phe; a substitution of Phe by Trp, Tyr, Met, Ile, or Leu; a substitution of Ser by Thr or Ala; a substitution of Thr by Ser or Ala; a substitution of Trp by Phe or Tyr; a substitution of Tyr by His, Phe, or Trp; and a substitution of Val by Met, Ile, or Leu. The aforementioned substitution, deletion, insertion, addition, or the like of an amino acid residue(s) also encompasses naturally occurring variations such as those based on individual differences between horseshoe crabs and the strain and species of the horseshoe crabs, from which the gene is derived.

[0030]

The Factor C of the present invention may also be a protein which has a homology or identity of 80% or higher, preferably 90% or higher, more preferably 95% or higher, still more preferably 97% or higher, particularly preferably 99% or higher with respect to the entire amino acid sequence of Factor C (e.g., the entire amino acid sequence shown in SEQ ID NO: 2 or 4) and which has activity of Factor C.

[0031]

The gene encoding the Factor C of the present invention is not particularly limited, so long as the gene codes for the aforementioned Factor C of the present invention. The gene encoding the Factor C of the present invention may be a DNA encoding a protein having activity of Factor C, wherein the DNA fragment is hybridized with a probe prepared from a

known gene sequence, e.g., with the complementary sequence to the entirety or a part of the nucleotide sequence shown SEQ ID NO: 1 or 3, under stringent conditions. As used herein, the term "stringent conditions" generally refer to the conditions under which a specific hybrid is formed but a non-specific hybrid is not formed. An example of such conditions is conditions under which DNAs having a high homology, e.g., a homology of 80% or higher, preferably 90% or higher, more preferably 95% or higher, still more preferably 97% or higher, particularly preferably 99% or higher, are hybridized with each other, and DNAs having a homology lower than the above level are not hybridized with each other. Another example of the conditions is conditions generally employed in washing of southern hybridization; i.e., washing once, preferably 2 to 3 times, at temperature and salt concentration corresponding to 60°C, 1×SSC, 0.1% SDS, preferably 60°C, 0.1×SSC, 0.1% SDS, more preferably 68°C, 0.1×SSC, 0.1% SDS.

[0032]

In the gene encoding the Factor C of the present invention, any codon may be substituted by its equivalent codon. For example, the codon combination of the gene encoding the Factor C of the present invention may be modified such that the codon combination is optimized for expression in a mammalian cell. The optimization may be performed through employment of, for example, a conventional contract service. Notably, the gene encoding the Factor C of the present invention may be a variant of a DNA in which the

codon combination is optimized for expression in a mammalian cell.

[0033]

The above descriptions about the variants of genes and proteins may also be applied *mutatis mutandis* to Factor B, Pro-clotting enzyme, and gene encoding the same.

[0034]

The Factor C of the present invention may have a molecular weight of 115 kDa to 140 kDa, 115 kDa to 130 kDa, 120 kDa to 130 kDa, or 120 kDa to 128 kDa, as measured through SDS-PAGE under a non-reducing condition. Also, the Factor C of the present invention may have a molecular weight of 127 kDa ±5 kDa, 128 kDa ±2 kDa, 127 kDa ±2 kDa, 126 kDa ±2 kDa, or 127 kDa ±1 kDa, as measured through SDS-PAGE under a non-reducing condition. Also, the Factor C of the present invention may have a molecular weight of 128 kDa or 126 kDa, as measured through SDS-PAGE under a non-reducing condition.

[0035]

The Factor C of the present invention may have low susceptibility to inhibition of activity by an ion. The expression "low susceptibility to inhibition of activity by an ion" refers to the state that the Factor C of the present invention exhibits a high residual activity in the presence of an ion, as compared with a control Factor C. Examples of the control Factor C include a recombinant Factor C expressed in insect cell Sf9 and a native Factor C. The meaning of the expression "exhibiting a high residual activity" is not

particularly limited, so long as the residual activity of the Factor C of the present invention is higher than that of the control Factor C. The expression "exhibiting a high residual activity" may refer to, for example, the state that the residual activity of the Factor C of the present invention is 1.1 times or higher, 1.2 times or higher, 1.3 times or higher, 1.5 times or higher, 2.0 times or higher, 2.5 times or higher, or 3.0 times or higher than the residual activity of the control Factor C. Specific examples of "low susceptibility to inhibition of activity by an ion" include such a property that the Factor C of the present invention exhibits a higher residual activity in the presence of 21 mM sodium citrate, as compared with the control Factor C; such a property that the Factor C of the present invention exhibits a higher residual activity in the presence of 52 mM sodium hydrogencarbonate, as compared with the control Factor C; such a property that the Factor C of the present invention exhibits a higher residual activity in the presence of 214 mM sodium chloride, as compared with the control Factor C; such a property that the Factor C of the present invention exhibits a higher residual activity in the presence of 16 mM magnesium sulfate, as compared with the control Factor C; and such a property that the Factor C of the present invention exhibits a higher residual activity in the presence of 2.5 mM calcium chloride, as compared with the control Factor C. Specific examples of "low susceptibility to inhibition of activity by an ion" further include such a property that the Factor C of the

present invention exhibits a residual activity of 10% or higher or 20% or higher in the presence of 21 mM sodium citrate, as compared with the control Factor C; such a property that the Factor C of the present invention exhibits a residual activity of 25% or higher or 35% or higher in the presence of 52 mM sodium hydrogencarbonate, as compared with the control Factor C; such a property that the Factor C of the present invention exhibits a residual activity of 25% or higher or 35% or higher in the presence of 214 mM sodium chloride, as compared with the control Factor C; such a property that the Factor C of the present invention exhibits a residual activity of 15% or higher or 25% or higher in the presence of 16 mM magnesium sulfate, as compared with the control Factor C; and such a property that the Factor C of the present invention exhibits a residual activity of 35% or higher or 45% or higher in the presence of 2.5 mM calcium chloride, as compared with the control Factor C. The Factor C of the present invention may have any one of the above properties singly or two or more of the above properties in combination. The "residual activity" is defined as a relative reactivity (%) observed for a measurement specimen (endotoxin concentration: 0.05 EU/mL) prepared by adding endotoxin to an ion-containing test specimen, with respect to the reactivity observed for another measurement specimen (endotoxin concentration: 0.05 EU/mL) prepared by adding endotoxin to injection water as 100%, in the assay system shown in Fig. 4.

[0036]

The Factor C of the present invention may be water-soluble. As used herein, the term "water-soluble" refers to the state that, when the Factor C of the present invention is expressed in an appropriate host cell, the thus-expressed Factor C is detected in a soluble fraction. The expression "detected in a soluble fraction" may refer to the state that 20% or more, 50% or more, 80% or more, 90% or more, or 95% or more of the expressed Factor C is detected in a soluble fraction.

[0037]

(2) Method for producing the Factor C of the present invention

The Factor C of the present invention may be produced through, for example, expression in a mammalian cell as a host. That is, the present invention provides a method for producing a horseshoe crab Factor C, the method comprising expressing the horseshoe crab Factor C in a mammalian cell as a host cell. This method may also be referred to as "the production method of the present invention." The Factor C producible through production method of the present invention is an embodiment of the Factor C of the present invention.

[0038]

The mammalian cell is not particularly limited, so long as the cell can functionally express a horseshoe crab Factor C. The term "functional expression" refers to the state that the expressed Factor C exhibits activity of Factor C.

Specifically, the mammalian cell may be a cell other than COS-1 cell. The mammalian cell is preferably a cell of a mammal selected from the group consisting of a rodent and a primate. Examples of the rodent include, but are not particularly limited to, Chinese hamster, hamster, mouse, rat, and guinea pig. Of these, Chinese hamster is preferred. Examples of the Chinese hamster cell include Chinese hamster ovary (CHO) cell line. Examples of CHO include CHO DG44 and CHO K1. Examples of the primate include, but are not particularly limited to, human, monkey, and chimpanzee. The primate may be a primate other than monkey. Of these, human is preferred. Examples of the human cell include human embryo kidney (HEK) cell line. Examples of HEK include HEK293.

[0039]

When the mammalian cell retains a gene encoding Factor C, the cell can express Factor C. The gene encoding Factor C may be retained in the mammalian cell, such that it can be expressed under the control of a promoter which functions in the cell. In the mammalian cell, the gene encoding Factor C, for example, may be present on a vector such as a plasmid which undergoes autonomous replication outside a chromosome, or may be introduced to a chromosome. The technique of introducing the gene encoding Factor C into a mammalian cell is not particularly limited. Examples of the vector which undergoes autonomous replication outside a chromosome include pCA7 (Makoto Takeda, *et al.*, J. Viol. 79(22): 14346-54

(2005)). Alternatively, the gene encoding Factor C may be introduced to a chromosome of a mammalian cell by use of pCI-Neo Vector (product of Promega). The mammalian cell may retain one copy of, or two or more copies of the gene encoding Factor C.

[0040]

The culture conditions under which a mammalian cell is cultured are not particularly limited, so long as the mammalian cell can proliferate. Conditions generally employed in culturing mammalian cells may be employed, after appropriate modification has been performed, if needed. A culture medium which is generally employed in culturing mammalian cells may be employed as the culture medium in the invention. Specifically, an RPMI 1640 medium (product of Sigma) or a DMEM medium (product of Sigma) may be used. Culturing can be performed, for example, as static culture at 36°C to 38°C under feeding of 5% CO_2.

[0041]

Whether or not Factor C has been functionally expressed can be confirmed by measuring activity of Factor C. Expression of Factor C may also be confirmed by measuring the amount of mRNA formed via transcription of a gene encoding Factor C, or detecting Factor C through western blotting by use of an antibody.

[0042]

The expressed Factor C can be recovered as a solution containing Factor C, and can be used as a component of the

endotoxin assay agent of the present invention. The solution containing Factor C may be, for example, a culture broth, a culture supernatant, a disrupted cell extract, a mixture thereof, or the like. Factor C may be used after being purified to a desired extent, or may be used without any purification. In the present invention, even when a cell culture supernatant containing expressed Factor C is used as it is without any purification, satisfactory endotoxin assay performance can be attained. Purification of Factor C may be performed through, for example, a known protein purification technique. Examples of such a technique include ammonium sulfate precipitation, gel filtration chromatography, ion exchange chromatography, hydrophobic interaction chromatography, hydroxyapatite chromatography, and so forth. In the case where a tag such as His-tag has been added to Factor C, the Factor C may be purified through affinity chromatography based on affinity to the tag. A solution containing Factor C may be used after being filtered by means of a filter. For example, a 0.22-μm filter can be used as the filter.

[0043]

(3) Endotoxin assay agent of the present invention

The Factor C of the present invention may be used in endotoxin assay. That is, the present invention provides an endotoxin assay agent containing the Factor C of the present invention. This endotoxin assay agent may also be referred to as an "endotoxin assay agent of the present invention."

The endotoxin assay agent of the present invention may further contain a factor or factors other than Factor C, depending on the mode of endotoxin assay.

[0044]

For example, the Factor C of the present invention may be used in combination with Factor B and Pro-clotting enzyme, for endotoxin assay. In other words, an embodiment of the endotoxin assay agent of the present invention is an endotoxin assay agent containing the Factor C of the present invention, Factor B, and Pro-clotting enzyme. In this case, endotoxin can be measured by detecting Clotting enzyme, which is the final product of the cascade reaction. The Factor B and the Pro-clotting enzyme contained in the embodiment of the endotoxin assay agent of the present invention may also be referred to as the "Factor B of the present invention" and the "Pro-clotting enzyme of the present invention", respectively.

[0045]

Alternatively, endotoxin can be measured by detecting an intermediate stage of the cascade reaction. In this case, it is sufficient that the endotoxin assay agent of the present invention contains a factor or factors involved in from the start of the cascade reaction to the relevant intermediate stage. Specifically, for example, when endotoxin is measured by detecting the activated Factor C, which is an intermediate of the cascade reaction, it is sufficient that the endotoxin assay agent of the present

invention contains Factor C.

[0046]

The Factor B of the present invention is a Factor B derived from a horseshoe crab. The Pro-clotting enzyme of the present invention is a Pro-clotting enzyme derived from a horseshoe crab. Examples of the horseshoe crab include *Tachypleus tridentatus* (Japanese horseshoe crab), *Limulus polyphemus* (American horseshoe crab), *Carcinoscorpius rotundicauda* (Southeast Asian (Singaporean) horseshoe crab), and *Tachypleus gigas* (Southeast Asian horseshoe crab). Among them, *Tachypleus tridentatus* (Japanese horseshoe crab) is preferred as a source of Factor B and Pro-clotting enzyme.

[0047]

The amino acid sequence of the Factor B of *Tachypleus tridentatus* and that of the Pro-clotting enzyme of *Tachypleus tridentatus* are shown in SEQ ID NOs: 6 and 8, respectively. The nucleotide sequence of a gene encoding the Factor B of *Tachypleus tridentatus* and that of a gene encoding the Pro-clotting enzyme of *Tachypleus tridentatus* are shown in SEQ ID NOs: 5 and 7, respectively.

[0048]

The Factor B of the present invention may be a variant of any of the aforementioned horseshoe crab Factor B proteins (e.g., a protein having the amino acid sequence shown in SEQ ID NO: 6), so long as the variant has activity of Factor B. Such a variant includes, for example, a homologue and an artificially modified product of the aforementioned Factor B.

The gene encoding the Factor B of the present invention is not particularly limited, so long as the gene codes for the Factor B of the present invention. The above descriptions about the variants of Factor C and the gene encoding Factor C may also be applied *mutatis mutandis* to the variants of Factor B and the gene encoding Factor B.

[0049]

The activity of Factor B refers to such an activity that the Factor B is activated in the presence of activated Factor C to convert Pro-clotting enzyme into the activated form thereof, i.e. the corresponding Clotting enzyme. Whether "the Factor B of the present invention has activity of Factor B" can be confirmed, for example, by combining the Factor B of the present invention with a suitable Factor C and a suitable Pro-clotting enzyme in the presence of endotoxin, and detecting the progress of the cascade reaction. Specifically, the protein of SEQ ID NO: 2 and the protein of SEQ ID NO: 8 may be used as a suitable Factor C and a suitable Pro-clotting enzyme, respectively. The progress of the cascade reaction may be determined by use of the below-mentioned substrate for detection.

[0050]

The Pro-clotting enzyme of the present invention may be a variant of any of the aforementioned horseshoe crab Pro-clotting enzymes (e.g., a protein having the amino acid sequence shown in SEQ ID NO: 8), so long as the variant has activity of Pro-clotting enzyme. Such a variant includes,

for example, a homologue and an artificially modified product of the aforementioned Pro-clotting enzyme. The gene encoding the Pro-clotting enzyme of the present invention is not particularly limited, so long as the gene codes for the Pro-clotting enzyme of the present invention. The above descriptions about the variants of Factor C and the gene encoding Factor C may also be applied *mutatis mutandis* to the variants of Pro-clotting enzyme and the gene encoding Pro-clotting enzyme.

[0051]

The activity of Pro-clotting enzyme is such an activity that the Pro-clotting enzyme is converted into the activated form thereof, i.e. the corresponding Clotting enzyme, in the presence of activated Factor B, to react with the below-mentioned substrate for detection. The activity of reacting with the detection substrate refers to, for example, an activity of reacting with a coagulogen to induce coagulation, or an activity of reacting with the below-described substrate represented by X-Y-Z (wherein X represents a protection group, Y represents a peptide, and Z represents a dye linked to Y via amide bond), to thereby release dye Z (e.g., an activity of reacting with Boc-Leu-Gly-Arg-pNA, to thereby release pNA). Whether "the Pro-clotting enzyme of the present invention has the activity of Pro-clotting enzyme" can be confirmed, for example, by combining the Pro-clotting enzyme of the present invention with a suitable Factor C and a suitable Factor B in the presence of endotoxin, and detecting the progress of the

cascade reaction. Specifically, the protein of SEQ ID NO: 2 and the protein of SEQ ID NO: 6 may be used as a suitable Factor C and a suitable Factor B, respectively. The progress of the cascade reaction may be measured by use of the below-mentioned substrate for detection.

[0052]

So long as the Factor B of the present invention and the Pro-clotting enzyme of the present invention has activity of Factor B and activity of Pro-clotting enzyme, respectively, the Factor B of the present invention and/or the Pro-clotting enzyme of the present invention may have any peptide or the like added thereto. Examples of such a peptide include tag sequences such as His-tag and V5-tag. Similar to Factor C of the present invention, the Factor B of the present invention and/or the Pro-clotting enzyme of the present invention may have no His-tag added to the C-terminus; may have no V5-tag added to the C-terminus; may have no peptide added to the C-terminus; may have no peptide added to the N-terminus; or may have no peptide added to any of the N-terminus and the C-terminus.

[0053]

In the gene encoding the Factor B of the present invention and/or the gene encoding the Pro-clotting enzyme of the present invention, any codon may be substituted by its equivalent codon. For example, the codon combination of the gene encoding the Factor B of the present invention and/or the gene encoding the Pro-clotting enzyme of the present

invention may be modified such that the codon combination is optimized for expression in a host cell. An example of DNA which code for Factor B of SEQ ID NO: 6 and whose codon combination is optimized for expression in an insect cell is a DNA of SEQ ID NO: 9. Notably, the gene encoding the Factor B of the present invention and/or the gene encoding the Pro-clotting enzyme of the present invention may be a variant of a DNA in which the codon combination is optimized for expression in a host cell.

[0054]

The Factor B of the present invention and the Pro-clotting enzyme of the present invention may each be a native protein or a recombinant protein.

[0055]

A native Factor B and a native Pro-clotting enzyme may each be obtained from the hematocyte extract of the aforementioned various horseshoe crabs. These factors may be used after being purified to a desired extent. Purification may be performed through, for example, a known technique (Nakamura T. *et al*., J. Biochem. 1986 Mar; 99(3): 847-57).

[0056]

The recombinant Factor B and the recombinant Pro-clotting enzyme may each be obtained through expression in a host cell. The host cell is not particularly limited, so long as a factor of interest can be expressed. For example, a host cell generally employed for heterologous protein expression may be used. Examples of the host cell include

insect cells, animal cells, plant cells, yeast cells, and bacterial cells. Among them, an eukaryotic cell is preferred from the viewpoint of modification after translation. Specifically, an insect cell and an animal cell are more preferred.

[0057]

Examples of the animal cells include mammalian cells. In the case where an animal cell such as a mammalian cell is used, the above descriptions about production of the Factor C of the present invention may also be applied *mutatis mutandis* to production of the Factor B and the Pro-clotting enzyme.

[0058]

Examples of the insect cells include Sf9, Sf21, SF+, and High-Five. Of these, Sf9 is preferred as the insect cell.

[0059]

The technique for expressing a factor of interest in an insect cell as a host is not particularly limited, so long as the factor can be expressed. For example, techniques generally employed for heterologous protein expression may be suitably used. For example, a factor of interest can be expressed by infecting an insect cell with a virus into which a gene encoding the factor has been incorporated (the technique being called a "virus method"). Also, for example, a factor of interest can be expressed by incorporating into an insect cell a vector into which a gene encoding the factor has been incorporated, to thereby incorporate the gene into a chromosome of the host cell (the technique being called a

"stably expressing cell line method").

[0060]

<Virus method>

The virus employed in the virus method is not particularly limited, so long as an insect cell can be infected with the virus, to thereby express a factor of interest. A virus generally employed for protein expression in insect cells may be suitably used. Examples of such viruses include Baculovirus. Baculovirus is preferably Nucleopolyhedrovirus (NPV). Examples of NPV include *Autographa californica* NPV (AcNPV) and *Bombyx mori* NPV (BmNPV). NPV is preferably AcNPV.

[0061]

Incorporation of a nucleic acid into a virus may be performed through a conventional method. For example, incorporation of a nucleic acid into a virus may be performed through homologous recombination by use of a transfer vector. Examples of the transfer vector include pPSC8 (product of Protein Sciences), pFastBac (product of Life technologies corporation), and pVL1393 (product of Pharmingen). Among them, pPSC8 is a preferable transfer vector.

[0062]

An insect cell can be infected, through a conventional technique, with the virus into which a gene encoding a factor of interest has been incorporated, to thereby obtain the insect cell which retain the virus and express the factor.

[0063]

<Stably expressing cell line method>

Through incorporation of a gene encoding a factor of interest into a chromosome of an insect cell, a stably expressing cell line which stably expresses the factor can be established. The method of establishing the stably expressing cell line is not particularly limited, and a conventional technique may be employed. For example, a stably expressing cell line can be established by use of pIZ-V5 (product of Life Technologies Corporation), according to a user's instruction attached thereto.

[0064]

The conditions under which an insect cell is cultured are not particularly limited, so long as the insect cell can proliferate. Conditions generally employed in culturing insect cells may be employed, after appropriate modification has been performed, if needed. A culture medium which is generally employed in culturing insect cells may be employed as the culture medium in the invention. Examples of such a culture medium include a commercially available serum-free medium for culturing insect cells. Specifically, an Sf900III serum-free medium (product of Life Technologies Corporation) or the like may be suitably used. Culturing can be performed, for example, as shaking culture at 27°C to 28°C.

[0065]

Whether or not a factor of interest has been expressed can be confirmed by measuring activity of the factor. Expression of a factor of interest may also be confirmed by

measuring the amount of mRNA formed via transcription of a gene encoding the factor, or detecting the factor through western blotting by use of an antibody.

[0066]

The expressed factor can be recovered as a solution containing the factor, and can be used as a component of the endotoxin assay agent of the present invention. The solution containing the factor may be, for example, a culture broth, a culture supernatant, a disrupted cell extract, a mixture thereof, or the like. The factor may be used after being purified to a desired extent, or may be used without any purification. In the present invention, even when a cell culture supernatant containing the expressed factor is used as it is without any purification, satisfactory endotoxin assay performance can be attained. Purification of the factor may be performed through, for example, a known protein purification technique. Examples of such a technique include ammonium sulfate precipitation, gel filtration chromatography, ion exchange chromatography, hydrophobic interaction chromatography, hydroxyapatite chromatography, and so forth. In the case where a tag such as His-tag has been added to the factor, the factor may be purified through affinity chromatography based on affinity to the tag. A solution containing the factor may be used after being filtered by means of a filter. For example, a 0.22-μm filter can be used as the filter.

[0067]

In the case each factor is produced through the virus method, the virus is preferably removed. The method of removing virus is not particularly limited, and a conventional technique may be employed. For example, the virus may be removed by means of a hollow fiber filtration membrane having a pore size of 500 kDa.

[0068]

In the present invention, the Factor C of the present invention, the Factor B of the present invention, and the Pro-clotting enzyme of the present invention may be separately expressed in individually established expression cells.

[0069]

The endotoxin assay agent of the present invention may contain, for example, only the Factor C of the present invention. Alternatively, the agent may contain, for example, only the Factor C of the present invention, the Factor B of the present invention, and the Pro-clotting enzyme of the present invention.

[0070]

The endotoxin assay agent of the present invention may further contain a substrate for detecting the progress of the cascade reaction. As used herein, such a substrate may be referred to as a detection substrate.

[0071]

Examples of the detection substrate include coagulogen. Coagulogen is a detection substrate with respect to Clotting

enzyme, which is the final product of the cascade reaction. When coagulogen comes into contact with Clotting enzyme, a coagulated product, coagulin, is formed. The progress of the coagulation reaction can be determined by measuring the turbidity of the reaction mixture. Coagulogen can be recovered from a hematocyte extract of a horseshoe crab (i.e., lysate). Since the nucleotide sequence of the gene encoding coagulogen has been determined (Miyata *et al*, Protein Nucleic acid Enzyme, extra issue, No. 29; p. 30-43; 1986), coagulogen can also be produced genetically according to a conventional technique.

[0072]

The detection substrate may be a synthetic substrate. The synthetic substrate is not particularly limited, so long as it has a property suitable for detection of the cascade reaction. Examples of the "property suitable for detection of the cascade reaction" include a property for detecting the presence of Clotting enzyme and a property for detecting an intermediate stage of the cascade reaction. Examples of the "property for detecting the presence of Clotting enzyme" include a property of coloring by catalytic reaction with the Clotting enzyme and a property of generating fluorescence by catalytic reaction with the Clotting enzyme. Examples of the "property for detecting an intermediate stage of the cascade reaction" include a property of coloring by catalytic reaction of a cascade reaction intermediate such as activated Factor C and a property of generating fluorescence by

catalytic reaction of a cascade reaction intermediate such as activated Factor C. Examples of the synthetic substrate include a substrate represented by formula X-Y-Z (wherein X represents a protection group, Y represents a peptide, and Z represents a dye linked to Y via amide bond). When endotoxin is present in the reaction system, an amide linkage Y-Z is cleaved by the catalytic reaction of Clotting enzyme or an intermediate, which is yielded by the cascade reaction, to release the dye Z, whereby coloring occurs or fluorescence is generated. The protective group X is not particularly limited, and a known protective group for peptide may be suitably used. Examples of such a protective group include a t-butoxycarbonyl group and a benzoyl group. The dye Z is not particularly limited, and it may be a dye detectable under visible light or may be a fluorescent dye. Examples of the dye Z include p-nitroaniline (pNA), 7-methoxycoumarin-4-acetic acid (MCA), 2,4-dinitroaniline (DNP), and Dansyl dyes. Examples of the peptide Y include Leu-Gly-Arg (LGR), Ile-Glu-Gly-Arg (IEGR) (SEQ ID NO: 10), Val-Pro-Arg (VPR), and Asp-Pro-Arg (DPR). A synthetic substrate which reacts with the Clotting enzyme or the intermediate may be appropriately selected as the synthetic substrate in accordance with the mode of endotoxin assay. For example, from the viewpoint of substrate specificity, a substrate comprising LGR as peptide Y can be suitably used for detecting the Clotting enzyme, and a substrate comprising VPR or DPR as peptide Y can be suitably used for detecting activated Factor C. The released

dye Z may be determined through a technique in accordance with the property of the target dye.

[0073]

The endotoxin assay agent of the present invention may further contain an additional component other than the relevant factors and a detection substrate, so long as the assay agent can be used for endotoxin assay. The additional component is not particularly limited, and it may be selected in consideration of storage stability, handling easiness, stability of the factors and detection substrate, and so forth.

[0074]

The endotoxin assay agent of the present invention may be formulated into any preparation such as solid, liquid, or gel. In the formulation process, additives such as an excipient, a binder, a disintegrant, a lubricant, a flavor/corrigent, a diluent, and a solvent, which are generally used in formulation, may be used. The endotoxin assay agent of the present invention as is may be used for endotoxin assay. Alternatively, the assay agent may be used for endotoxin assay after being diluted with or dispersed or dissolved in water, physiological saline, buffer, etc. Needless to say, the endotoxin assay agent of the present invention also encompasses such diluted, dispersed, dissolved, or similarly processed form of the agent.

[0075]

In the endotoxin assay agent of the present invention,

the factors and other components may be present as a mixture or present separately. For example, the factors may be mixed at any proportions in formulation, or individual factors may be formulated separately.

[0076]

The concentrations of the factors and other components contained in the endotoxin assay agent of the present invention are not particularly limited. However, each concentration is preferably adjusted so as to fall within the below-mentioned suitable concentration range for assaying endotoxin. The concentration of each factor in the endotoxin assay agent of the present invention (as a solution prepared before contact with a test specimen) may be, for example, 5 to 200 μg/mL, and is preferably 20 to 100 μg/mL, more preferably 40 to 80 μg/mL, particularly preferably about 60 μg/mL. Also, the concentration of each factor in the endotoxin assay agent of the present invention may be adjusted in accordance with the method of producing the factor. When Factor C is produced with a rodent cell as a host, the concentration of Factor C in the endotoxin assay agent of the present invention (as a solution prepared before contact with a test specimen) can also be, for example, 5 to 200 μg/mL, 10 to 50 μg/mL, or 15 to 40 μg/mL. When Factor C is produced with a primate cell as a host, the concentration of Factor C in the endotoxin assay agent of the present invention (as a solution prepared before contact with a test specimen) can also be, for example, 5 to 200 μg/mL, 50 to 150

μg/mL, or 80 to 120 μg/mL. The above-exemplified concentrations may be calculated, for example, on the assumption that all the protein species present in the filtrate of a culture supernatant which has been obtained through expression of a factor of interest via the above-exemplified technique consists of the factor of interest.

[0077]

The endotoxin assay agent of the present invention may be provided as an endotoxin assay kit. The endotoxin assay kit is not particularly limited, so long as the kit comprises the endotoxin assay agent of the present invention. The endotoxin assay kit may further comprise, for example, one or more elements selected from an endotoxin standard, a reaction container (e.g., a tube or a microplate), an instruction, and the like.

[0078]

(4) Endotoxin assay method of the present invention

When a test specimen contains endotoxin, the cascade reaction proceeds by mixing the endotoxin assay agent of the present invention with the test specimen. Through measuring the progress of the cascade reaction, endotoxin contained in the test specimen can be measured. That is, the present invention provides a method for measuring endotoxin present in a test specimen, the method comprising a step of mixing the endotoxin assay agent of the present invention and the test specimen, and a step of measuring the progress of a cascade reaction. The method is also referred to as the

"endotoxin assay method of the present invention."

[0079]

In an embodiment of the endotoxin assay method of the present invention (hereinafter may be referred to as the "first embodiment"), all the factors are mixed with the test specimen. In the first embodiment, the factors contained the endotoxin assay agent of the present invention may be contained in the reaction system from the start of the step of mixing the endotoxin assay agent of the present invention with the test specimen, or may be successively added to the reaction system.

[0080]

For example, the step of mixing the endotoxin assay agent of the present invention with the test specimen may comprise the following steps (A) to (C):

(A) a step of adding the Factor C of the present invention to the reaction system;

(B) a step of adding the Factor B of the present invention to the reaction system; and

(C) a step of adding the Pro-clotting enzyme to the reaction system.

[0081]

The steps (A) to (C) may be performed separately, partially simultaneously, or totally simultaneously. The steps (A) to (C) may be performed in any order. For example, the step (B) may be performed after the step (A), and the step (C) may be performed after the step (B).

[0082]

In the first embodiment, the progress of the cascade reaction can be measured by adding a detection substrate to the reaction system and measuring the response (coloring, coagulation, etc.) of the substrate. The detection substrate may be contained in the reaction system from the start of the step of mixing the endotoxin assay agent of the present invention with the test specimen, or may be added to the reaction system during the progress of the step or after completion of the step. Needless to say, the first embodiment also encompasses an endotoxin assay method employing the endotoxin assay agent of the present invention which contains a detection substrate in advance.

[0083]

In the endotoxin assay method of the present invention, so long as the cascade reaction proceeds when the test specimen contains endotoxin, the Factor B and the Pro-clotting enzyme of the present invention *per se* are not need to be in contact with the test specimen. That is, another embodiment of the endotoxin assay method of the present invention (hereinafter may be referred to as a "second embodiment") is a method for measuring endotoxin present in a test specimen, the method comprising the following steps (A) to (D):

(A) a step of mixing the Factor C of the present invention with the test specimen;

(B) a step of mixing the Factor B of the present

invention with the Factor C after mixing of step (A);

(C) a step of mixing the Pro-clotting enzyme of the present invention with the Factor B after mixing of step (B); and

(D) a step of measuring the progress of a cascade reaction.

[0084]

In the second embodiment, the steps (A) to (D) may be performed separately, partially simultaneously, or totally simultaneously. For example, step A may be initiated, and then the Factor B and the Pro-clotting enzyme may be added to the reaction system during the progress of the step A or after completion of step A. Also, step B may be initiated, and then the Pro-clotting enzyme may be added to the reaction system during the progress of the step B or after completion of step B. Alternatively, all three factors may be incorporated into the reaction system from the start of step A. Still alternatively, the Factor C after the contact of step A may be recovered and used in step B, or the Factor B after the contact of step B may be recovered and used in step C.

[0085]

In the second embodiment, the progress of the cascade may be measured by adding a detection substrate to the reaction system and measuring the response (coloring, coagulation, etc.) of the substrate. The detection substrate may be contained in the reaction system from the start of

step A, or may be added to the reaction system during the progress of any step or after completion of any step.

[0086]

Alternatively, endotoxin can be measured by detecting an intermediate stage of the cascade reaction. Specifically, endotoxin can be measured by detecting activated Factor C, which is an intermediate of the cascade reaction. That is, still another embodiment of the endotoxin assay method of the present invention (hereinafter may be referred to as a "third embodiment") may be a method for measuring endotoxin present in a test specimen, the method comprising a step of mixing the Factor C of the present invention with the test specimen and a step of detecting activated Factor C. In the third embodiment, the activated Factor C can be detected by adding a detection substrate to the reaction system and measuring the response (coloring, etc.) of the substrate. The detection substrate may be contained in the reaction system from the start of the step of mixing the Factor C of the present invention with a test specimen, or may be added to the reaction system during the progress of the step or after completion of the step.

[0087]

The endotoxin assay method of the present invention, so long as the cascade reaction proceeds when the test specimen contains endotoxin, may further comprise any other arbitrary step. For example, the endotoxin assay method of the present invention may comprise a step of adding a detection substrate

to the reaction system, or a step of mixing Clotting enzyme or an intermediate, which was yielded by the cascade reaction, with the detection substrate. Also, for example, the endotoxin assay method of the present invention may comprise a step of calculating the endotoxin level of the test specimen based on the reaction of the detection substrate.

[0088]

In the endotoxin assay method of the present invention, reaction is preferably carried out in an aqueous medium such as water or a buffer.

[0089]

In the assay method of the present invention, the concentration of each factor in the reaction mixture is not particularly limited, so long as the cascade reaction proceeds when the test specimen contains endotoxin. The concentration of each factor may be appropriately tuned in accordance with properties of the factor and so forth. For example, the concentration of each factor, as a final concentration, may be, for example, 2.5 to 100 μg/mL, and is preferably 10 to 50 μg/mL, more preferably 20 to 40 μg/mL, particularly preferably about 30 μg/mL. Also, the concentration of each factor in the reaction mixture may be adjusted in accordance with the method of producing the factor. When Factor C is produced with a rodent cell as a host, the concentration of Factor C in the reaction mixture, as a final concentration, may also be, for example, 2.5 to 100 μg/mL, 5 to 25 μg/mL, or 7.5 to 20 μg/mL. When Factor C

is produced with a primate cell as a host, the concentration of Factor C in the reaction mixture, as a final concentration, may also be, for example, 2.5 to 100 μg/mL, 25 to 75 μg/mL, or 40 to 60 μg/mL. The above-exemplified concentrations may be calculated, for example, on the assumption that all the protein species present in the filtrate of a culture supernatant which has been obtained through expression of a factor of interest via the above-exemplified technique consists of the factor of interest.

[0090]

In the assay method of the present invention, the concentration of the detection substrate in the reaction mixture is not particularly limited, so long as the cascade reaction proceeds when the test specimen contains endotoxin. The concentration of the detection substrate may be appropriately tuned in accordance with properties of the detection substrate and so forth. In the case where the detection substrate is a synthetic substrate, the concentration of the detection substrate is, for example, generally 0.001 mM to 100 mM, preferably 0.01 mM to 10 mM, as a final concentration.

[0091]

In any embodiment, so long as the cascade reaction proceeds when the test specimen contains endotoxin, the reaction system may further contain any other arbitrary component, in addition to the factors, the detection substrate, and the test specimen.

[0092]

The pH of the reaction mixture is not particularly limited, so long as the cascade reaction proceeds when the test specimen contains endotoxin. The pH of the reaction mixture may be appropriately tuned in accordance with properties of the factors. The pH of the reaction mixture is, for example, generally 5 to 10, preferably 7 to 8.5.

[0093]

The reaction temperature is not particularly limited, so long as the cascade reaction proceeds when the test specimen contains endotoxin. The reaction temperature may be appropriately tuned in accordance with properties of the factors. The reaction temperature is, for example, generally 10°C to 80°C, preferably 20°C to 50°C. For example, the reaction temperature may be 37°C.

[0094]

The reaction time is not particularly limited. The reaction time may be appropriately tuned in accordance with various conditions such as properties of the factors and reaction temperature. The reaction time is, for example, generally 5 minutes to 2 hours, preferably 15 minutes to 90 minutes. The reaction time may be, for example, 30 minutes to 40 minutes.

[0095]

In any embodiment, the test specimen, the factors, and other components may be further added singly or in any combination to the reaction system during the course of

reaction. These components may be added once or a plurality of times, or in a continuous manner. The reaction conditions may be constant from the start to the end of the reaction, or may be varied in the course of the reaction.

[0096]

The progress of the cascade reaction attributable to the presence of endotoxin can be measured by measuring the response (coloring, coagulation, etc.) of the detection substrate, whereby endotoxin in the test specimen can be measured. The response (coloring, coagulation, etc.) of the detection substrate may be measured through a technique in accordance with the property of the detection substrate employed.

[0097]

In the case of quantitatively measuring endotoxin, correlation data between endotoxin level and the extent of response (coloring, coagulation, etc.) of the detection substrate are obtained by use of an endotoxin standard sample with known concentration, and then the amount of endotoxin present in the specimen can be quantitatively determined on the basis of the obtained correlation data. The correlation data is, for example, a calibration curve. The quantitation may be carried out through a kinetic method or an end-point method.

[0098]

The test specimen which can be subjected to endotoxin assay is not particularly limited. Examples of the specimen

include water for medical use, pharmaceutical products, infusion solutions, blood products, medical apparatuses, medical instruments, cosmetics, foods and beverages, environmental specimens (e.g., air, river water, and soil), biological specimens (e.g., blood, body fluid, and tissue), native proteins, recombinant proteins, nucleic acids, and saccharides. The test specimen may be, for example, an injection. An injection refers to an agent that can be administered to the biological body via injection. Such an injection is not necessarily fluid during commercial distribution, and may be a lyophilized product. The test specimen itself, or an extract or wash liquid thereof may be mixed, dispersed, or dissolved in the reaction system, to thereby be subjected to endotoxin assay.

[0099]

As described above, the Factor C of the present invention may have low susceptibility to inhibition of activity by an ion. Thus, in an embodiment of the present invention, endotoxin present in a test specimen containing an ion can be suitably measured.

[0100]

In other words, the test specimen of the invention may be a test specimen containing an ion. The expression "containing an ion" refers to the state that the test specimen contains a substance which is present in the form of an ion during endotoxin assay. That is, "ion" referred to in the present invention may be species which are present

inherently in an ionized form in the test specimen, or may be species which are present inherently in a salt form in the test specimen but are ionized during endotoxin assay. The type of ions is not particularly limited. An example of the ion is a cation, and an example of the cation is a metal ion. Examples of the metal ion include an alkali metal ion and an alkaline earth metal ion. Specific examples of the alkali metal ion and the alkaline earth metal ion include sodium ion, potassium ion, calcium ion, and magnesium ion. The contained amount of the ion in the test specimen may be such an amount that, for example, the concentration of the cation derived from the test specimen becomes 1 mM or higher, 2 mM or higher, 5 mM or higher, 10 mM or higher, 20 mM or higher, 50 mM or higher, or 100 mM or higher, as a final concentration in the reaction system after mixing the test specimen with the endotoxin assay agent. Specific examples of the salt include sodium chloride, magnesium sulfate, sodium hydrogencarbonate, sodium citrate, calcium chloride, potassium chloride, sodium iothalamate, calcium disodium edetate, and dihydrogen sodium phosphate. The contained amount of the salt in the test specimen may be such an amount that, for example, the concentration of the salt derived from the test specimen becomes 1 mM or higher, 2 mM or higher, 5 mM or higher, 10 mM or higher, 20 mM or higher, 50 mM or higher, or 100 mM or higher, as a final concentration in the reaction system after mixing the test specimen with the endotoxin assay agent. The test specimen may contain one kind of ion (or salt) or two or

more kinds of ions (or salts).

Examples

[0101]

The present invention will next be described more particularly by way of examples. However, these are merely examples of the present invention, and the scope of the present invention is not limited to these.

[0102]

1. Preparation of Factor C proteins

(1) Expression of Factor C in insect cells (Sf9)

In this section, Factor C was prepared by use of insect cell Sf9 as a host in the following manner.

[0103]

A DNA encoding Factor C of Japanese horseshoe crab, *Tachypleus tridentatus*, was inserted between the *Eco*RV recognition site and the *Mlu*I recognition site of pIZ-V5 (product of Life Technologies Corporation), which is a vector for expression in insect cells, to thereby construct a Factor C expression plasmid. The nucleotide sequence of the DNA is shown in SEQ ID NO: 1, and the amino acid sequence of Factor C encoded by the DNA is shown in SEQ ID NO: 2 (the same applies throughout the Example).

[0104]

The Factor C expression plasmid was introduced into cultured Sf9 cells through transfection by use of Celfectin reagent (product of Life Technologies Corporation). The Sf9

cells were statically cultured at 28°C in an Sf900 III serum-free medium (product of Life Technologies Corporation) containing 300 to 600 μg/mL Zeocin (product of Life Technologies Corporation), whereby Sf9 strains into which the genome the expression plasmid had been incorporated were selected.

[0105]

Candidates for Sf9 in which the Factor C gene had been stably expressed were isolated through the cloning cylinder technique, and a plurality of Sf9 cell lines was cloned. The thus-obtained clones were individually cultured, to thereby obtain culture supernatants. Secretion of the recombinant Factor C protein in each culture supernatant was confirmed through western blotting by use of an anti-Factor C antibody (2C12 antibody; Yoshiki Miura, *et al.*, J. Biochem. 112: 476-481 (1992)). Also, activity of Factor C (property of being activated by endotoxin) in the culture supernatant was confirmed through activity measurement by use of a synthetic substrate Boc-LGR-pNA. Notably, the procedure of activity measurement is described in "4. Activity measurement" below (the same applies throughout the Example). Based on the results of western blotting and activity measurement, an Sf9 cell line highly expressing Factor C was selected.

[0106]

The Sf9 cell line highly expressing Factor C was suspension-cultured (28°C) in an Sf900 III medium containing 1× penicillin/streptomycin (product of Life Technologies

Corporation) and 50 μg/mL Zeocin to a later phase of the logarithmic growth phase (6 to 8×10^6 cells/mL). Then, the culture was centrifuged (3,000×g, 30 minutes, 4°C), to thereby recover a culture supernatant. The supernatant was filtered through a 0.22-μm filter, and the thus-recovered fraction (i.e. filtrate) was employed as recombinant Factor C (Sf9).

[0107]

(2) Expression of Factor C in mammalian cells (CHO DG44)

In this section, Factor C was prepared by use of CHO DG44, which is a Chinese hamster ovary cell line, as a host through the following procedure.

[0108]

A DNA encoding Factor C of Japanese horseshoe crab, *Tachypleus tridentatus*, was inserted between the *Eco*RI recognition site and the *Xba*I recognition site of pCI-neo (product of Promega), which is a vector for expression in mammalian cells, to thereby construct a Factor C expression plasmid. A kozak sequence (GCCACC) was added just before the start codon of Factor C.

[0109]

The Factor C expression plasmid and a dhfr expression plasmid were incorporated into cultured CHO DG44 cells through simultaneous transfection using a lipofection reagent (Lipofectamine LTX) (product of Life Technologies Corporation). The CHO DG44 cells were statically cultured at 37°C under feeding of 5% CO_2 in an RPMI 1640 medium (5%

dialyzed serum, containing 1× penicillin/streptomycin) (product of Sigma) added with 1 mg/mL Geneticin (product of Life Technologies Corporation), whereby CHO DG44 strains into which the genome the expression plasmids had been incorporated were selected.

[0110]

Fifteen days after the simultaneous transfection, MTX (methotrexate) (product of Wako Pure Chemical Industries, Ltd.) was added to the culture medium. The MTX concentration was elevated stepwise from 50 nM to 5,000 nM over three months, and CHO DG44 cell lines highly expressing Factor C were selected as polyclones. Effective amplification of the incorporated Factor C gene by virtue of MTX was confirmed by detecting Factor C in a culture supernatant through western blotting using a 2C12 antibody, and quantitating the transcriptional amount of mRNA of Factor C through real-time PCR.

[0111]

The group of polyclonal CHO DG44 cell lines highly expressing Factor C was diluted by the medium to obtain a cell concentration of 0.5 cells/well, and the dilute was inoculated to a 96-well plate. From about 10 to 20 days after inoculation, the formed cell colonies were successively subjected to expansion culture, whereby CHO DG44 cell lines highly expressing Factor C were cloned. The transcriptional amount of mRNA of Factor C was confirmed through real-time PCR. The amount of Factor C protein secreted to the medium

was confirmed through western blotting using a 2C12 antibody. The Factor C activity (property of being activated by endotoxin) in the culture supernatant was confirmed through activity measurement using a synthetic substrate Boc-LGR-pNA. Based on these results, a monoclonal CHO DG44 cell line highly expressing Factor C was selected.

[0112]

The CHO DG44 cell line highly expressing Factor C (monoclonal) was conditioned in a serum-free complete synthetic medium containing 5 μM MTX and thus floated. The cell line was grown at 37°C under feeding of 5% CO_2 to a later phase of the logarithmic growth phase. The thus-obtained floating culture broth was centrifuged (3,000×g, 30 minutes, 4°C), to thereby recover a culture supernatant. The supernatant was filtered through a 0.22-μm filter, and the thus-recovered fraction (i.e. filtrate) was employed as recombinant Factor C (CHO).

[0113]

(3) Expression of Factor C in mammalian cells (HEK293)

In this section, Factor C was prepared by use of HEK293, which is a human embryo kidney cell line, as a host through the following procedure.

[0114]

A DNA encoding Factor C of a Japanese horseshoe crab, *Tachypleus tridentatus*, was inserted between the *Eco*RI recognition site and the *Xho*I recognition site of pCA7 (Makoto Takeda, *et al*., J. Viol. 79 (22): 14346-54 (2005)),

which is a vector for expression in mammalian cells, to thereby construct a Factor C expression plasmid. A kozak sequence (GCCACC) was added just before the start codon of Factor C.

[0115]

The Factor C expression plasmid was incorporated into cultured HEK293 cells through transfection using polyethylenimine (product of Sigma) (Hashiguti *et al.*, Expression of Recombinant Protein Using Cultured Human Cells -Standard Protocol by 293-type cells-, PSSJ Archives, 1, e017 (2008); available on the world wide web at pssj.jp/archives/Protocol/Expression/293_01/293_01_01.html. HEK293 cells were statically cultured in a DMEM medium (containing 10% fetal bovine serum and 2mM L-glutamine) (product of Sigma) at 37°C under feeding of 5% CO_2 without replacement of the medium. Four days after transfection, the culture broth was recovered, and was centrifuged (3,300×g, 30 minutes, 4°C), to thereby recover a culture supernatant. The supernatant was filtered through a 0.22-μm filter, and the thus-recovered fraction (i.e. filtrate) was employed as recombinant Factor C (HEK).

[0116]

(4) Preparation of native Factor C (derived from amebocyte of *Tachypleus tridentatus*)

A fraction containing Factor B and Factor C was recovered from an amebocyte extract of Japanese horseshoe crab, *Tachypleus tridentatus*, through column chromatography

by use of dextran sulfate resin (Takanori Nakamura, *et al.*, Eur. J. Biochem. 154: 511-521 (1986)). The fraction (5 mL) was subjected to gel filtration by means of a column (ϕ 2.2 cm × 97 cm) filled with Sepharose CL6B gel (product of GE Healthcare). Gel filtration was performed under almost the same conditions as employed in a known technique (Jeak Ling Ding and Bow Ho, US 5,712,144; Jeak L. Ding, *et al.*, Biochem. Biophys. Acta 1202: 149-156). The flow rate was adjusted to 15 mL/h, and 50 mM Tris buffer (pH 8.0) containing 154 mM NaCl, 1 mM EDTA, and 5% DMSO was employed as a solvent.

[0117]

Gel filtration eluted fractions (3.7 mL/tube, 150 tubes) were subjected to SDS-PAGE followed by CBB staining and western blotting using a 2C12 antibody, and activity measurement using a synthetic substrate Boc-LGR-pNA, whereby fractions containing Factor C protein were identified. These fractions containing the purified Factor C protein were employed as native Factor C (TAL).

[0118]

2. Preparation of recombinant Factor B and recombinant Pro-clotting enzyme

A DNA encoding Factor B of Japanese horseshoe crab, *Tachypleus tridentatus*, was inserted between the *Eco*RV recognition site and the *Mlu*I recognition site of pIZ-V5 (product of Life Technologies Corporation), which is a vector for expression in insect cells, to thereby construct a Factor B expression plasmid. The nucleotide sequence of the DNA is

shown in SEQ ID NO: 9, and the amino acid sequence of Factor B encoded by the DNA is shown in SEQ ID NO: 6. Notably, the nucleotide sequence shown in SEQ ID NO: 9 is a nucleotide sequence optimized for expression in insect cells.

[0119]

A DNA encoding Pro-clotting enzyme of Japanese horseshoe crab, *Tachypleus tridentatus*, was inserted between the *Eco*RV recognition site and the *Mlu*I recognition site of pIZ-V5 (product of Life Technologies Corporation), which is a vector for expression in insect cells, to thereby construct a Pro-clotting enzyme expression plasmid. The nucleotide sequence of the DNA is shown in SEQ ID NO: 7, and the amino acid sequence of Pro-clotting enzyme encoded by the DNA is shown in SEQ ID NO: 8.

[0120]

Through the same method as employed in the case of Factor C, the expression plasmids were each incorporated into Sf9 cells, high-expression clones were selected, and culture supernatants thereof were prepared. The culture supernatants were individually filtered through a 0.22-μm filter, and the obtained fractions (i.e. filtrates) were employed as a recombinant Factor B and a recombinant Pro-clotting enzyme. Recovery of the factors was confirmed through western blotting employing an antibody specific to each factor and activity measurement.

[0121]

3. Comparison of molecular weights of Factor C proteins (1)

The molecular weights of the above-prepared four types of Factor C proteins were compared with one another through SDS-PAGE and western blotting.

[0122]

Firstly, each Factor C in an amount shown in Fig. 2 was subjected to SDS-PAGE under a non-reducing condition employing a 5 to 20% gradient gel. In Fig. 2, Sf9, CHO, and HEK denote the Factor C expressed in Sf9 cells, CHO DG44 cells, and HEK293 cells, respectively, and TAL refers to native Factor C (hereinafter the same applies). Notably, Factor C is cleaved into an H chain and an L chain under reducing conditions. In the present Example, SDS-PAGE was carried out under a non-reducing condition in order to compare the molecular weights of Factor C in an intact state.

[0123]

After SDS-PAGE, proteins were transferred to a PVDF membrane (product of Bio RAD) by means of a semi-dry blotter. The PVDF membrane was recovered and blocked with 5% skim milk, and then sequentially treated with a primary antibody (2C12 antibody) and a secondary antibody (HRP-labeled anti-mouse goat antibody) (product of Dako). Bands attributed to Factor C were made to emit light with a detection reagent, SuperSignal West Dura (product of Thermo Scientific), and the light emission was recorded by a CCD camera.

[0124]

The four types of Factor C were found to have different molecular weights, and the molecular weight was the greatest

in the case of CHO, followed by TAL, HEK, and Sf9 in the descending order (Fig. 2).

[0125]

Regarding Singaporean horseshoe crab, *Carcinoscorpius rotundicauda*, it has been reported that all of the native Factor C protein purified from an amebocyte extract, and recombinant Factor C proteins produced in insect cells Sf9 and S2, and in African green monkey kidney cell line COS-1 as a host cell have the same molecular weight of 132 kDa (Jeak Ling Ding and Bow Ho, US 5,712,144, and Jeak L. Ding, *et al.*, Biochem. Biophys. Acta 1202: 149-156 (1993) (for native Factor C protein); Jing Wang, *et al.*, J. Biol. Chem. 277(39): 36363-72 (2002) (for Sf9 and S2); and Roopashree S. Dwarakanath, *et al.*, Biotechnology letters 19(4): 357-361 (1997) (for COS-1)).

[0126]

Thus, among the above-prepared four types of Factor C, the molecular weight of each of CHO and TAL was correctly measured. Firstly, recombinant Factor C (CHO) was purified in a manner similar to that employed in the case of native Factor C (TAL). Then, electrophoresis (15% gel, LMW Marker kit (product of GE Healthcare)) was performed under the same conditions as employed in a known technique (Jeak Ling Ding and Bow Ho, US 5,712,144; and Jeak L. Ding, et al., Biochem. Biophys. Acta 1202: 149-156), and the molecular weight of each Factor C was then determined through Ferguson plotting. As a result, recombinant Factor C (CHO) was found to have a

molecular weight of 126 kDa, and native Factor C (TAL) was found to have a molecular weight of 123 kDa (Fig. 3). Thus, native Factor C (TAL) and recombinant Factor C (CHO) derived from Japanese horseshoe crab, *Tachypleus tridentatus*, were found to have a molecular weight smaller than that of the Factor C derived from Singaporean horseshoe crab, *Carcinoscorpius rotundicauda* (132 kDa).

[0127]

The difference in molecular weight among the above-prepared four types of Factor C is conceived to be attributed to the difference in post-translation modification (sugar chain modification) depending on the type of host cells employed. For example, it has been known that sialic acid is linked to the N-type sugar chain after translation in mammalian cells, whereby a recombinant protein produced in mammalian cells has dimensions larger than those of a recombinant protein produced in insect cells (Robert L. Harrison and Donald L. Jarvis, Methods in Molecular Biology 338: 341-356 (2007)). This supports the results of the present Example (Fig. 2).

[0128]

4. Activity measurement

Each of the above-prepared four types of Factor C was combined with other factors, and activity measurement was carried out through the following procedure. Fig. 4 shows the scheme of activity measurement.

[0129]

As shown in Fig. 4, common materials other than Factor C (a recombinant Factor B, a recombinant Pro-clotting enzyme, a synthetic substrate Boc-LGR-pNA, and Tris buffer) were mixed together, to thereby prepare a common liquid mixture. To the common liquid mixture, Factor C in an amount specified in Fig. 6 was added, and the total volume was adjusted to 50 μL. The thus-obtained mixture was mixed with 50 μL of a specimen (water containing United States Pharmacopoeia standard endotoxin (USP-RSE) (product of Seikagaku corporation)), and the resultant mixture was heated at 37°C for 30 minutes. In this assay system, Factor C is activated by endotoxin, to thereby form activated Factor C; Factor B is activated by the activated Factor C, to thereby form activated Factor B; Pro-clotting enzyme was activated by the activated Factor B, to thereby form a corresponding Clotting enzyme; and the Clotting enzyme cleaves a synthetic substrate Boc-LGR-pNA, whereby p-nitroaniline is released (Fig. 5). The change in absorbance (A 405 nm) attributed to formation of p-nitroaniline was measured in a time-dependent manner, and the absorbance change rate per unit time (mAbs/min) was calculated and employed as Factor C activity. The Measurement was conducted thrice for each sample.

[0130]

In the above assay system, the final concentration of each factor was as follows: recombinant Factor B 30 μg/mL, recombinant Pro-clotting enzyme 30 μg/mL, recombinant Factor C (Sf9) 11.3 μg/mL, recombinant Factor C (CHO) 12 μg/mL,

recombinant Factor C (HEK) 51.5 μg/mL, and Factor C (TAL) 0.25 μg/mL. The above-exemplified concentrations (except for the case of TAL) were calculated, on the assumption that all the protein species present in the sample (i.e. filtrate) prepared through expression of a factor of interest consisted of the factor of interest. The Factor C (TAL) concentration was measured after purification of Factor C (TAL).

[0131]

As a result, reconstituted systems each exhibiting almost equivalent activity were successfully constituted for the four types of Factor C (Fig. 6). In each reconstituted system, the activity was increased with an increase in endotoxin concentration (0.05 and 0.1 EU/mL) of a specimen.

[0132]

The recombinant Factor C derived from *Carcinoscorpius rotundicauda* and produced in mammalian cell COS-1 (132 kDa) is reported to be recovered in an insoluble fraction (Roopashree S. Dwarakanath, *et al.*, Biotechnology letters 19(4): 357-361 (1997); and Jing Wang, Bow Ho and Jeak L. Ding, Biotechnology letters 23: 71-76 (2001)). Also, the recombinant Factor C derived from *Carcinoscorpius rotundicauda* and produced in insect cell S2 (132 kDa) is reported to exhibit no protease activity in response to endotoxin (Jing Wang, *et al.*, J. Biol. Chem. 277(39): 36363-72 (2002)). Lack of activity of the latter Factor C (produced in S2) is thought to be attributed to the difference in sugar chain structure from the native Factor C

derived from *Carcinoscorpius rotundicauda* and the recombinant Factor C produced in insect cell Sf9, due to difference in reactivity to lectin.

[0133]

Meanwhile, in the present Example, it was found that the recombinant Factor C derived from *Tachypleus tridentatus* (Sf9, CHO, and HEK) exhibited an endotoxin-dependent activity, even though the molecular weight thereof differs from that of native Factor C (TAL); i.e., even though the sugar chain structure thereof differs from that of native Factor C (TAL).

[0134]

5. Inhibition of activity by injection

Endotoxin is a strong immune reaction-inducing substance. Thus, when an injection contaminated with endotoxin is administered to the body, a grave side effect occurs. Therefore, in production of injections, detection of endotoxin by use of a limulus amebocyte lysate reagent is obligated by the pharmacopoeia of the relevant country, to thereby confirm no endotoxin contamination.

[0135]

Thus, the above-prepared four types of Factor C were compared in terms of reactivity to endotoxin in various injections.

[0136]

As a control specimen, injection water containing endotoxin (endotoxin concentration: 0.05 EU/mL) was used. Measurement specimens used were as follows: 4-fold dilute of

10 w/v% sodium chloride injection (10% sodium chloride injection, product of Otsuka Pharmaceutical Co., Ltd.); 16-fold dilute of 0.5 M magnesium sulfate injection (Mg sulfate corrective injection 1 mEq/mL, product of Otsuka Pharmaceutical Co. Ltd.); undiluted solution of physiological saline (Otsuka isotonic sodium chloride solution, product of Otsuka Pharmaceutical Co., Ltd.); 8-fold dilute of 7 w/v% sodium hydrogencarbonate injection (Meylon injection 7%, product of Otsuka Pharmaceutical Co., Ltd.); 8-fold dilute of 10 w/v% sodium citrate injection (sodium citrate injection for transfusion, product of Fuso Pharmaceutical Industries, Ltd.); 100-fold dilute of 0.5 M calcium chloride injection (Ca chloride corrective injection 1 mEq/mL, product of Otsuka Pharmaceutical Co., Ltd.); undiluted solution of Ringer's solution (Ringer's solution "Fuso", product of Fuso Pharmaceutical Industries, Ltd.); and 16-fold dilute of sodium iothalamate injection (Conray 400 injection, product of Daiichi Sankyo Co., Ltd.), each spiked with endotoxin (endotoxin concentration: 0.05 EU/mL). When the measurement specimen was 8-fold dilute of 10 w/v% sodium citrate injection, the final sodium citrate concentration was about 21 mM. When the measurement specimen was 8-fold dilute of 7 w/v% sodium hydrogencarbonate injection, the final sodium hydrogencarbonate concentration was about 52 mM. When the measurement specimen was 4-fold dilute of 10 w/v% sodium chloride injection, the final sodium chloride concentration was about 214 mM. When the measurement specimen was

undiluted solution of physiological saline, the final sodium chloride concentration was about 77 mM. When the measurement specimen was 16-fold dilute of 0.5 M magnesium sulfate injection, the final magnesium sulfate concentration was about 16 mM. When the measurement specimen was 100-fold dilute of 0.5 M calcium chloride injection, the final calcium chloride concentration was about 2.5 mM. Activity of Factor C was measured through the procedure shown in Fig. 4. A residual activity was defined as a relative reactivity (%) for endotoxin in an injection specimen, with respect to the reactivity for endotoxin in the corresponding control specimen as 100%. The measurement was carried out four times for each injection specimen.

[0137]

Fig. 7 shows the results. When a typical injection was employed as a measurement specimen, reaction with endotoxin was inhibited, as compared with the case in which injection water was used. The degree of inhibition varied in accordance with the type of injection and the type of host for expressing Factor C. Recombinant Factor C (CHO) and recombinant Factor C (HEK) showed a tendency to have low susceptibility to reaction inhibition to all the tested injections, as compared with native Factor C (TAL). Particularly, recombinant Factor C (CHO) exhibited the highest residual activity in many cases. In contrast, recombinant Factor C (Sf9) was most susceptible to reaction inhibition in many injections (7 injections except sample No.

6).

[0138]

The test results indicate that Factor C expressed in mammalian cells can have low susceptibility to activity inhibition by ions. Such a property is particularly advantageous for detection of endotoxin contamination in production of injections.

[0139]

6. Preparation of L chain-specific Factor C protein antibody

A Factor C protein is known to be present as a single chain (Full chain) under non-reducing conditions, and to be cleaved into two chains (H chain and L chain) under reducing conditions. The anti-Factor C monoclonal antibody (2C12 antibody) is reported to recognize the Full chain and the H chain (Yoshiki Miura, *et al.*, J. Biochem. 112: 476-481(1992)). Thus, a peptide antigen present in the L chain was produced. A rabbit was immunized with the peptide antigen through a conventional method, whereby a polyclonal antibody which can recognize the L chain and the Full chain (FCL antibody) was produced (Fig. 10).

[0140]

7. Purification of recombinant Factor C proteins

According to the method employed in "(4) Preparation of native Factor C (derived from *Tachypleus tridentatus*)" of "1. Preparation of Factor C proteins", three types of recombinant Factor C proteins were purified from culture supernatants of mammalian cells (CHO DG44, and HEK293) and insect cells (Sf9).

Briefly, each supernatant (100 to 300 mL) was subjected to column chromatography by use of dextran sulfate resin, and elution fractions containing the corresponding recombinant Factor C were isolated through gel filtration. Purification of the recombinant Factor C proteins was confirmed through SDS-PAGE and CBB staining, western blotting employing the 2C12 antibody, and activity measurement.

[0141]

8. Comparison of molecular weights of Factor C proteins (2)

The molecular weights of the above-purified four types of Factor C proteins were compared again with one another through SDS-PAGE and western blotting.

[0142]

The four types of purified Factor C proteins, derived from a native horseshoe crab (TAL), a culture supernatant of CHO DG44 (CHO), a culture supernatant of HEK293 (HEK), and a culture supernatant of Sf9 (Sf9), were applied to a single gel (15% gel) for SDS-PAGE, and subjected to CBB staining (Fig. 11). Each of the four types of purified Factor C proteins presented as a single chain (Full chain) under non-reducing conditions, and was cleaved into two chains (H chain and L chain) under reducing conditions. After transfer to the PVDF membrane, western blotting was performed by use of the 2C12 antibody and the FCL antibody (Fig. 12). In relation to antibody recognition sites (Fig. 10), the 2C12 antibody specifically recognized the Full chain and the H chain, and the FCL antibody specifically recognized the Full

chain and the L chain. The CBB-stained bands shown in Fig. 11 were identified to bands derived from Factor C. Regarding molecular weight of Factor C, CHO had the largest molecular weight; TAL and HEK had almost the same molecular weight; and Sf9 has the smallest molecular weight, in all cases of the Full chain under non-reducing conditions and of the H-chain and L chain under reducing conditions.

[0143]

In order to more precisely compare the molecular weights of the four types of purified Factor C, electrophoresis (15% gel, LMW marker (product of GE Healthcare)) was performed under the same conditions as employed in a known technique (Jeak Ling Ding and Bow Ho, US 5,712,144; and Jeak L. Ding, *et al.*, Biochem. Biophys. Acta 1202: 149-156), followed by CBB staining, and the molecular weights were then determined through Ferguson plotting.

[0144]

Table 1 shows the results. Under non-reducing conditions, TAL had a molecular weight of 124 kDa, which is almost equivalent to 123 kDa reported by the reference. The molecular weight of CHO was higher, 128 kDa, and that of HEK was 124 kDa, which is equivalent to that of TAL. The molecular weight of Sf9 was 109 kDa, which is smaller than that of TAL. As a result, when recombinant Factor C was expressed in insect cells (Sf9), the molecular weight thereof was found to be smaller than that of native Factor C (TAL), whereas when recombinant Factor C was expressed in mammalian

cells (CHO, HEK), the molecular weight thereof was found to be equivalent to or larger than that of native Factor C (TAL). This tendency was also confirmed in the case of the H chain or the L chain under reducing conditions.

[0145]

The molecular weight of native Factor C derived from *Carcinoscorpius rotundicauda* (Singaporean horseshoe crab) and that of recombinant Factor C thereof obtained via gene expression in insect cells have been reported to be the same value, 132 kDa, under non-reducing conditions. The four types of purified Factor C derived from *Tachypleus tridentatus* (Japanese horseshoe crab) and obtained in the present Example were found to have a molecular weight smaller than 132 kDa (Table 1).

[0146]

[Table 1]

Species of horseshoe crab	Source	Mol. wt. (kDa)			Reference
		Full	H	L	
Tachypleus tridentatus	Natural lysate	124	85	42	Present Example
Tachypleus tridentatus	CHO DG44	128	88	43	Present Example
Tachypleus tridentatus	HEK293	124	85	42	Present Example
Tachypleus tridentatus	Sf9	109	75	39	Present Example
Tachypleus tridentatus	Natural lysate	123	80	43	Takanori Nakamura, *et al.*, Eur. J. Biochem. 154: 511-521 (1986)
Carcinoscorpius rotundicauda	Natural lysate	132	80	52	Jeak L. Ding, *et al.*, Biochem. Biophys. Acta 1202: 149-156 (1993)
Carcinoscorpius rotundicauda	Sf9 (+baculo virus)	132	No data	No data	Jing Wang, *et al.*, J. Biol. Chem. 277 (39): 36363-72 (2002)
Carcinoscorpius rotundicauda	S2	132	No data	No data	Jing Wang, *et al.*, J. Biol. Chem. 277 (39): 36363-72 (2002)

[0147]

9. Confirmation of N-type sugar chain modification

The genes employed in the present Example for expressing the recombinant Factor C were sequences all derived from *Tachypleus tridentatus*, and the expression plasmids were designed so that no tag was added to any terminus of each sequence. Therefore, the translated amino acid sequences of the four types of Factor C obtained in the present Example are thought to be the same one. In addition,

the partial sequences of the N-terminal amino acid residues of the recombinant Factor C (HEK, Sf9) were determined. These amino acid sequences are identical to the sequence of the native Factor C of *Tachypleus tridentatus*, reported by the reference (data not shown).

[0148]

Generally, the molecular weight of protein is thought to vary considerably by modification after translation. In addition, it has also been reported that the structure of an N-type sugar chain added to protein through modification after translation differs between the case of insect cells and the case of mammalian cells. Thus, in order to confirm whether the above-observed difference in molecular weight among the purified Factor C proteins is attributed to the difference in N-type sugar chain structure, the Factor C proteins each were treated with glycopeptidase F. This enzyme is known to cleave and remove an N-type sugar chain added to protein. Therefore, when the purified Factor C proteins have the same molecular weight after treatment with the enzyme, N-type sugar chain is conceived to induce the difference in molecular weight among the Factor C proteins.

[0149]

According to an attached instruction, each purified Factor C protein (1 μg) was treated with 1 mU glycopeptidase F (product of Takara) under modification conditions in the presence of SDS and a reducing agent. The thus-treated samples were separated through SDS-PAGE, and then analyzed

through western blotting. Fig. 13 shows the results. In all cases of the four types of Factor C, the molecular weight of H chain decreased by the glycopeptidase treatment and became the same value. By contrast, although the molecular weight of L chain decreased after the glycopeptidase treatment in all cases of the four types of Factor C, the molecular weight became the same value only in the cases of CHO, HEK, and TAL. Specifically, among four types of L chains, the L chain of the Factor C derived from Sf9 was found to have a molecular weight larger than that of the other three cases, after the glycopeptidase treatment. Therefore, in the L chain of the Factor C derived from Sf9, a certain modification other than N-type sugar chain modification was conceived to occur, after translation.

[0150]

Thus, the aforementioned analysis indicates that the difference in mode of N-type sugar chain after translation plays a main role in providing the difference in molecular weight of Factor C.

[0151]

10. Analysis of N-type sugar chain of Factor C

In N-type sugar chain modification of a protein in mammalian cells, sugar molecules are stepwise added to an asparagine residue of the protein, and eventually, a sugar chain containing terminal sialic acid (i.e., complex-type) is formed. In contrast, in insect cells, sialic acid is not generally added, and a sugar chain having a mannose terminus

(i.e., paucimannose-type) is formed (Robert L. Harrison and Donald L. Jarvis, Methods in Molecular Biology 338: 341-356 (2007)). In order to confirm the difference in N-type sugar chain structure among the four types of purified Factor C proteins, lectin blotting was performed by use of various lectins such as terminal sialic acid-specific lectins (e.g., *Maackia amurensis* agglutinin (MAM) and *Sambucus sieboldiana* agglutinin (SSA)).

[0152]

Dilution series (100, 25, and 6 ng) samples of each of the four types of purified Factor C and bovine fetuin protein as a positive control were dot-blotted onto a nitrocellulose membrane, and the membrane was blocked with 3% BSA. Then, the membrane was sequentially reacted with biotin-labeled lectin (product of J-Oil Mills, Inc.) and streptavidin-labeled HRP (product of Thermo Scientific). Light emission attributed to decomposition of the HRP substrate (product of Thermo Scientific) was detected. Instead of lectin, a 2C12 anti-Factor C antibody was used as a control, to thereby confirm that the same amounts of four types of Factor C were blotted.

[0153]

Fig. 14(a) shows the results of lectin blotting, and Fig. 14(b) shows the binding specificity of lectins employed. *Lens culinaris* agglutinin (LCA) (fucose-specific lectin) and *Canavalia ensiformis* agglutinin (Con A) (bi-antenna-type, high-mannose-type, and complex-type sugar chain-specific

lectin) were bonded to all the samples; i.e., four types of Factor C and fetuin. In contrast, MAM ((α-2,3)-linked terminal sialic acid-specific lectin) was strongly bonded to CHO and less strongly bonded to HEK. TAL and fetuin have almost the same weak reactivity, but did not react with Sf9. SSA ((α-2,6)-linked terminal sialic acid-specific lectin) reacted with HEK, TAL, and fetuin, but did not react with CHO and Sf9.

[0154]

The above analysis indicates that the N-type sugar chain of recombinant Factor C expressed in mammalian cells (CHO and HEK) contains (α-2,3)-linked terminal sialic acid, and that the N-type sugar chain of recombinant Factor C expressed in insect cells (Sf9) contains no (α-2,3)-linked terminal sialic acid. Fig. 14(c) shows schematic structures of the N-type sugar chain of recombinant Factor C, estimated by the results of the above experiments and reports by the reference. The results of the above experiments support that the difference in mode of N-type sugar chain modification after translation is mainly attributed to the difference in molecular weight of Factor C.

Industrial Applicability

[0155]

According to the present invention, a recombinant horseshoe crab Factor C can be efficiently produced. In an embodiment of the present invention, endotoxin present in a

test specimen containing an ion can be measured, while inhibition of reaction is reduced.

[0156]

Sequence listing

SEQ ID NO: 1: DNA sequence of the Factor C gene of Japanese horseshoe crab

SEQ ID NO: 2: Amino acid sequence of the Factor C of Japanese horseshoe crab

SEQ ID NO: 3: DNA sequence of the Factor C gene of Southeast Asian (Singaporean) horseshoe crab

SEQ ID NO: 4: Amino acid sequence of the Factor C of Southeast Asian (Singaporean) horseshoe crab

SEQ ID NO: 5: DNA sequence of the Factor B gene of Japanese horseshoe crab

SEQ ID NO: 6: Amino acid sequence of the Factor B of Japanese horseshoe crab

SEQ ID NO: 7: DNA sequence of the Pro-clotting enzyme gene of Japanese horseshoe crab

SEQ ID NO: 8: Amino acid sequence of the Pro-clotting enzyme of Japanese horseshoe crab

SEQ ID NO: 9: DNA sequence of a Factor B gene of Japanese horseshoe crab, the gene having a codon optimized for expression in insect cells

SEQ ID NO: 10: Peptide sequence

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	Q246419
	Application Number	Not Yet Assigned
Title of Invention	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN	

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				
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Inventor 3				
Legal Name				
Prefix	**Given Name**	**Middle Name**	**Family Name**	**Suffix**

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	Q246419
	Application Number	Not Yet Assigned
Title of Invention	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN	

	Shun-ichiro		KAWABATA	
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City	Fukuoka-shi, Fukuoka	**State/Province**	
Postal Code	812-8581	**Country**	JP

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	23373
Email Address	sughrue@sughrue.com

Application Information:

Title of the Invention	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN		
Attorney Docket Number	Q246419	**Small Entity Status Claimed**	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	10	**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

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	Q246419
	Application Number	Not Yet Assigned
Title of Invention	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN	

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:

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	23373		

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		
Application Number	**Continuity Type**	**Prior Application Number**	**Filing or 371(c) Date (YYYY-MM-DD)**
	Continuation of	15/983,725	2018-05-18
Prior Application Status	Pending		
Application Number	**Continuity Type**	**Prior Application Number**	**Filing or 371(c) Date (YYYY-MM-DD)**
15/983,725	Continuation of	14/650,767	2015-06-09
Prior Application Status	Pending		
Application Number	**Continuity Type**	**Prior Application Number**	**Filing or 371(c) Date** (YYYY-MM-DD)
14/650,767	A 371 of international	PCT/JP2013/083082	2013-12-10

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	Q246419
	Application Number	Not Yet Assigned
Title of Invention	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN	

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) 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).

Application Number	Country	Filing Date (YYYY-MM-DD)	Access Code (if applicable)
2012-269840	JP	2012-12-10	

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: 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.

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

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	Q246419
	Application Number	Not Yet Assigned
Title of Invention	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN	

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.

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.

☑ 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.	☑	

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Phone Number		**Fax Number**	

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	Q246419
	Application Number	Not Yet Assigned
Title of Invention	NOVEL RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN	

Email Address	

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.			
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.	☑		
Organization Name	SEIKAGAKU CORPORATION		
Mailing Address Information For Assignee including Non-Applicant Assignee:			
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Address 2			
City	Tokyo	**State/Province**	
Country	JP	**Postal Code**	100-0005
Phone Number		**Fax Number**	
Email Address			

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	/markboland/	**Date (YYYY-MM-DD)**	2019-04-30
Name	Mark Boland	**Registration Number**	32,197

FEE TRANSMITTAL

Complete if Known	
Application Number	Not Yet Assigned
Filing Date	**April 30, 2019**
First Named Inventor	Hikaru MIZUMURA
Examiner Name	Not Yet Assigned
Art Unit	Not Yet Assigned
Docket No.	Q246419

☐ 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 attached or have been submitted previously.

TOTAL AMOUNT OF PAYMENT ($/EFS - 1,720

METHOD OF PAYMENT (check all that apply)

☐ Check ☑ Credit Card ☐ Money Order ☐ None ☐ Other (please identify):

☐ Deposit Account Deposit Account Number: 19-4880 Deposit Account Name: Sughrue Mion, PLLC

For the above-identified deposit account, the Director is hereby authorized to (check all that apply):

☐ Charge fee(s) indicated below ☐ Charge fee(s) indicated below, **except for the Issue Fee and the Publication Fee**

☐ Charge fee(s) indicated below, **except for the filing fee, the Issue Fee and the Publication Fee**

☑ Charge any additional fee(s), except for the Issue Fee and the Publication Fee, or underpayments of fee(s) under 37 CFR 1.16 and 1.17 ☑ Credit any overpayment of fees(s)

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES (U = undiscounted fee; S = small entity fee; M = micro entity fee)

	FILING FEES			SEARCH FEES			EXAMINATION FEES			
Application Type	**U($)**	**S($)**	**M($)**	**U($)**	**S($)**	**M($)**	**U($)**	**S($)**	**M(&)**	**Fees Paid ($)**
Utility	300	150*	75	660	330	165	760	380	190	1,720
Design	200	100	50	160	80	40	600	300	150	
Plant	200	100	50	420	210	105	620	310	155	
Reissue	300	150	75	660	330	165	2200	1100	550	
Provisional	280	140	70	0	0		0	0	0	

* The $150 small entity status filing fee for a utility application is further reduced to $75 for a small entity status applicant who files the application via EFS-Web.

2. EXCESS CLAIM FEES

Fee Description	**Undiscounted Fee ($)**	**Small Entity Fee ($)**	**Micro Entity Fee ($)**
Each claim over 20 (including Reissues)	100	50	25
Each independent claim over 3 (including Reissues)	460	230	115
Multiple dependent claims	820	410	205

Total Claims		**Extra Claims**		**Fee ($)**		**Fee Paid ($)**
18	- 20 or HP =	0	X	100	=	0

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims		**Extra Claims**		**Fee ($)**		**Fee Paid ($)**
2	- 3 or HP =	0	X	460	=	0

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims	
Fee($)	**Fee Paid ($)**
820	0

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $400 ($200 for small entity)($ 100 for micro entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets		**Extra Sheets**		**Number of each additional 50 or fraction thereof**		**Fee($)**		**Fee Paid ($)**
115	- 100 =	15	/ 50 =	1	(round **up** to a whole number) x	400	=	400
115 *.75	86 - 100 =	-14	/ 50 =	0	EFS Fee Discount	400	=	0

4. OTHER FEE(S) Fees Paid ($)

Non-English Specification, $140 fee ($ 70 for small entity or $35 for micro entity)

Non-electronic filing fee under 37 CFR 1.16(t) for a utility application, $400 fee ($200 small or micro entity)

Other (e.g., late filing surcharge):

SUBMITTED BY

Signature	/markboland/	Registration No. (Attorney/Agent) 32,197	Telephone: 202.293.7060
Name (Print/Type)	Mark Boland		Date: April 30, 2019

DocCode – SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: 16399386 Document Date: 04/30/2019

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