United States Patent and Trademark Office

Before the Patent Trial and Appeal Board

Charles River Laboratories, Inc.,

(Petitioner)

v.

Seikagaku Corporation

(Patent Owner)

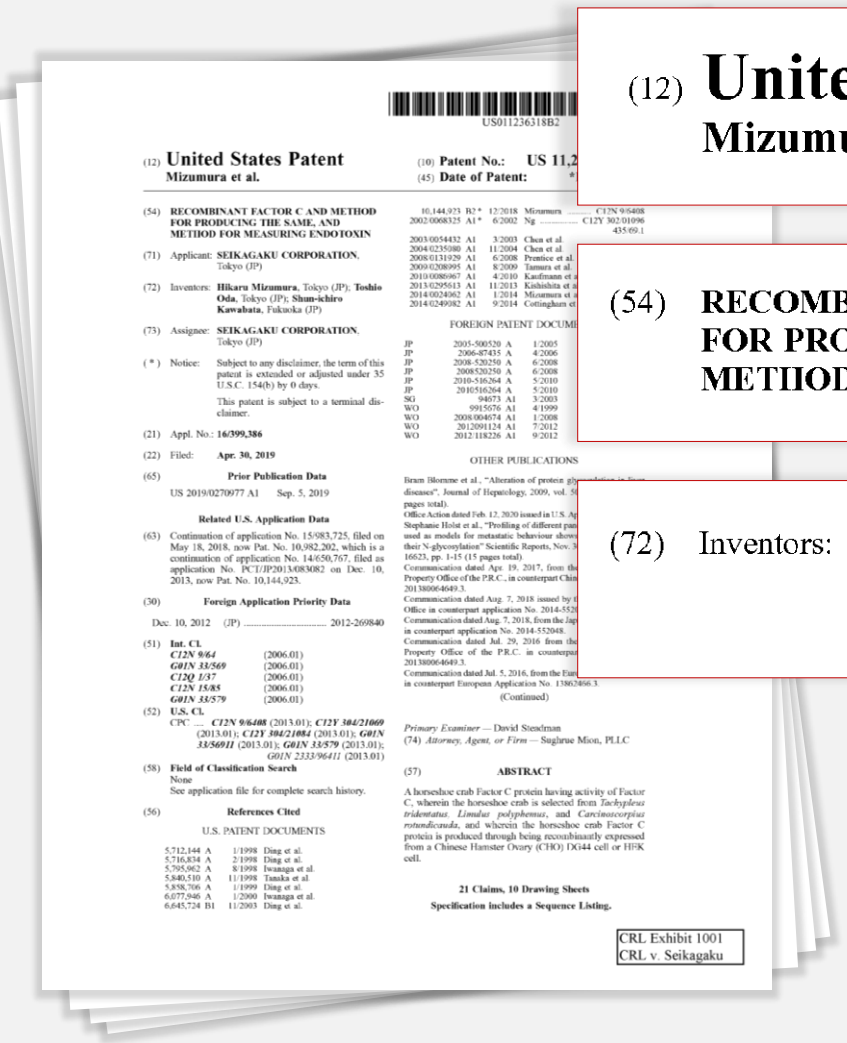
IPR2025-00440 | **Patent No. 11,236,318**

CRL Exhibit 1077
CRL v. Seikagaku
PGR2025-00023

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Challenged Patent - U.S. Patent No. 11,236,318

EX1001



(12) **United States Patent**
Mizumura et al.

(10) **Patent No.: US 11,236,318 B2**
(45) **Date of Patent: *Feb. 1, 2022**

(54) **RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN**

(72) Inventors: **Hikaru Mizumura**, Tokyo (JP); **Toshio Oda**, Tokyo (JP); **Shun-ichiro Kawabata**, Fukuoka (JP)

(21) Appl. No.: **16/399,386**

(22) Filed: **Apr. 30, 2019**

(65) **Prior Publication Data**

US 2019/0270977 A1 Sep. 5, 2019

Related U.S. Application Data

(63) Continuation of application No. 15/983,725, filed on May 18, 2018, now Pat. No. 10,982,202, which is a continuation of application No. 14/650,767, filed as application No. PCT/JP2013/083082 on Dec. 10, 2013, now Pat. No. 10,144,923.

(30) **Foreign Application Priority Data**

Dec. 10, 2012 (JP) 2012-269840

EX1001

Overview of Petition Grounds

Petition at 8

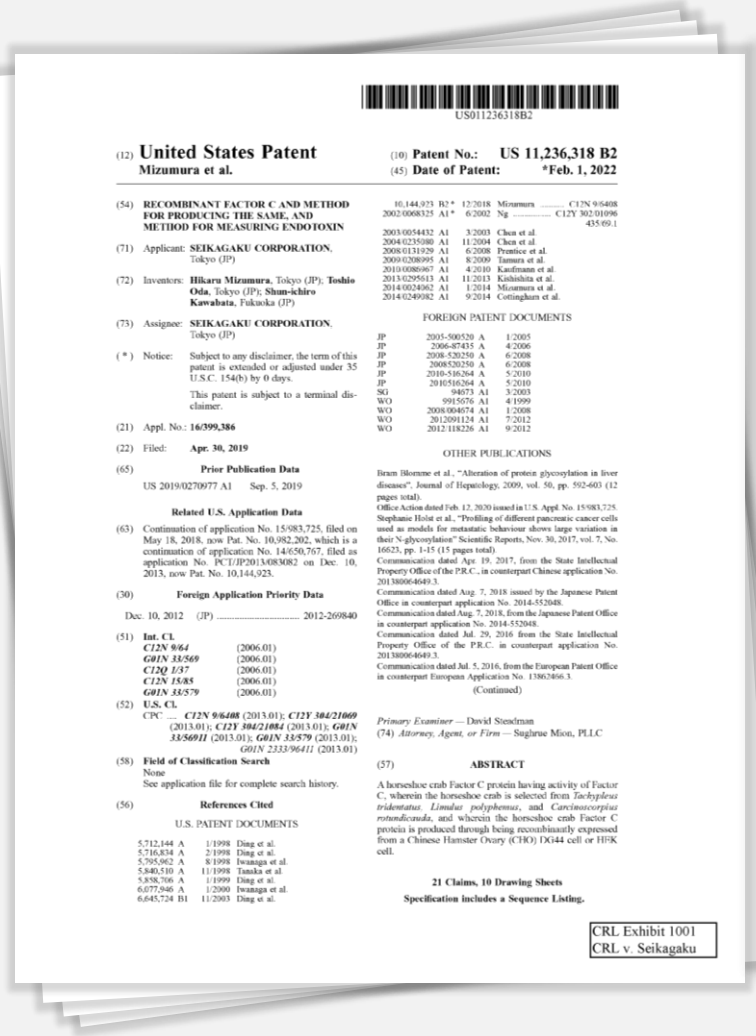
Ground Number and Reference(s)	Claim	Basis
1. **Mizumura** in view of the **'498 PCT**	**21**	**35 U.S.C. § 103 (post-AIA)**
2. **Mizumura** in view of **McClymont**	**21**	**35 U.S.C. § 103 (post-AIA)**

No dispute that post-AIA law applies to patentability of claim 21. Petition at 8, n. 2

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Challenged Claim 21

EX1001



US011236318B2

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(45) **Date of Patent: *Feb. 1, 2022**

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(72) Inventors: **Hikaru Mizumura**, Tokyo (JP); **Toshio Oda**, Tokyo (JP); **Shun-ichiro Kawabata**, Fukuoka (JP)

(73) Assignee: **SEIKAGAKU CORPORATION**, Tokyo (JP)

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G01N 33/569 (2006.01)
C12Q 1/37 (2006.01)
C12N 15/85 (2006.01)
G01N 33/579 (2006.01)

(52) **U.S. Cl.**
CPC ***C12N 9/6488*** (2013.01); ***C12Y 304/21069*** (2013.01); ***C12Y 304/21084*** (2013.01); ***G01N 33/56911*** (2013.01); ***G01N 33/579*** (2013.01); *G01N 2333/96411* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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JP	2008-520250	A	6/2008
JP	2008520250	A	6/2008
JP	2010-516264	A	5/2010
JP	2010516264	A	5/2010
SG	94073	A1	3/2003
WO	9915676	A1	4/1999
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WO	2012/118226	A1	9/2012

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(Continued)

Primary Examiner — David Steadman
(74) *Attorney, Agent, or Firm* — Sughrue Mion, PLLC

(57) **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.

21 Claims, 10 Drawing Sheets
Specification includes a Sequence Listing.

CRL Exhibit 1001
CRL v. Seikagaku

21. 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:

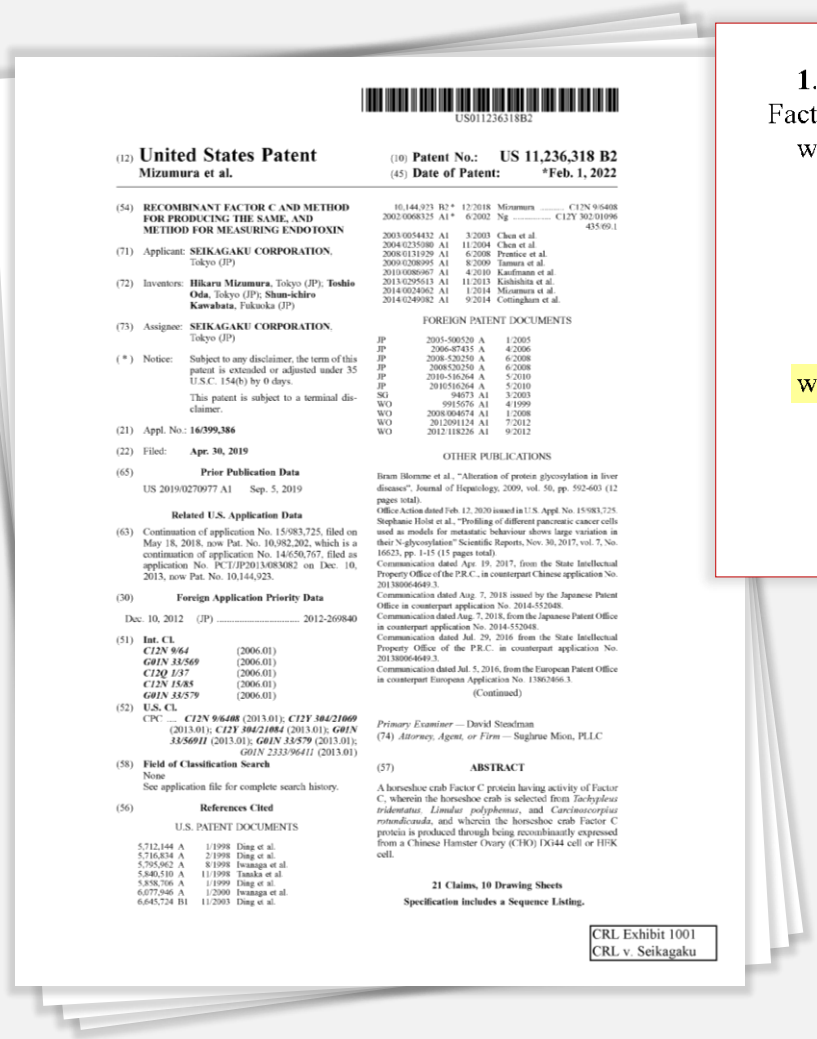
(1) 10% or higher in the presence of 21 mM sodium citrate; and

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

EX1001, 76:50-61

Challenged Patent – U.S. Patent No 11,236,318

Petition 12-13, 16-17



US011236318B2

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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.

21 Claims, 10 Drawing Sheets

Specification includes a Sequence Listing.

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wherein the horseshoe crab is selected from the group consisting of *Tachypleus tridentatus*, and *Limulus polyphemus*, 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. 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

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

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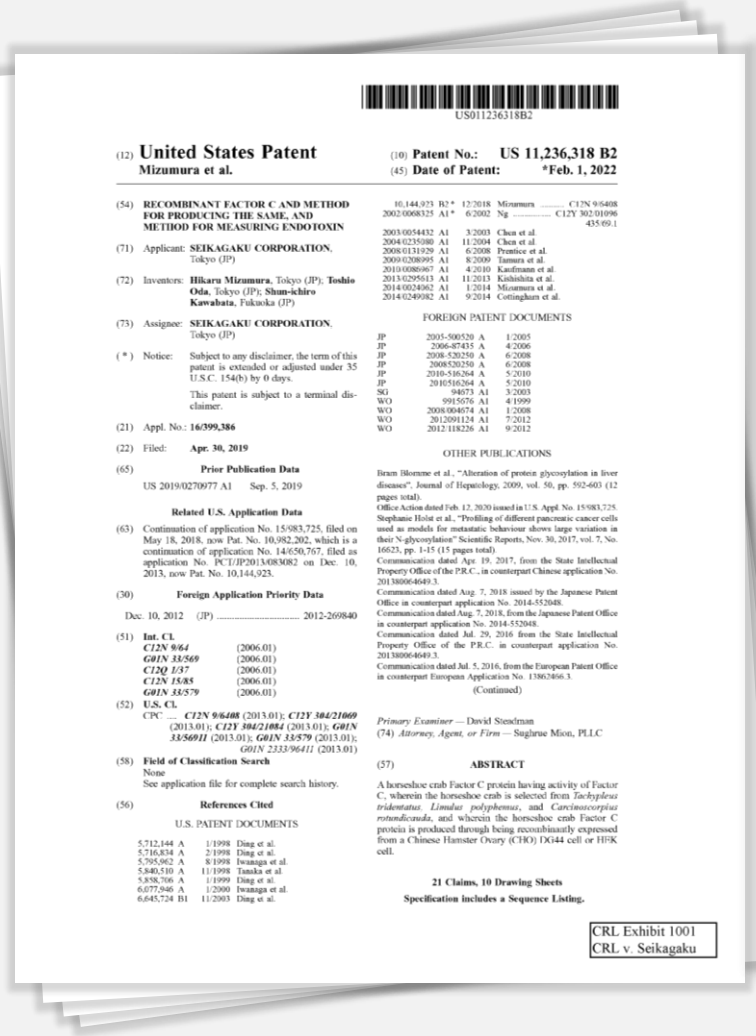
EX1001, 75:2-43

Challenged Claim 21

EX1001



US011236318B2

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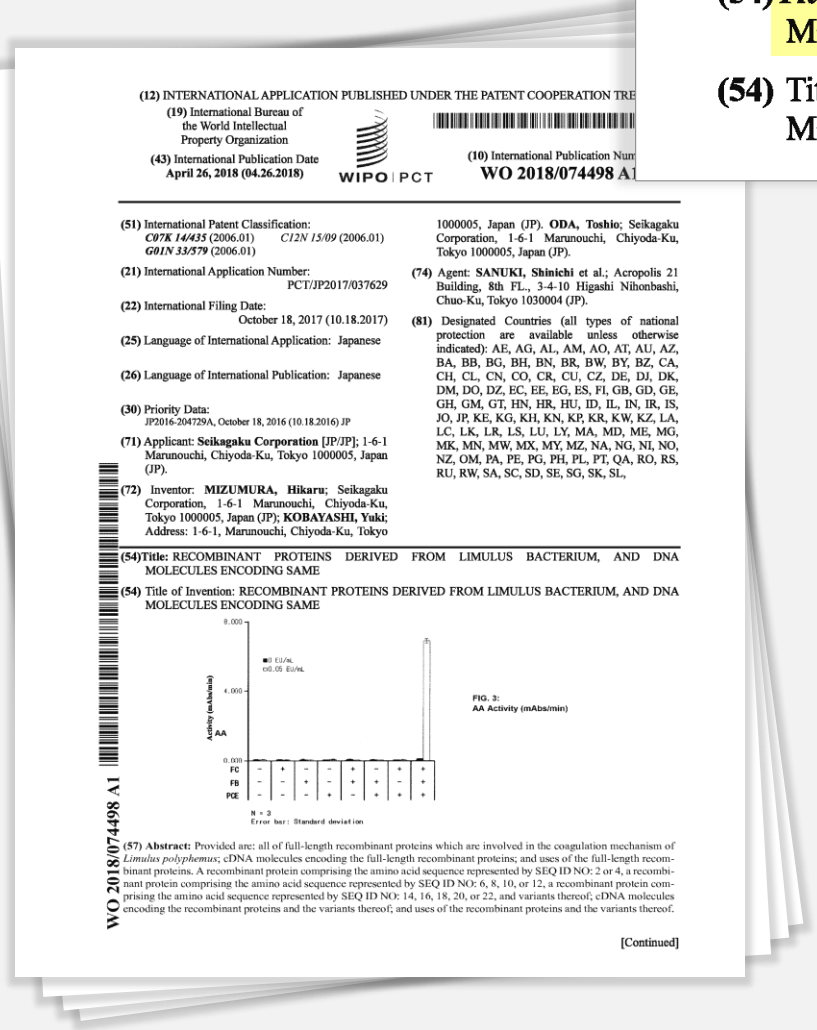
EX1001, 76:50-61

October 18, 2016 – ’498 PCT

EX1005

’498 PCT



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TRE

(19) International Bureau of the World Intellectual Property Organization

(43) International Publication Date
April 26, 2018 (04.26.2018)

WIPO | PCT

(10) International Publication Num
WO 2018/074498 A1

(51) International Patent Classification:
C07K 14/435 (2006.01) *C12N 15/09* (2006.01)
G01N 33/579 (2006.01)

(21) International Application Number:
PCT/JP2017/037629

(22) International Filing Date:
October 18, 2017 (10.18.2017)

(25) Language of International Application: Japanese

(26) Language of International Publication: Japanese

(30) Priority Data:
JP2016-204729A, October 18, 2016 (10.18.2016) JP

(71) Applicant: **Seikagaku Corporation** [JP/JP]; 1-6-1 Marunouchi, Chiyoda-Ku, Tokyo 1000005, Japan (JP).

(72) Inventor: **MIZUMURA, Hikaru**; Seikagaku Corporation, 1-6-1 Marunouchi, Chiyoda-Ku, Tokyo 1000005, Japan (JP); **KOBAYASHI, Yuki**; Address: 1-6-1, Marunouchi, Chiyoda-Ku, Tokyo 1000005, Japan (JP). **ODA, Toshio**; Seikagaku Corporation, 1-6-1 Marunouchi, Chiyoda-Ku, Tokyo 1000005, Japan (JP).

(74) Agent: **SANUKI, Shinichi** et al.; Acropolis 21 Building, 8th FL., 3-4-10 Higashi Nihonbashi, Chuo-Ku, Tokyo 1030004 (JP).

(81) Designated Countries (all types of national protection are available unless otherwise indicated): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL,

(54)Title: RECOMBINANT PROTEINS DERIVED FROM LIMULUS BACTERIUM, AND DNA MOLECULES ENCODING SAME

(54) Title of Invention: RECOMBINANT PROTEINS DERIVED FROM LIMULUS BACTERIUM, AND DNA MOLECULES ENCODING SAME

FIG. 3:
AA Activity (mAbs/min)

N = 3
Error bar: Standard deviation

WO 2018/074498 A1

(57) **Abstract:** Provided are: all of full-length recombinant proteins which are involved in the coagulation mechanism of *Limulus polyphemus*; cDNA molecules encoding the full-length recombinant proteins; and uses of the full-length recombinant proteins. A recombinant protein comprising the amino acid sequence represented by SEQ ID NO: 2 or 4, a recombinant protein comprising the amino acid sequence represented by SEQ ID NO: 6, 8, 10, or 12, a recombinant protein comprising the amino acid sequence represented by SEQ ID NO: 14, 16, 18, 20, or 22, and variants thereof; cDNA molecules encoding the recombinant proteins and the variants thereof; and uses of the recombinant proteins and the variants thereof.

[Continued]

(54)Title: RECOMBINANT PROTEINS DERIVED FROM LIMULUS BACTERIUM, AND DNA MOLECULES ENCODING SAME

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EX1005

October 18, 2016 – ’498 PCT

Petition at 4, 32-33

’498 PCT

[0008] In order to protect horseshoe crabs, there is an idea to artificially produce proteins in amebocyte lysate using recombinant technology and prepare reagents (Patent Documents 1 to 8). In fact, products utilizing recombinant proteins, such as PyroGene (trademark) (Lonza), Endo Zyme (trademark) (HygLos), and PyroSmart (trademark) (Seikagaku Corporation), have been commercialized.

[0009] However, all of these methods use recombinant proteins derived from organisms of the genus Tachypleus. There have been no reports of a reagent using recombinant proteins derived from Limulus polyphemus, which is widely used as a lysate reagent.

[0010] The reason is that in Limulus polyphemus, the proteins and their genes involved in the mechanism of coagulation caused by endotoxin have not been completely identified structurally or functionally.

EX1005, ¶¶0008-10

[0012] However, in Limulus polyphemus, even though more than 20 years have passed since the report in the genus Tachypleus, complete identification of these proteins and genes in terms of both structure and function has not been achieved. This is because due to the various differences and diversity of biomolecules depending on the genus and species of horseshoe crab, it has not been possible to accurately determine the full-length sequence structure, expression, and function of these proteins and genes using conventional methods.

[0013] In fact, a comprehensive analysis of the genes of Limulus polyphemus is being conducted as part of the invertebrate genome project using next-generation sequencers (http://genome.wustl.edu/genomes/detail/limulus-polyphemus/). The full-length sequences of all proteins and genes involved in the coagulation mechanism of Limulus polyphemus have not yet been elucidated. This fact also demonstrates the difficulty in accurately determining the full-length sequence structures of these proteins and genes in Limulus polyphemus.

EX1005, ¶¶0012-13

October 18, 2016 – ’498 PCT

Petition at 4, 33

’498 PCT

[0017] The present inventors have succeeded in obtaining full-length cDNAs encoding all of the protein factors involved in the coagulation mechanism of Limulus polyphemus, something that no one had succeeded in doing for many years, and in expressing all of the recombinant proteins, and have further discovered that these can be used for a variety of purposes, thereby completing the present invention.

EX1005, ¶0017

[0023] The recombinant first protein may more specifically be a recombinant protein that is any of the following:

(A) A recombinant protein comprising the amino acid sequence represented by SEQ ID NO: 2 or 4.

* * *

[0025] This protein was obtained for the first time in the world by the method disclosed in the Embodiments of this Specification, and its full-length sequence, structure and function have been elucidated for the first time. Its function is to become active in the presence of endotoxin and to convert proteins having factor B activity, such as the second protein described below, into their active form (referred to as "factor C activity" in the present application). The first recombinant protein may exhibit Factor C activity at least in combination with a second protein, such as the second recombinant protein.

EX1005, ¶¶0023-25

October 18, 2016 – ’498 PCT

Petition at 49, 58-59

’498 PCT

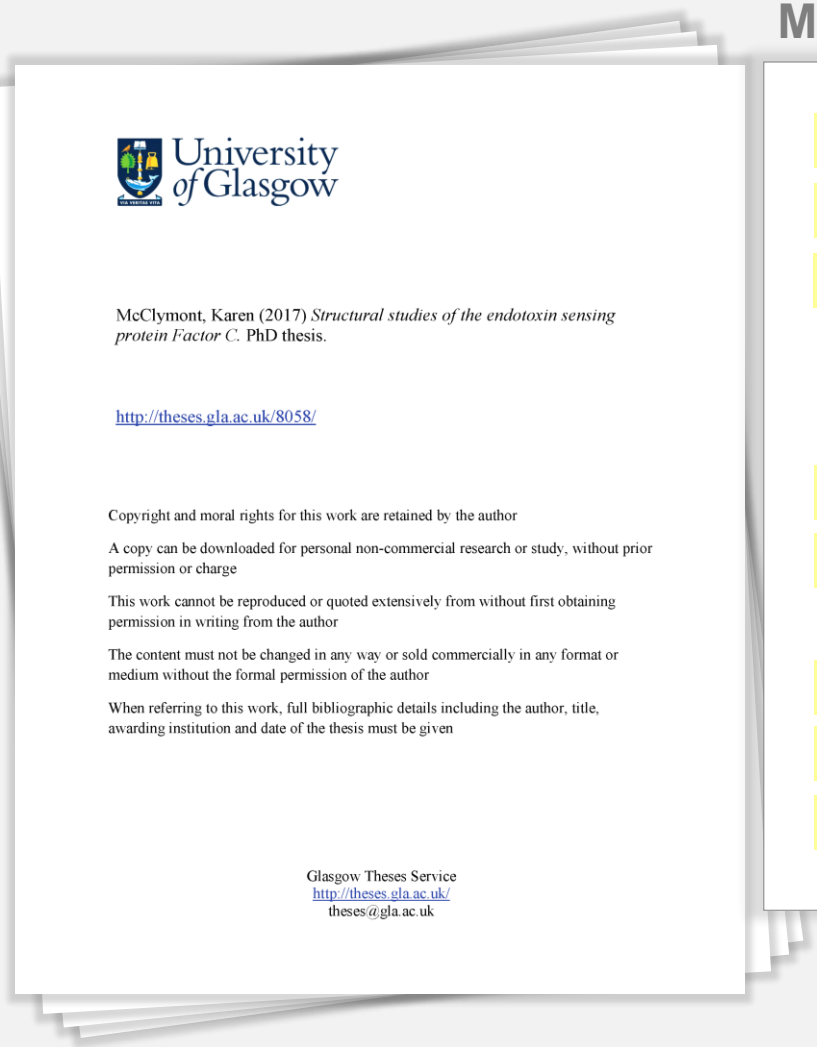
[0180] As a result, there was success for the first time in expressing and obtaining a protein from Limulus polyphemus that may have factor C activity (sequence number 2), a protein that may have factor B activity (sequence number 6), and a protein that may have pro-clotting enzyme activity (sequence number 14). The activity analysis of these proteins will be described later.

EX!005, ¶0180

[0183] Based on this, the expressed protein which may have factor C activity, the protein which may have factor B activity, and the protein which may have pro-clotting enzyme activity have factor C activity, factor B activity, and pro-clotting enzyme activity, respectively, and this demonstrated for the first time that contact with endotoxin triggers a cascade reaction. It was also demonstrated for the first time that these recombinant proteins can be used to detect endotoxin.

EX1005, ¶0183

March 2017 - McClymont Thesis



University of Glasgow

McClymont, Karen (2017) *Structural studies of the endotoxin sensing protein Factor C.* PhD thesis.

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McClymont 2017

Studies to understand the reason Factor C is so sensitive to minute quantities of LPS have been hindered by the difficulties faced with the production of biologically active recombinant Factor C, along with the fact LPS molecules separate from Factor C after activation. The recognition of the lipid A component of LPS by Factor C is very specific, and details surrounding this type of interaction are lacking. However, attempts have been made to determine the nature and exact site of LPS-binding to Factor C, which inspired the experimental approach taken in this project.

Contradictory results as to the location of the LPS-binding site have been published by Tan *et al.* and Koshiba *et al.* after both groups carried out experiments on different fragments of Factor C (Koshiba *et al.*, 2007; Tan *et al.*, 2000). Tan *et al.* led investigations

EX1010, 32

March 2017 - McClymont Thesis

Reply at 6-7

McClymont 2017

1.6 Experimental Approach

The sequence of *Limulus polyphemus* Factor C is not publicly available. For this reason, it was necessary to assemble the lpFC gene from the raw data kindly provided from Washington University, St Louis, in order to synthesise full-length lpFC. Three different eukaryotic expressions systems were chosen to test synthetic production of the full-length protein, to establish which was the most suitable to produce lpFC to fulfil the aim of carrying out a combination of biophysical techniques including circular dichroism, analytical ultracentrifugation and small angle X-ray scattering, to elicit its ligand-free and LPS-bound conformations in solution.

Despite the fact that other Factor C based tests use protein from the *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda* species, the commercial LAL test is made from *Limulus* Factor C. As there are known variances between different LPS binding sites, it is beneficial to study lpFC to identify any differences that may arise between the species in terms of sensitivity or specificity of binding. Additionally, Marine Biotech Limited, the industrial sponsor for this project, use *Limulus polyphemus* for their investigations, and so experiments were carried out using lpFC to coincide with their studies.

2.2 Choice of Factor C Sequence

The most widely used amebocyte lysate tests are produced from *Limulus polyphemus* (lp) blood, while the commercially available recombinant Factor C tests use proteins based on the previously determined *Tachypleus tridentatus* (tt) and *Carcinoscorpius rotundicauda* (cr) sequences. Given published differences in LPS binding sites, in order to assess whether differences in sensitivity of current synthetic Factor C based endotoxin reagents exist due to variations in the sequences, it was considered desirable to obtain the *Limulus polyphemus* Factor C sequence. This would allow for a direct comparison of the Factor C genes between the three species.

EX1010, 36, 38

March 2017 - McClymont Thesis

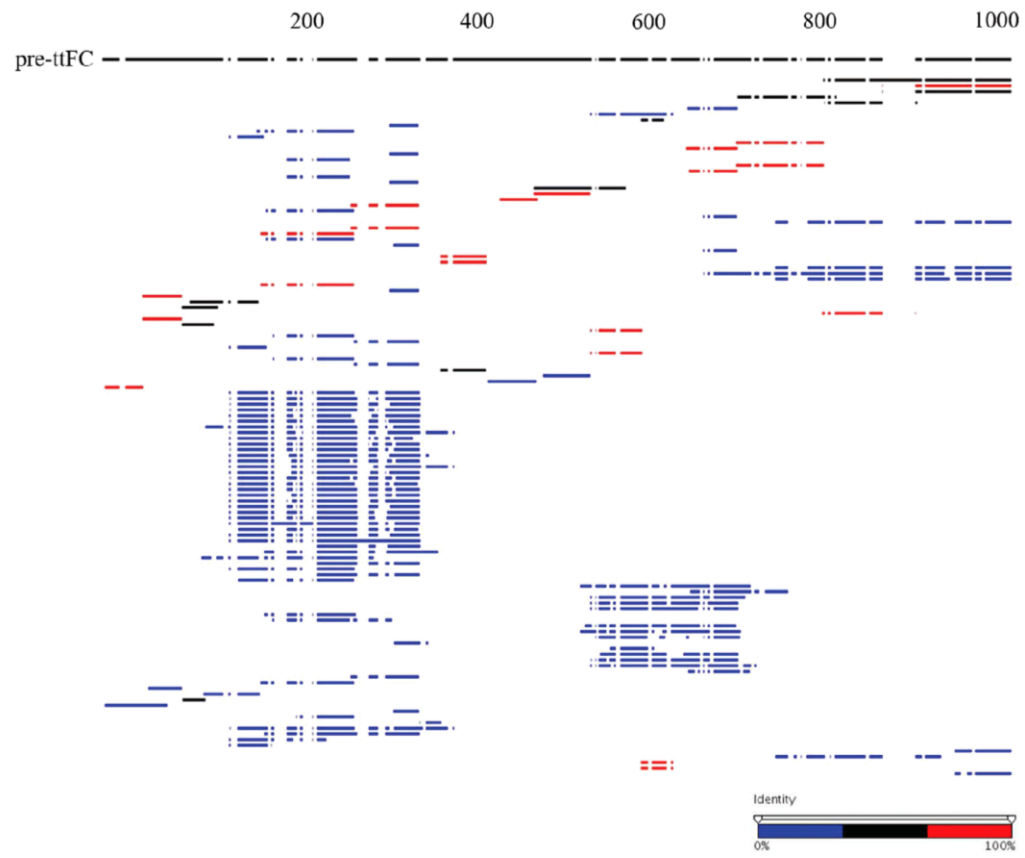

Figure 2-2: Contigs BLAST hits against pre-ttFC sequence. The black bar along the top represents the pre-ttFC sequence, with contig hits shown as bars of varying colour depending on sequence similarity. Each line represents a hit within a specific contig and in this case, there are a number of hits of more than 60% sequence identity. There are often multiple hits to different regions of Factor C in any one contig meaning a contig may appear in more than one line. A full list of the contigs unique identifiers are outlined in Appendix A.

McClymont 2017

For the assembly of the lpFC cDNA, pairs of the selected contigs encoding adjacent regions of the protein coding sequence presumed to represent exons were compared in an attempt to identify common sequences that would allow the intervening intron sequences to be identified. However, this approach was unsuccessful in assembling the gene.

As a result of failing to produce a sensible construct the contigs identified in the tblastn search were assembled using the ttFC cDNA sequence as a guide in the CLC "assemble sequences to template" tool. The resulting assembly was examined and the regions of each contig contributing to the consensus coding sequence were adjusted by hand to likely splice sites following the consensus splicing rules where the intron sequence typically starts before the GT at the 5' end and after the AG at the 3' end (Figure 2-3).

EX1010, 42

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

September 9, 2020 – Mizumura Declaration

App. No. 16/399,386

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 C

known to express LPS-induced protease activity in common[4, 6, 7].

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 2020

Hikaru Mizumura

Dr. Hikaru MIZUMURA

EX1004, 245, 248

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

August 24, 2021 – Amendment to the Claims

App. No. 16/399,386

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

AMENDMENT UNDER 37 C.F.R. § 1.111 Attorney Docket No.: Q246419
Appln. No.: 16/399,386

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.

EX1004, 68-73

May 9, 2025 - PO Defense of EP670 (’498 PCT Progeny)

Reply at 6

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Patent- und Rechtsanwälte
Partnerschaftsgesellschaft mbB
Arabellastraße 30
81925 München
Deutschland | Germany
T +49. (0)89. 92 409. 0
F +49. (0)89. 91 83 56
pm@hoffmanneitle.com
www.hoffmanneitle.com

Munich, May 9, 2025

Our Ref.: **270 325 n7/n31**

Opposition proceedings against

European Patent 3 530 670 B1 / 17 862 926.7

METHOD FOR PRODUCING ENDOTOXIN DETECTING AGENT COMPRISING RECOMBINANT LIMULUS FACTOR C AND USE THEREOF

On behalf of the Proprietor

Seikagaku Corporation
6-1 Marunouchi 1-chome
Chiyoda-ku
Tokyo 100-0005 / JP

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CRL Exhibit 1043
CRL v. Seikagaku
IPR2025-00440

Reply to Opposition to EP670

3.4.1.2 The claimed subject matter is not obvious

50 Because O1 has formulated the objective technical problem incorrectly, its assessment of obviousness is riddled with the sort of *ex post facto* analysis that should be avoided when assessing Article 56 EPC. There is no suggestion in the prior art that recombinant LpFC would have a higher activity than recombinant TtFC. Thus, the subject matter of claim 1 is not obvious.

51 O1 has provided a lengthy explanation as to all the experiments that the skilled person could have done prior to the effective filing date to determine the sequence of LpFC. However, this is not the correct standard. In the third stage of the problem-solution approach, the could-would approach should be applied and O1 has failed to demonstrate that there was any motivation to arrive at the claimed subject matter with the expectation of some advantage. See GL, G-VII, 5.3:

> *"In other words, the point is not whether the skilled person could have arrived at the invention by adapting or modifying the closest prior art but whether the skilled person **would have done** so because the prior art provided motivation to do so in the expectation of some improvement or advantage (see T 2/83)."*

52 D1 discloses in paragraph [0151] that *Limulus polyphemus* is an example of a horseshoe crab. D1 then states in paragraph [0151] that CrFC and, particularly, TtFC are preferred. D1 also discloses that CrFC, a sequence with 97.7% sequence homology to TtFC (D24: abstract), can be complicated to express recombinantly (D1: para [0265]). It was inactive when expressed in S2 cells. Thus, there were no reasons to speculate that LpFC would behave better than TtFC in a recombinant system.

53 D3, including D3a-c, were not available at the effective filing date and, thus, cannot be relevant for the interpretation of D1 at the effective filing date. Regardless of their availability, the applicable standard at the USPTO for determining whether an Applicant is in possession of the full scope of the claims is not the same as required by the EPO's could-would approach.

EX1043, ¶¶50-53

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

May 9, 2025 - PO Defense of EP670 (’498 PCT Progeny)

Reply to Opposition to EP670

55 O1 has also attempted to mischaracterize D3a. In D3a, Dr. Mizumura stated on page 4, paragraph 2 that “*a person skilled in the art would readily recognize that the presently claimed invention could also work when Factor C is the Factor C of Limulus Polyphemus*” (emphasis added). There is no indication in D3a that Dr. Mizumura believed that the skilled person would have been motivated to employ LpFC for the purpose of obtaining the recombinant Factor C having a higher enzymatic activity than TtFC.

56 Dr. Mizumura then stated in D3a, paragraph bridging pages 4 and 5 “*the above-described evidence supports that the Factor C of Limulus polyphemus would also have unexpectedly superior property of being less susceptible to inhibition by ions, as exhibited by Factor C of Tachypleus tridentatus*” (emphasis added). The evidence that Dr. Mizumura referred to was an alignment of the sequence of LpFC with TtFC provided on pages 2-4 of D3a. The sequence of LpFC was not publicly disclosed prior to the effective filing date of the Patent. Thus, the alignment was also not part of the prior art.

EX1043, ¶¶55, 56

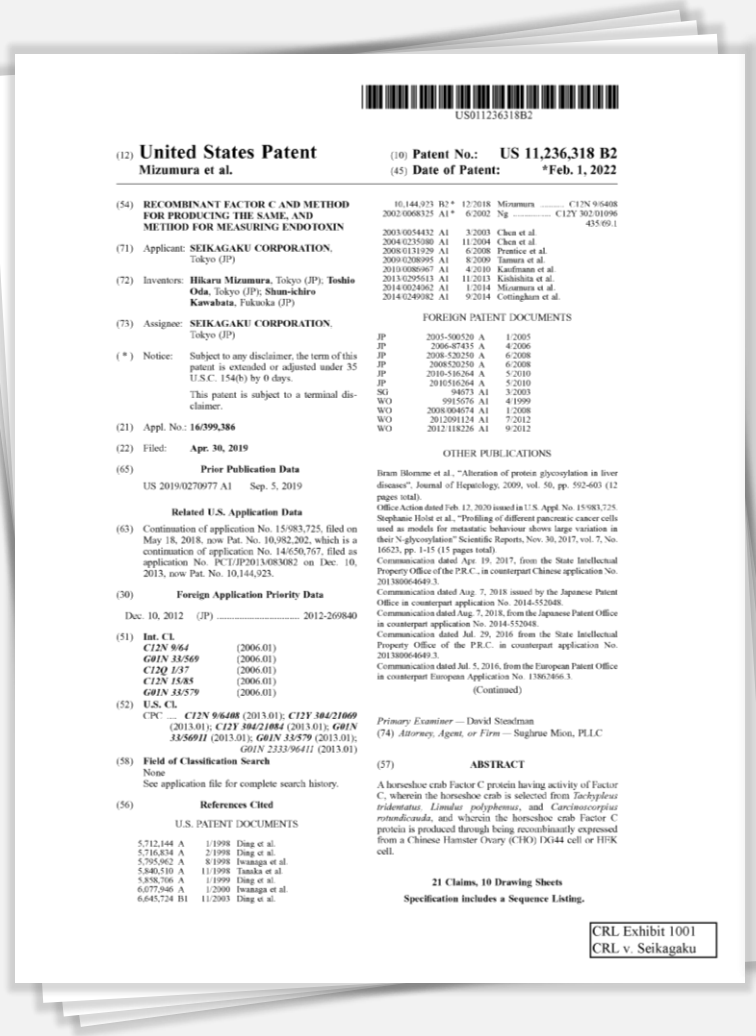
57 We note that O1 has conveniently omitted “*the above-described evidence supports that*” when it misquoted D3a (see O1’s statement of grounds for opposition, paragraph 54). It is not acceptable to misquote Dr. Mizumura and then allege that Dr. Mizumura believed it was obvious that recombinant LpFC would be associated with a superior property based solely on what was known prior to the effective filing date of the Patent. This is not the case. Dr. Mizumura based their conclusion on the “*above-described evidence*” provided in D3a. Said evidence included an alignment of a previously unknown sequence. The reality is that Dr. Mizumura acknowledges in D33 that the higher activity of LpFC was surprising (D33: paras 22 and 23) and the Proprietor maintains the position that the claimed subject matter involves an inventive step.

EX1043, ¶57

“effective filing date of the Patent” = October 18, 2016

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Challenged Claim 21



US011236318B2

(12) **United States Patent**
Mizumura et al.

(10) **Patent No.: US 11,236,318 B2**
(45) **Date of Patent: *Feb. 1, 2022**

(54) **RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN**

(71) Applicant: **SEIKAGAKU CORPORATION**, Tokyo (JP)

(72) Inventors: **Hikaru Mizumura**, Tokyo (JP); **Toshio Oda**, Tokyo (JP); **Shun-ichiro Kawabata**, Fukuoka (JP)

(73) Assignee: **SEIKAGAKU CORPORATION**, Tokyo (JP)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **16/399,386**

(22) Filed: **Apr. 30, 2019**

(65) **Prior Publication Data**

US 2019/0270977 A1 Sep. 5, 2019

Related U.S. Application Data

(63) Continuation of application No. 15/983,725, filed on May 18, 2018, now Pat. No. 10,982,202, which is a continuation of application No. 14/650,767, filed as application No. PCT/JP2013/083082 on Dec. 10, 2013, now Pat. No. 10,144,925.

(30) **Foreign Application Priority Data**

Dec. 10, 2012 (JP) 2012-269840

(51) **Int. Cl.**
C12N 9/64 (2006.01)
G01N 33/569 (2006.01)
C12Q 1/37 (2006.01)
C12N 15/85 (2006.01)
G01N 33/579 (2006.01)

(52) **U.S. Cl.**
CPC ***C12N 9/6488*** (2013.01); ***C12Y 304/21069*** (2013.01); ***C12Y 304/21084*** (2013.01); ***G01N 33/56911*** (2013.01); ***G01N 33/579*** (2013.01); *G01N 2333/96411* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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Communication dated Jul. 5, 2016, from the European Patent Office in counterpart European Application No. 13862466.3.
(Continued)

Primary Examiner — David Steadman
(74) *Attorney, Agent, or Firm* — Sughrue Mion, PLLC

(57) **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.

21 Claims, 10 Drawing Sheets
Specification includes a Sequence Listing.

CRL Exhibit 1001
CRL v. Seikagaku

21. 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:

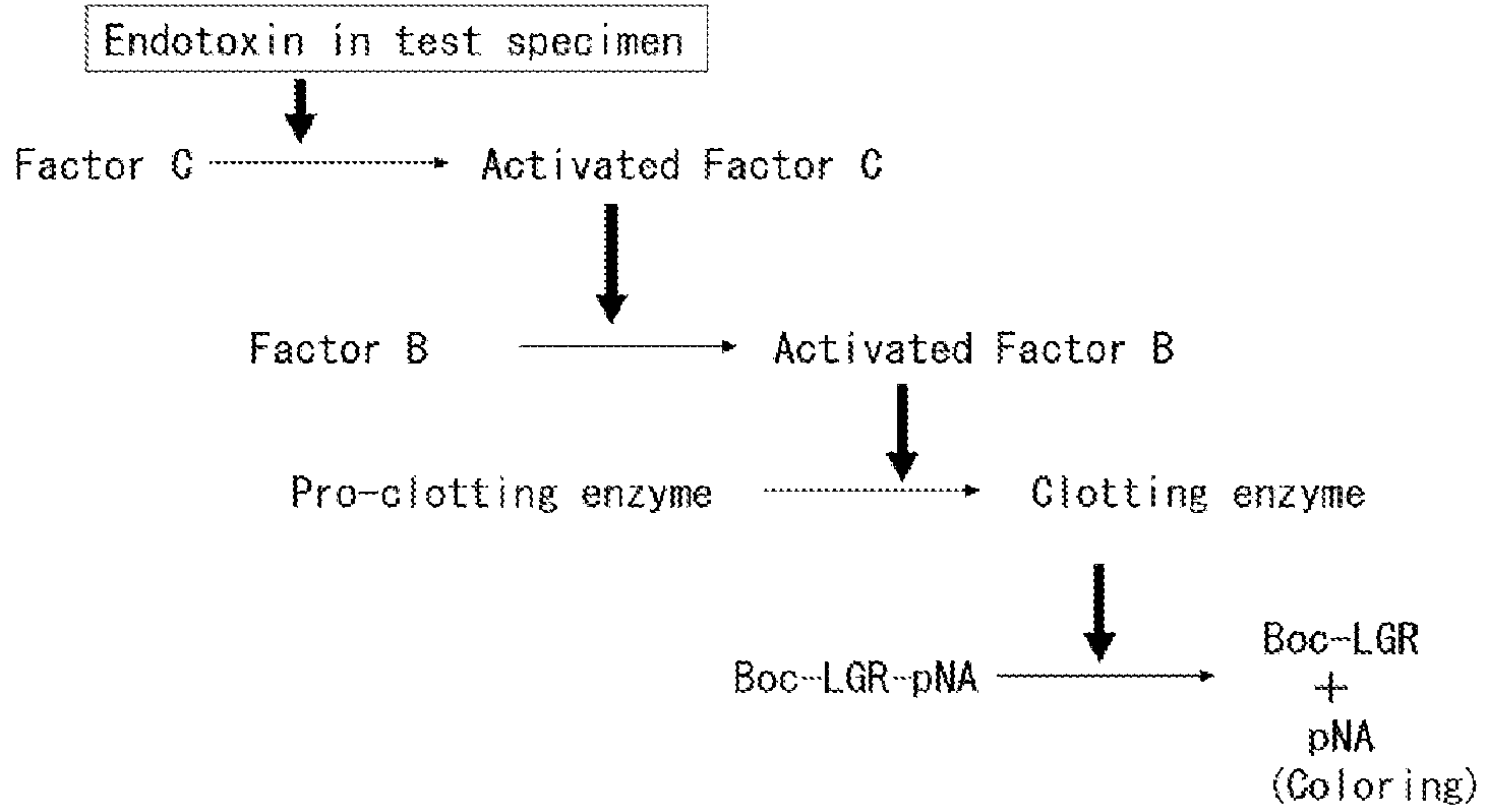
(1) 10% or higher in the presence of 21 mM sodium citrate; and

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

EX1001, 76:50-61

’767 Application

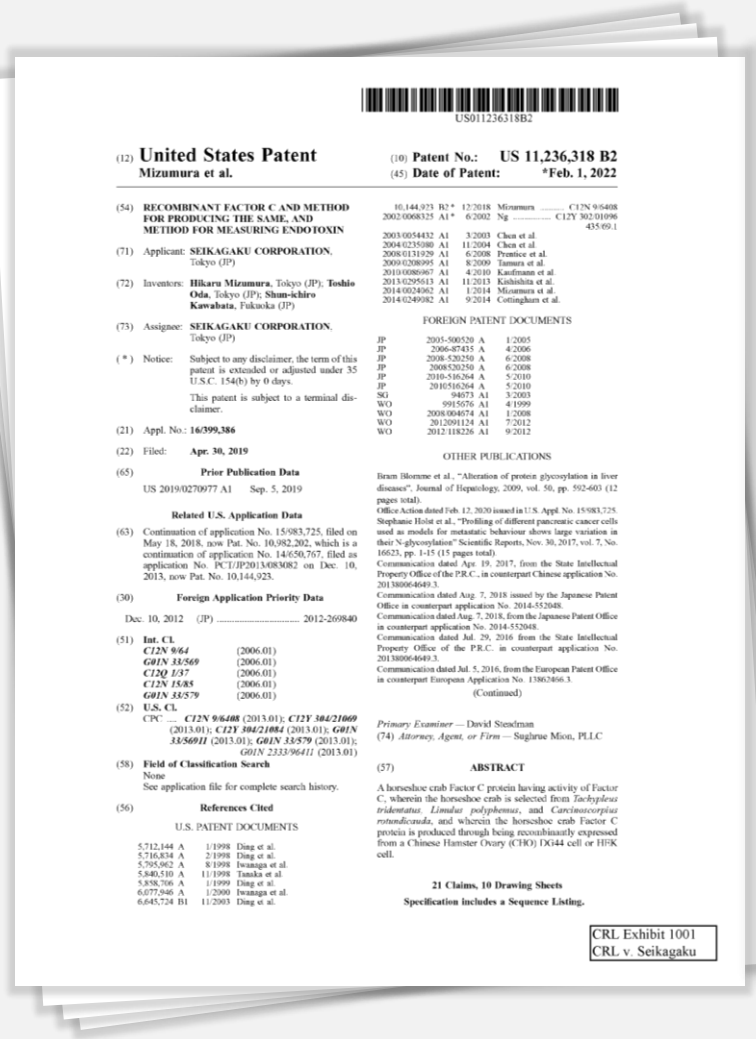
[Fig. 1]



EX1006, 118, FIG. 1

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Challenged Claim 21



US011236318B2

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Mizumura et al.

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(45) **Date of Patent: *Feb. 1, 2022**

(54) **RECOMBINANT FACTOR C AND METHOD FOR PRODUCTING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN**

(71) Applicant: **SEIKAGAKU CORPORATION**, Tokyo (JP)

(72) Inventors: **Hikaru Mizumura**, Tokyo (JP); **Toshio Oda**, Tokyo (JP); **Shun-ichiro Kawabata**, Fukuoka (JP)

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C12Q 1/37 (2006.01)
C12N 15/85 (2006.01)
G01N 33/579 (2006.01)

(52) **U.S. Cl.**
CPC ***C12N 9/6488*** (2013.01); ***C12Y 304/21069*** (2013.01); ***C12Y 304/21084*** (2013.01); ***G01N 33/56911*** (2013.01); ***G01N 33/579*** (2013.01); *G01N 2333/96411* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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Communication dated Aug. 7, 2018 issued by the Japanese Patent Office in counterpart application No. 2014-552048.
Communication dated Aug. 7, 2018, from the Japanese Patent Office in counterpart application No. 2014-552048.
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Communication dated Jul. 5, 2016, from the European Patent Office in counterpart European Application No. 13862466.3.
(Continued)

Primary Examiner — David Steadman
(74) *Attorney, Agent, or Firm* — Sughrue Mion, PLLC

(57) **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.

21 Claims, 10 Drawing Sheets
Specification includes a Sequence Listing.

CRL Exhibit 1001
CRL v. Seikagaku

21. 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.

EX1001, 76:50-61

Prosecution History of ’318 Patent

Petition at 13-14

U.S. App. No. 16/399,386

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.

EX1004, 235

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).

EX1004, 75-76

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Prosecution History of ’318 Patent

Petition at 13-14

U.S. App. No. 16/399,386

> 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.

EX1004, 177

> 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.

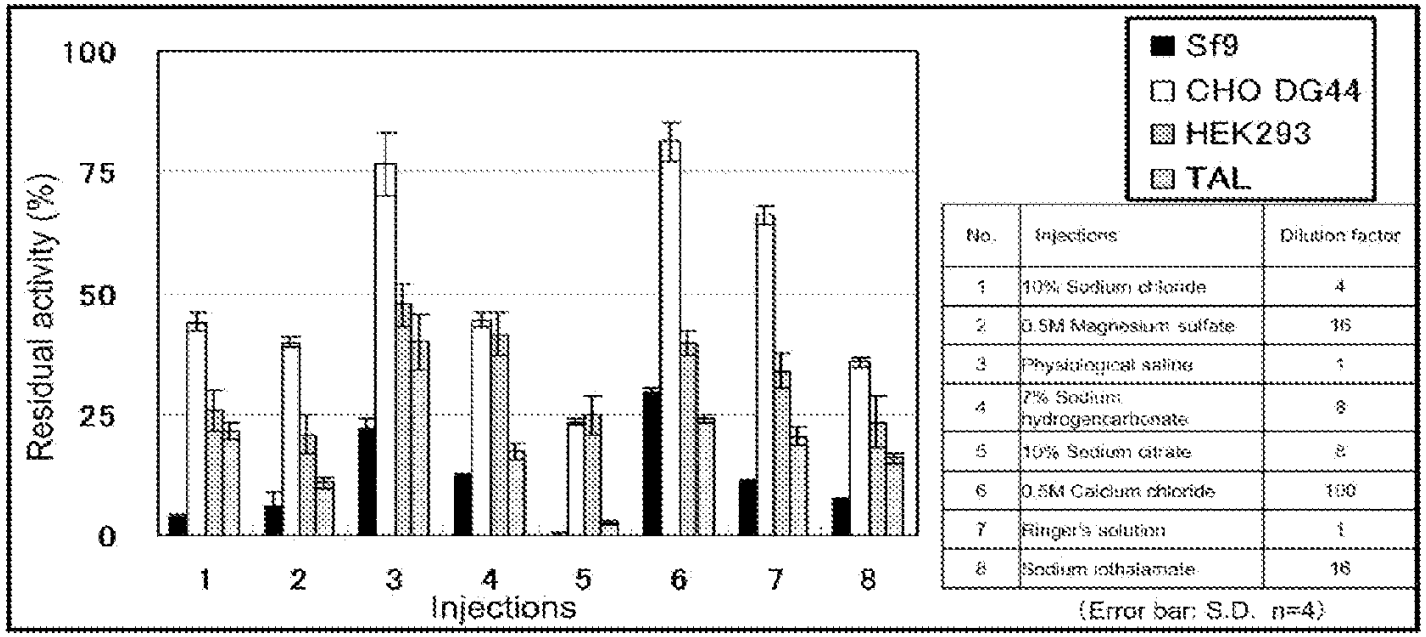
EX1004, 78

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

’767 Application Specification

Petition at 11, 23, 29-30

[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 iothalamate	16

(Error bar: S.D. n=4)

EX1006, 122

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Dr. Siddhartha Jain, Ph.D., PMP

Petition at 24, 27

Jain Declaration (EX1002)

84. The species delineation of these horseshoe crabs is based on geography (Japanese, Singaporean, North American species). Given this geographical separation, a POSA would have expected each species to have evolved distinct characteristics, including differences in certain protein functions. Ex. 1038, 6-7, 10-16. While a POSA in 2013 may have expected the native members of these varied horseshoe crab species to behave similarly in terms of their hemolymph ability to detect endotoxin, recombinant generation of the proteins responsible for such endotoxin detection function and the specifics of how such recombinant proteins would function on more specific features such as "residual activity" would have been highly speculative and not amenable to extrapolation between species.

EX1002, ¶84

88. Where geography separated these species of crabs (belonging to distinct taxonomic families Limulidae and Tachypleinae), giving rise to genetic and morphological distinctness, and where existing and limited sequence data for the *Limulus polyphemus* crab showed ***differences*** between its Factor C amino acid sequence and those of other species (discussed below), the failure to provide any experimental example or verified structural link between species giving rise to such similar function only confirms a lack of possession Ex. 1038, 6-7, 10-16 (discussing the geographic and genetic discreteness of Family Limulidae and Family Tachypleinae); Ex. 1022, 157.

EX1002, ¶88

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

“Genetic Differentiation”

Petition at 27, 38

Taxonomically the four extant species (Figs. 1 and 2) are assigned to three genera in two families in the Superfamily Limulacea:

Family Limulidae
Limulus polyphemus (Linnaeus)

Family Tachypleinae
Tachypleus tridentatus (Leach 1819)
Tachypleus gigas (Muller 1785)
Carcinoscorpius rotundicauda (Latreille 1802)

Yamasaki (1988) proposed renaming *C. rotundicauda* as *Tachypleus rotundicauda* (Latreille 1802) n. comp. – with which we agree, but this has to be brought to the International Commission on Zoological Nomenclature for adjudication. Meanwhile, *Carcinoscorpius,* an apt descriptor, is in general use.

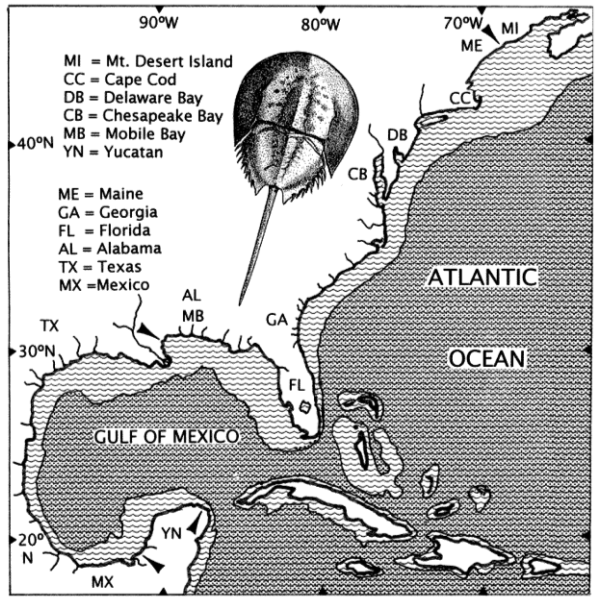


Fig. 5 Breeding populations of the American horseshoe crab, *Limulus polyphemus*, are distributed, intermittently, on the shore of Yucatan and from the Mississippi delta eastward to the Florida Keys and then along the Atlantic coast, Florida to Maine (from about 21°N to 44°N), with occasional strays reported in Texas and Canadian waters. Except for a narrow continental shelf along the southeast coast of Florida, the shelf is broad elsewhere

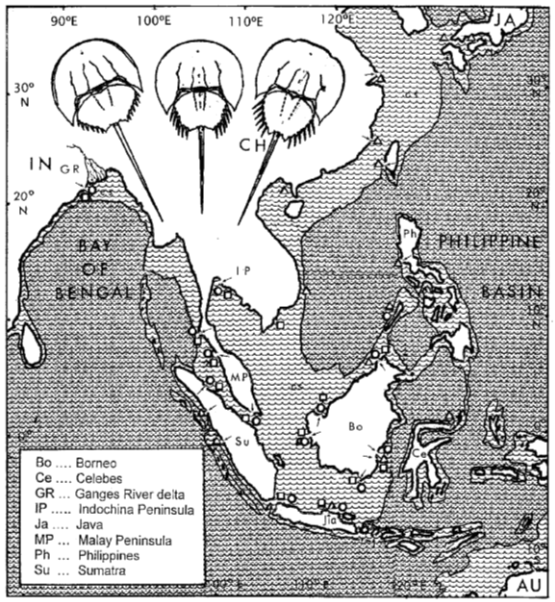


Fig. 6 The continental shelf (cs) is the avenue of distribution for horseshoe crabs. The three Indo-Pacific species are widely distributed with their ranges overlapping in many locations (modified from Anderson and Shuster in Shuster, Barlow and Brockmann 2003, based on Sekiguchi 1988). One or more species were found near most research sites (*small arrows*); *triangles* = *Tachypleus tridentatus* (dorsal view to right in masthead); *squares* = *Tachypleus gigas* (middle of masthead), and *circles* = *Carcinoscorpius rotundicauda* (*left* in masthead); JA = Japan CH = China, IN = India, and AU = Australia

2.5 Genetic Considerations

Genetic studies substantiate the existence of discrete populations and lend credence to the physiological races concept. Selander et al. (1970) established that electrophoretic variations between four populations of *Limulus polyphemus* were comparable to those of other animals and concluded that the stabilization of their external morphology for at least 200 million years was due to the temporal uniformity of the organic environment and/or genetic homeostasis. Sokoloff (1978) studied eye color and concluded that there were physiological races of *Limulus*. Microsatellite DNA analyses of several *Limulus* populations, from the Gulf of Mexico to New England (Saunders et al. 1986), revealed distinct populations. Pierce et al. (2000) showed that the smaller-sized *Limulus* from the upper Chesapeake Bay were also genetically different from the larger-sized Delaware Bay horseshoe crabs. The latest evidence for genetic differentiation in *Limulus* was provided by a large-scale study of populations throughout their range (King et al. 2003). The extent of the available genetic information leaves little doubt as to the physiological differences of the populations.

Other evidences of relationships between the extant species were demonstrated in a series of developmental and biochemical studies by Sekiguchi and colleagues (1988). Experimental hybridization using reciprocal fertilization of gametes produced viable, swimming larvae only in crosses between *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda*; reciprocal crosses between the three Indo-Pacific species and *Limulus* were non-fertile (Sugita et al. 1988). The diploid number of chromosomes is 26 (*T. tridentatus*), 28 (*T. gigas*), 32 (*C. rotundicauda*), and 52 (*L. polyphemus*) (Iwasaki et al. 1988). Extensive biochemical and immunological studies on the hemocyanin also yielded specific relationships among the species (Sugita et al. 1988). Serological correspondence studies yielded immunological distances that placed the separation of the Indo-Pacific species and *Limulus* at some 130 million years ago while *T. gigas* separated from the other two about 20mya and *T. tridentatus* and *C. rotundicauda* about 10 mya (Shishikura et al. 1982).

EX1038, 6-7, 10-15

Dr. Siddhartha Jain, Ph.D., PMP

Jain Declaration (EX1002)

91. Indeed, POSAs at the time would have been familiar with the cautionary concept that "function similarity generally increases with sequence similarity but with a high degree of variability." Ex. 1021, 1; Ex. 1008, 2781. Without the full gene and amino acid sequence information for *Limulus polyphemus* Factor C in 2013 from which ***any comparison*** could even be made to any other species, any view about the resulting functional characteristics (specifically, residual activity) of a recombinantly produced protein from *Limulus polyphemus* would have been mere speculation.

EX1002, ¶¶91-93

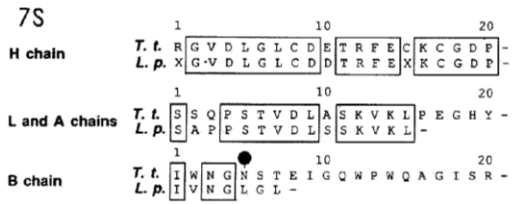
92. A POSA would not expect that such protocols would yield recombinant proteins in soluble and functional form from a ***different*** species of crab (whose full sequence information was not even known as of 2013) and that too with a similar residual activity falling in that range without performing an experimental verification. For example, Wang et. al. (2002) demonstrated that the mutation of only two amino acids in the *Carcinoscorpius rotundicauda* Factor C impaired their expression in the insect cells. Ex. 1036, 36365-36366.

subject to error. Even a single amino acid substitution can be critical enough to change the function and biophysical-biochemical properties of a protein such as in the case of hemoglobin that results in sickle cell disease with a single amino acid substitution. Ex. 1037, 239-241. Hence, a POSA could not have been assured of functional similarity between Factor C from different species of horseshoe crab without experimental verification, especially in the absence of complete *Limulus polyphemus* sequence information or any experimental data in the '767 Application to be able to apply the residual activity data across species.

Dr. Siddhartha Jain, Ph.D., PMP

Jain Declaration (EX1002)

96. For example, Supplementary Figure 7S from Tokunaga 1991 provides a side-by-side comparison of a partial amino acid sequence of Factor C for *Limulus polyphemus* (labeled as L.p.) and corresponding regions of *Tachypleus tridentatus* (labeled as T.t.).



97. As was known by December 2013, the middle panel (L and A chains) depicts the region immediately after the Proline-rich region (PRR) and the last panel (B chain) depicts the serine protease region immediately after the autocatalytic cleavage site. Referencing the list of conservative substitutions in the '318 patent, a POSA would see that the partial amino acid sequence contains non-conservative mutations—the middle panel has a Q → P (Gln → Pro) mutation and the last panel has W → V (Trp → Val) mutation.

98. These two non-conservative mutations would have been reasonably expected by a POSA to cause differences in protein structure. Since prolines are known to disrupt the secondary structures, Q → P may be expected to contribute to a structural change. Similarly, since tryptophan is a bulky and aromatic amino acid residue compared to valine, W → V is also expected to have contributed to a structural difference. Notably, W → V mutation is present adjacent to the Factor C autocatalytic cleavage site, which is a critical region for the activation Factor C and a structural difference at the cleavage site could translate to a functional difference. The last panel also shows a change from NST → LGL (Asn-Ser-Thr → Leu-Gly-Leu) which are not only non-conservative mutations but also removes an N-glycosylation site (NST).

EX1002, ¶¶96-98

Prosecution history of ’318 Patent

Petition at 11-14

U.S. App. No. 16/399,386

Apr. 30, 2019

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.

EX1004, 515

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.

EX1004, 517

Feb. 3, 2021

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.

EX1004, 168

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.

EX1004, 234

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Prosecution history of ’318 Patent

Petition at 11-14

U.S. App. No. 16/399,386

> 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.

EX1004, 236

> 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.

EX1004, 179

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Patent Owner Expert Dr. Tina Morris

PO's Expert Testimony

A. The molecules we are talking about are large molecules that have large charge-attracting surfaces. Bacterial endotoxins are known to be actively binding molecules themselves. So we are talking about an assay environment where large molecules are interacting with each other in an environment where a lot of salt ions are available.

And in many different ways, those can create what is called suppression, masking effects. And that's what we are talking about here.

* * *

In my scientific opinion, the interference and interaction that occurs in such assays is multifactorial and very difficult to attribute to a single -- to a single origin.

EX1069, 57:1-58:10

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Patent Owner Expert Dr. Tina Morris

PO Expert Testimony

Q. Okay. So is it fair to say that this ion inhibition phenomenon is -- has potentially many causes and is -- could not just be attributed to posttranslation sialic acid addition?

A. Yes, it is multifactorial. And the carbohydrate structure or terminal -- the carbohydrate or glycosylation structure or function or relationship is often much more complicated than the terminal sialic acids.

EX1069, 58:21-59:6

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

EP670 Claim 1

Notice of Opposition

8. **Independent claim 1** is reproduced below, with points (i)-(iii.a) itemizing features of the claim:

(i) ***A method for producing an endotoxin detecting agent***, *comprising*

(ii) *a step of* ***artificially expressing a protein***

(ii.a) ***using a cDNA***, *and*

(iii) ***collecting the expressed recombinant protein as a solution fraction*** *containing the expressed recombinant protein,*

(ii.b) *wherein* ***the cDNA encodes any one of the following****:*

(A) a recombinant protein expressed as containing the amino acid sequence represented by SEQ ID NO: 2 or 4;

(B) a recombinant protein expressed as 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 has factor C activity; and

(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

(iii.a) wherein ***the solution fraction*** *containing the expressed recombinant protein* ***is a culture solution, or a culture supernatant****.*

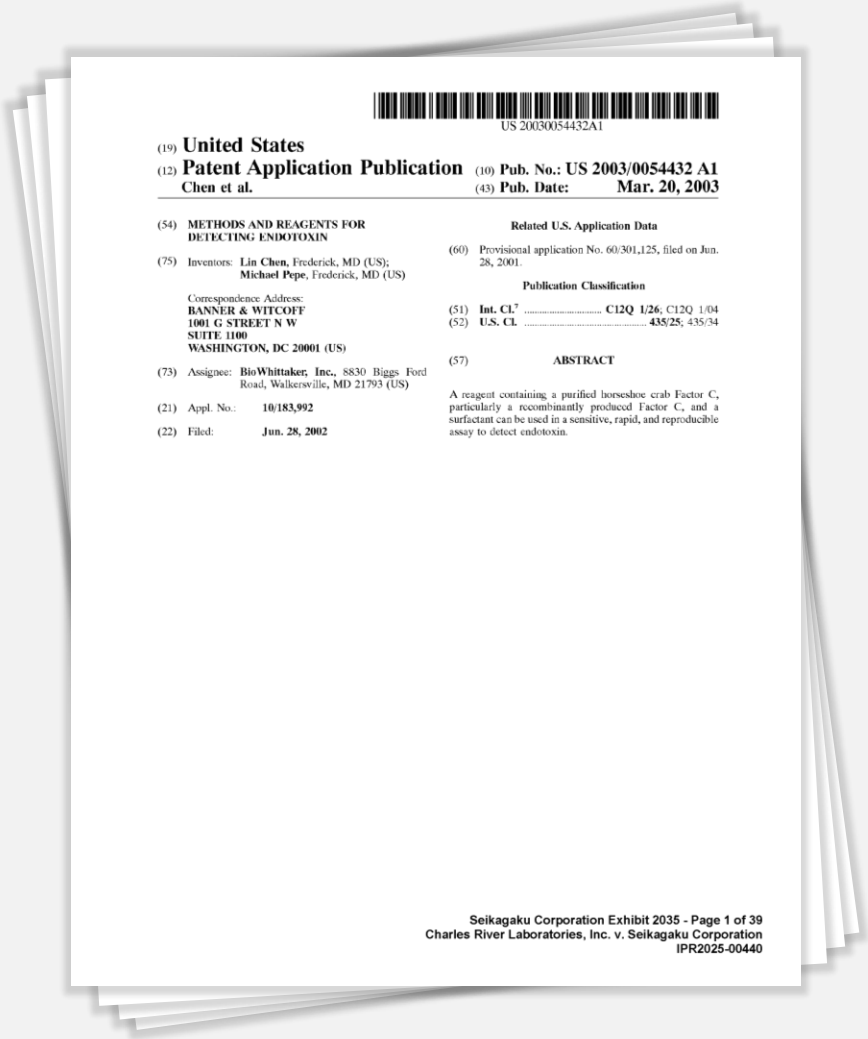
EX1048, ¶8

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

’432 Publication

Reply at 14-15

’432 Publication (2003)



US 20030054432A1

(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2003/0054432 A1**

Chen et al. (43) **Pub. Date: Mar. 20, 2003**

(54) **METHODS AND REAGENTS FOR DETECTING ENDOTOXIN**

(75) Inventors: **Lin Chen**, Frederick, MD (US); **Michael Pepe**, Frederick, MD (US)

Correspondence Address:
BANNER & WITCOFF
1001 G STREET N W
SUITE 1100
WASHINGTON, DC 20001 (US)

(73) Assignee: **BioWhittaker, Inc.**, 8830 Biggs Ford Road, Walkersville, MD 21793 (US)

(21) Appl. No.: **10/183,992**

(22) Filed: **Jun. 28, 2002**

Related U.S. Application Data

(60) Provisional application No. 60/301,125, filed on Jun. 28, 2001.

Publication Classification

(51) **Int. Cl.**[7] **C12Q 1/26**; C12Q 1/04

(52) **U.S. Cl.** **435/25**; 435/34

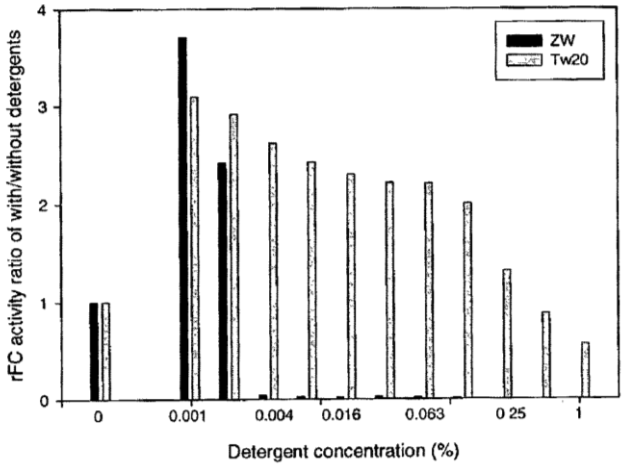
(57) **ABSTRACT**

A reagent containing a purified horseshoe crab Factor C, particularly a recombinantly produced Factor C, and a surfactant can be used in a sensitive, rapid, and reproducible assay to detect endotoxin.

Seikagaku Corporation Exhibit 2035 - Page 1 of 39
Charles River Laboratories, Inc. v. Seikagaku Corporation
IPR2025-00440

rFC (*C. rotundicuada*)

FIG. 1. Zwittergent 3-14 and Tween 20

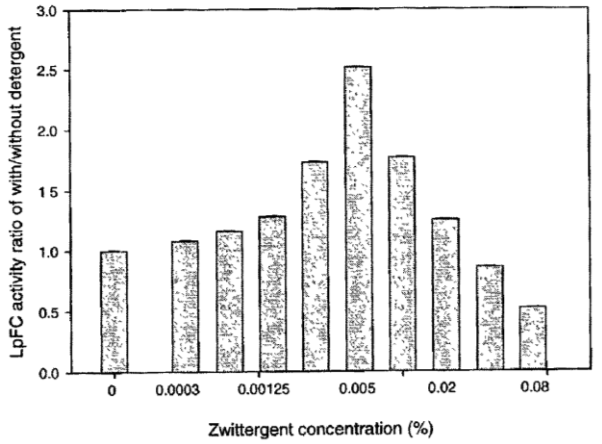


[0030] The invention also encompasses use of purified naturally and non-naturally occurring, i.e., recombinantly produced, Factor C variants, provided that the variants retain a Factor C enzyme activity. Factor C enzyme activity can be assessed using any assay for Factor C enzyme activity known in the art. See, e.g., Tokunaga et al., *J. Biochem.* 109,150-57 and Nakamura et al., *Eur. J. Biochem.* 176,89-94,1988. The endotoxin assays described in Examples 1 and 13, below, also can be used. Naturally occurring Factor C variants include, for example, products of Factor C mRNA splice variants or mutated Factor C genes.

Native *L. polyphemus*

FIG. 6

Limulus Factor C activity in different Zwittergent concentration



[0129] Results

[0130] As shown in FIGS. **1-3** and **9-11**, low concentrations of Zwittergent 3-14, Tween 20, Tween 80, Triton X-114, OTG, Genapol C-100 and Triton X-100 enhanced the rFC assay 2-7 fold. The enhancing concentration range for Zwittergent 3-14, Triton X-114, OTG and Triton X-100 is narrow, while the enhancing concentration range for Tween 20, Tween 80 and Genapol C-100 is wide.

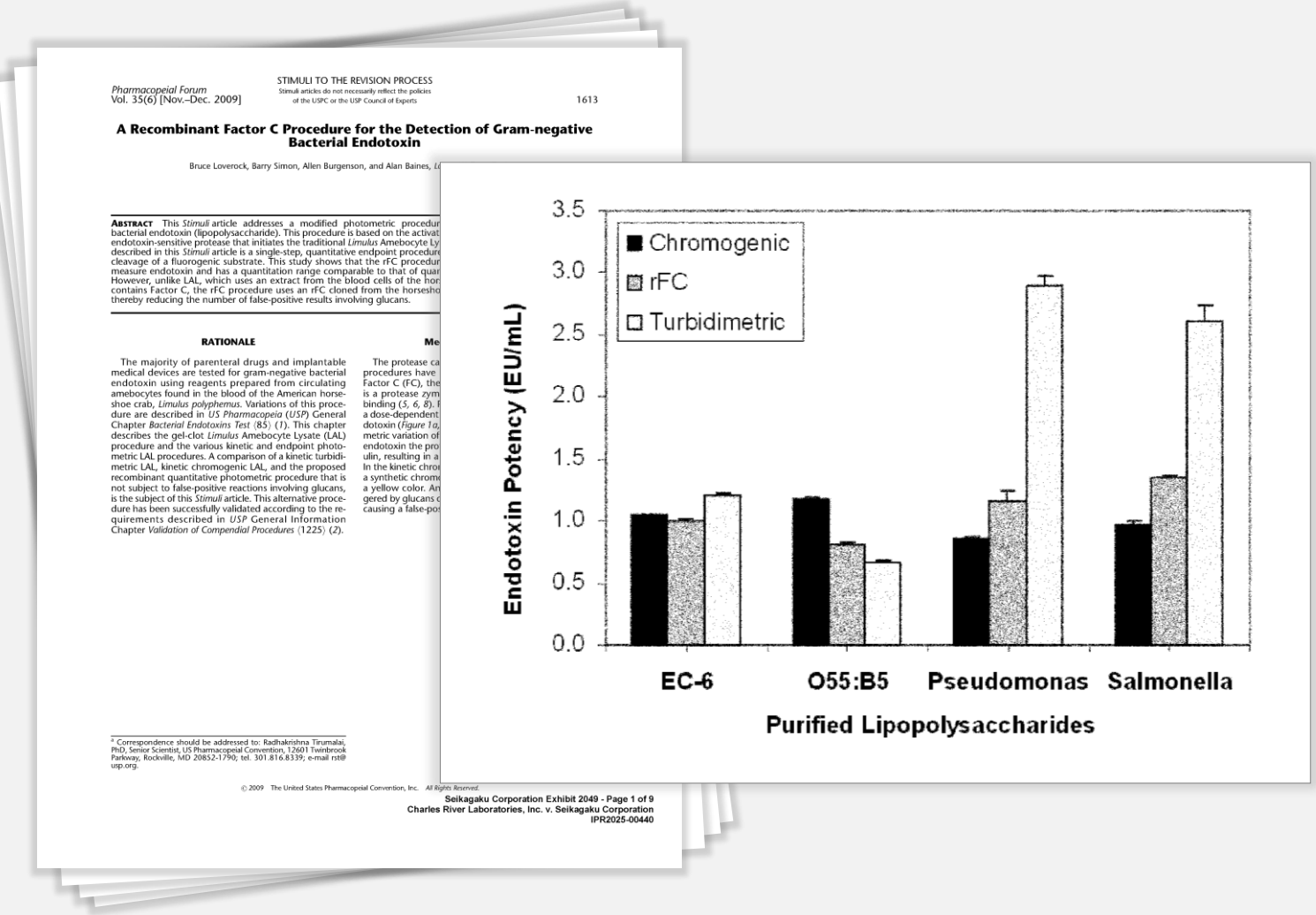
[0132] The enhancing/inhibiting effect of Zwittergent 3-14 was also tested using purified Limulus factor C (**FIG. 6**). A similar enhancing/inhibiting effect was observed, although the relative factor C activity ratio, the enhancing and inhibiting detergent concentrations were different from those in the recombinant Factor C assay.

EX2035, ¶¶35, 130, 132

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Loverock et al.

Loverock et al. (2009)



Pharmacopeial Forum
Vol. 35(6) [Nov.–Dec. 2009]

STIMULI TO THE REVISION PROCESS
Stimuli articles do not necessarily reflect the policies of the USPC or the USP Council of Experts

1613

A Recombinant Factor C Procedure for the Detection of Gram-negative Bacterial Endotoxin

Bruce Loverock, Barry Simon, Allen Burgenson, and Alan Baines, *La*

ABSTRACT This *Stimuli* article addresses a modified photometric procedur bacterial endotoxin (lipopolysaccharide). This procedure is based on the activat endotoxin-sensitive protease that initiates the traditional *Limulus* Amebocyte Ly described in this *Stimuli* article is a single-step, quantitative endpoint procedur cleavage of a fluorogenic substrate. This study shows that the rFC procedur measure endotoxin and has a quantitation range comparable to that of quar However, unlike LAL, which uses an extract from the blood cells of the hor contains Factor C, the rFC procedure uses an rFC cloned from the horsesho thereby reducing the number of false-positive results involving glucans.

RATIONALE

The majority of parenteral drugs and implantable medical devices are tested for gram-negative bacterial endotoxin using reagents prepared from circulating amebocytes found in the blood of the American horseshoe crab, *Limulus polyphemus*. Variations of this procedure are described in *US Pharmacopeia* (*USP*) General Chapter *Bacterial Endotoxins Test* ⟨85⟩ (*1*). This chapter describes the gel-clot *Limulus* Amebocyte Lysate (LAL) procedure and the various kinetic and endpoint photometric LAL procedures. A comparison of a kinetic turbidimetric LAL, kinetic chromogenic LAL, and the proposed recombinant quantitative photometric procedure that is not subject to false-positive reactions involving glucans, is the subject of this *Stimuli* article. This alternative procedure has been successfully validated according to the requirements described in *USP* General Information Chapter *Validation of Compendial Procedures* ⟨1225⟩ (*2*).

Me

The protease ca procedures have Factor C (FC), the is a protease zym binding (*5, 6, 8*). I a dose-dependent dotoxin (*Figure 1a,* metric variation of endotoxin the pro ulin, resulting in a In the kinetic chro a synthetic chrom a yellow color. An gered by glucans causing a false-po

* Correspondence should be addressed to: Radhakrishna Tirumalai, PhD, Senior Scientist, US Pharmacopeial Convention, 12601 Twinbrook Parkway, Rockville, MD 20852-1790; tel. 301.816.8339; e-mail rst@usp.org.

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Seikagaku Corporation Exhibit 2049 - Page 1 of 9
Charles River Laboratories, Inc. v. Seikagaku Corporation
IPR2025-00440

The two LAL-based procedures gave statistically significant different mean responses for all four endotoxin sources. The turbidimetric response was greater than the chromogenic response for all sources except O55:B5, where the chromogenic response was greater.

For the O55:B5, *Pseudomonas*, and *Salmonella* sources the rFC mean response was significantly different from the two LAL-based procedures but fell between the two. With EC-6 there was not a significant difference between the rFC and chromogenic procedures, but both of these were significantly less than the turbidimetric results although the magnitude of the difference was small.

Recombinant FC recognizes endotoxin from different source materials as do other photometric procedures, demonstrating equivalency to the LAL-based procedures.

EX2049, 4-5

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Alwis et al.

Alwis et al. (2006)

AMERICAN JOURNAL OF INDUSTRIAL MEDICINE 49:296–300 (2006)

Recombinant Factor C Assay for Measuring Endotoxin in House Dust: Comparison With LAL, and (1 → 3)-β-D-Glucans

K. Udeni Alwis, PhD and Donald K. Milton, MD, DrPH*

Background *Measurement of exposure to environmental endotoxin is frequently performed using a Limulus amebocyte lysate (LAL) based assay. Recently, a new method has become available with similar sensitivity and potentially greater specificity using recombinant Factor C (rFC) from the horseshoe crab Carcinoscorpius rotundicauda. A preliminary study was carried out to determine the comparability of LAL and rFC in measuring endotoxins in house dust for large scale epidemiologic studies.*

Methods *House dust samples were collected from family rooms by vacuuming 1 m^2 of the center of the room. Sixty sieved house dust samples were assayed for endotoxin by LAL (Cambrex, KQCL lysate) and rFC (Pyrogene, Cambrex) and for (1 → 3)-β-D-glucans by ELISA. The resistant parallel line estimation was used for data analysis of LAL and rFC. A four-parameter logistic fit with inverse prediction was used to calculate (1 → 3)-β-D-glucan levels of the samples.*

Results *The spike recovery was 113.63% (95% CI = 101.69, 125.57%) for LAL and 99.69% (95% CI = 90.14, 109.24%) for rFC assays. The LAL assay gave higher endotoxin estimates compared with rFC. The LAL and rFC estimates were highly correlated ($r = 0.86$, $P < 0.0001$). The difference between LAL and rFC endotoxin estimates correlated with the LAL estimates ($r = 0.51$, $P < 0.0001$). However, the difference was not correlated with (1 → 3)-β-D-glucans.*

Conclusion *LAL and rFC gave comparable results, hence either assay can be used for studies of endotoxin exposure. The current study shows that (1 → 3)-β-D-glucan is not a major factor interfering with endotoxin measurements in house dust using a Cambrex KQCL LAL preparation.* Am. J. Ind. Med. 49:296–300, 2006. © 2006 Wiley-Liss, Inc.

KEY WORDS: endotoxin; LAL; rFC; (1 → 3)-β-D-glucan; house dust

Department of Environmental Health, Exposure, Epidemiology and Risk Assessment Program, Harvard School of Public Health, Landmark Center West, Boston, Massachusetts

Presented in a Pre-Conference Session: "Exposure to Endotoxin and the Lung", at the Fifth International Symposium, Future of Rural Peoples: Rural Economy, Healthy People, Environment, Rural Communities, October 16, 2004, Saskatoon, Saskatchewan, Canada.
Contract grant sponsor: NIEHS; Contract grant numbers: ES07036, ES07456, 2P30ES00002.

*Correspondence to: Donald K. Milton, Department of Work Environment University of Massachusetts Lowell, 1 University Avenue, Lowell, MA 01854.
E-mail: Donald_Milton@umledu

Accepted 14 December 2005
DOI 10.1002/ajim.20254. Published online in Wiley InterScience (www.interscience.wiley.com)

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INTRODUCTION

Endotoxins are biologically active lipopolysaccharides, which compose the outer membrane of the gram-negative bacteria. Endotoxins are released into the environment after lysis of the bacterial cells, during active cell growth, and when bacterial cells are taken up by macrophages and processed. Gram-negative bacteria and their endotoxins are ubiquitous colonizers of soil, water, and living organisms.

Endotoxins are potent biological agents associated with bioaerosol-induced respiratory symptoms observed in both indoor [Wan and Li, 1999; Park et al., 2001] and occupational

Seikagaku Corporation Exhibit 2036 - Page 1 of 5
Charles River Laboratories, Inc. v. Seikagaku Corporation
IPR2025-00440

LAL estimates were higher compared with rFC estimates for all the samples we assayed, and larger differences between LAL and rFC were significantly correlated with larger LAL estimates of endotoxin content. A possible explanation may be differences in the Factor C of the horseshoe crabs that were the source of reagents. For the LAL assay the reagent is extracted from wild North American horseshoe crabs, *Limulus polyphemus*. The rFC was produced from the cDNA of the Singaporean horse shoe crab, *Carcinoscorpius rotundicauda* cloned in *Baculovirus* expression system in SF9 cells [Ding et al., 1995]. The genetically engineered rFC produced from *Carcinoscorpius rotundicauda* is reported to be 97.7% homologous (sequence identity) to that of *Tachypleus tridentatus* the Japanese horse shoe crab [Nakamura et al., 1988; Ding et al., 1993, 1995]. An unpublished study found that the Factor C of *Carcinoscorpius rotundicauda* has 89.3% homology with that of *Limulus polyphemus*, North American horse shoe crab Factor C [Lin Chen, personal communications]. Thus, subtle differences in responsiveness of the Factor C from these species may account for their small but systematically different results.

EX2036, 4

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Patent Owner Expert Dr. Brian Schaefer

PO's Expert Testimony

Q. Quantitatively, the LAL procedures had higher endotoxin potency metrics; right?

A. In the specific assay that they ran in Figure 3, with seemingly a single unnamed concentration that was tested, they got distinct results.

They got distinct results, although the results were not impressively different between the first two sources, really; it's just the second two sources, the pseudomonas and the salmonella. And only when the turbidometric assay was used and not the chromogenic assay. So I'm hesitant to draw global solutions about LAL versus the recombinant based on that.

Q. I'm not asking you to draw global solutions or conclusions. I'm just saying you are concluding that these are functionally, what do you say, comparable; right?

A. Functionally comparable. That doesn't mean you can't come up with an assay and assay conditions here and there where you get a numerical output that is somewhat different. But, again, we know from the pharmacopeias that both the recombinant assay and the assay -- the purified enzymes are accepted, and not only are they accepted, they're understood to give you comparable, real-world working values.

EX1067, 177:20-178:21

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

The Full Scope of Claim 21 is Not Enabled

Petition at 37-42

- **How would a POSA make and use recombinant Factor C proteins across the full scope of this claim?**
- **Applicants abandoned only structural feature providing explanation for observed function.**

Challenged Claim 21

21. 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.

EX1001, 76:50-61

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Wands Factor 8 – Claim 21 is Broad

- **No dispute that claim 21 recites a broad genus of recombinant Factor C proteins with any residual activity above a lower bound for either one of two ionic solutions.**

See EX1069, 108:9-109:8, 64:14-65:14, 66:8-19; EX1067, 202:11-203:16

PO's Expert Testimony

Q. And you agree that a residual activity of even greater than 100 percent, in the presence of sodium citrate, would meet that claim; right?

A. Well, again, I will recite the claim. The claim says 10 percent or higher in the presence of 20 millimolar sodium citrate. So it's a threshold value, and any value that's higher than threshold value is within the claim.

EX1067, 203:9-16

Wands Factors 4, 5, 7 – Unpredictable nature of invention

Petition at 38-39

Jain Declaration (EX1002)

137. In addition, while recombinant technology was not brand new as of 2013, a significant amount of research and experimentation was still required to generate a particular protein, with a particular functional capability, across a number of different expression systems, through screening of expression systems and optimization of cell culture conditions (including cell culture media composition). Ex. 1031, 16 ("Even when hundreds of proteins are produced at commercial scale, the production of recombinant proteins still constitutes a challenge in many cases… A necessary condition for adequate recombinant protein production is the efficient expression of the gene of interest. However, expression can be lost due to structural changes in the recombinant gene or disappearance of the gene from host cells."); Ex. 1030, Abstract ("PTMs directly influence the 2-DE pattern, and knowledge of the rules of protein separation is required in order to understand the protein distribution in a 2-DE gel. Glycosylation is the most common PTM and can modify both the molecular weight and the pI of a protein. In particular, the effect of charged monosaccharides (mainly sialic acids, SAs) on the 2-DE pattern of a protein is of major interest since changes in sialylation are regularly observed in comparative studies. Little is known about the pI shift of a glycoprotein induced by the presence of SAs, or whether this shift is the same for all glycoproteins."). Ex. 1039, 936 (Noting that "[t]here have been numerous reports on the effects of cell type, culture process, and culture media toward the resulting glycoform profile" and summarizing same).

EX1002 (Jain), ¶137

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Wands Factor 6 – Level of Ordinary Skill in Art is High

Petition at 39-40

Jain Declaration (EX1002)

140. However, designing, expressing, and testing recombinant proteins is laborious, time-consuming, complicated, and highly unpredictable due to the many effects that variations in amino acid sequence (and subsequent post-translational modifications) can have on protein structure and function. Moreover, raw material variability impacting protein glycosylation has been well-known and documented such that even replicating an expression process for the same recombinant protein and same cell line but different batch of cell culture media (or serum, if applicable) may lead to differing structure and functional activity. In the event of such raw material variability between two expression processes, a POSA would not expect the same residual activity for both proteins. Additionally, making a recombinant

Factor C protein that actually has ***any*** desired activity (i.e., ability to detect endotoxin) was known to be difficult in view of challenges, for example, with getting such proteins to be secreted by the host cells themselves. Ex. 1026, 360; Ex. 1027, 71. In view of this, making a recombinant Factor C protein with endotoxin detection function ***at or above a particular level*** was highly speculative.

EX1002 (Jain), ¶140

Wands Factors 1-3 – No Guidance or Examples

Petition at 40-42

Jain Declaration (EX1002)

144. As of 2013, the generation of recombinant Factor C proteins from a different species of crab and across a wide range of functional capabilities would have required literature review, the obtaining of sequences, the preparation of precursors, optimization of the experimental conditions, the obtaining and purifying of Factor C with various levels of sialic acid, and the verification of residual activity at lower, moderate, and higher values with then claimed "10% or higher" and "15% or higher" thresholds. However, the '767 Application makes no mention of what logic or protocol a POSA would use to generate recombinant Factor C proteins with the functional characteristics across the full scope of the alleged claimed invention (e.g., 90% or 95%).

Ex.1002 (Jain), ¶144.

145. In addition, the function of residual activity in claim 21, as explained through the '767 Application, is calculated based on measurement of the product of the full cascade reaction shown in FIG. 4. Ex. 1006, 52. Thus, in addition to providing no explanation of how to express recombinant *Limulus polyphemus* Factor C in HEK293 cells to achieve proteins with residual activity function across the full scope of the claimed range, the '767 Application provides no guidance whatsoever on how to express *Limulus polyphemus* Factor B and Pro-clotting enzyme to generate the cascade from which residual activity can be measured. Moreover, not only does the '767 Application not provide gene or amino acid sequence information for Factor B and Pro-clotting enzyme for *Limulus polyphemus* horseshoe crabs, it also provides no evidence or discussion about whether and how the claimed *Limulus polyphemus* Factor C would react with Factor B and Pro-clotting enzyme from *Tachypleus tridentatus* horseshoe crabs (produced in insect cells, as the '767 Application describes) to trigger the cascade reaction required to measure the claimed "residual activity." The inventors undoubtedly left it to the POSA to complete the invention defined by claim 21.

EX1002 (Jain), ¶145

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

United States Patent and Trademark Office

Before the Patent Trial and Appeal Board

Charles River Laboratories, Inc.,

(Petitioner)

v.

Seikagaku Corporation

(Patent Owner)

Petitioner's Opposition to Patent Owner's Revised Motion to Amend

IPR2025-00440 | **Patent No. 11,236,318**

June 9, 2026

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

New Claim 22 – Rearrangement of Claim 21

Opposition to RMTA at 1, 3

’318 Patent

21. 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.

EX1001, 76:50-61

PO’s Revised Contingent Motion to Amend

Claim 22. [Proposed substitute for claim 21] 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]] a residual activity in the presence of 21 mM sodium citrate that is:

(i) higher than the residual activity in the presence of 21 mM sodium citrate of a corresponding *Limulus polyphemus* Factor C protein expressed in Sf9 as a host cell, or

(ii) higher than the residual activity in the presence of 21 mM sodium citrate of a native *Limulus polyphemus* Factor C protein,

wherein the horseshoe crab Factor C protein is not a horseshoe crab Factor C protein having less than 10% residual activity in the presence of 21 mM sodium citrate; and

(2) [[15% or higher in the presence of 16 mM magnesium sulfate]] a residual activity in the presence of 16 mM magnesium sulfate that is:

(i) higher than the residual activity in the presence of 16 mM magnesium sulfate of a corresponding *Limulus polyphemus* Factor C protein expressed in Sf9 as a host cell, or

(ii) higher than the residual activity in the presence of 16 mM magnesium sulfate of a native *Limulus polyphemus* Factor C protein,

wherein the horseshoe crab Factor C protein is not a horseshoe crab Factor C protein having less than 15% residual activity in the presence of 16 mM magnesium sulfate.

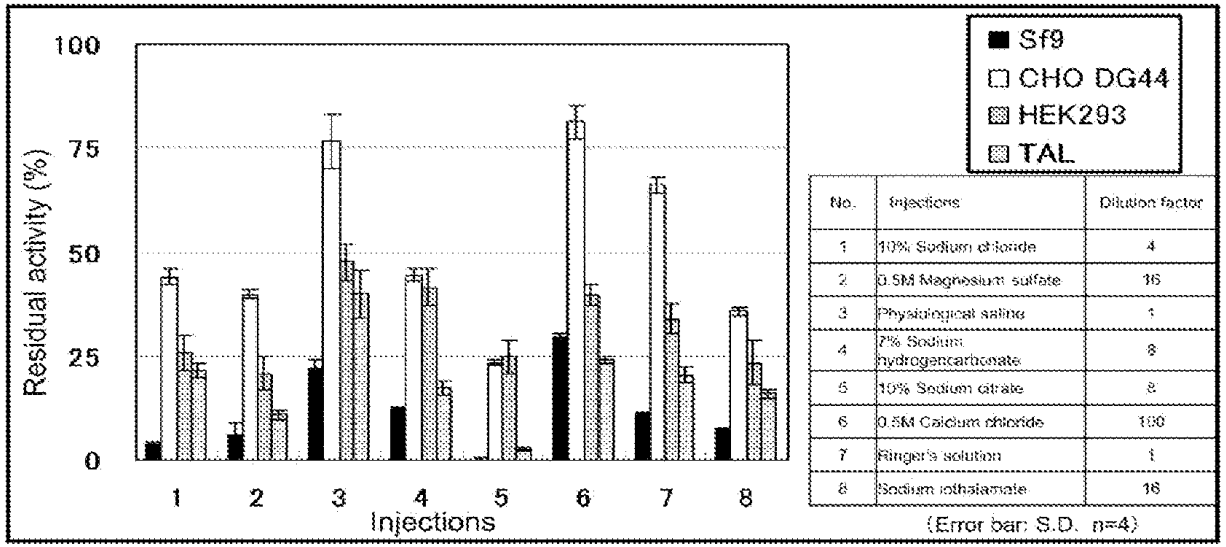
Patent Owner’s Revised Contingent Motion to Amend, Appx. A.

“Higher Than” Limitations Fail to Narrow

Opposition to RMTA at 3-4
Sur-Reply to RMTA at 5

’767 Application

[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 iothalamate	16

EX1006, 122

- For 16 mM magnesium sulfate – **both comparators lower than 15% threshold**
- For 21 mM sodium citrate – **both comparators lower than 10% threshold**
- No Factor C proteins that would be within scope of claim 21 but not claim 22

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Same Evidence Offered for Claim 22 as for Claim 21

POR

The '767 Application provides the requisite description such that a POSA would have understood the inventors had possession of the invention of claim 21 as of at least the 2013 filing date for the '767 Application.[2] Ex. 2038 ¶¶ 52-60.

First, in a section titled "***Modes for Carrying Out the Invention,***" the '767 Application states that the "Factor C of the present invention is a ***Factor C derived from a horseshoe crab***" and specifically identifies *Limulus polyphemus* as a known species. Ex. 1006 at 40.[3] Thus, the '767 Application identifies the specifically claimed *Limulus polyphemus* species as part of its invention.

Second, the specification includes a specific example titled "Expression of Factor C in mammalian cells (HEK 293)," which describes steps to produce Factor C using HEK293 cells as the host cell—the same host cell claim 21 recites. Ex. 1006 at 87.

Third, the '767 Application includes an example titled "Inhibition of activity by injection" that details measuring the residual activity of Factor C recombinantly expressed in HEK293 cells in the claimed salt solutions of 21 mM sodium citrate and 16 mM magnesium sulfate. Ex. 1006 at 96-99. The '767 Application not only discloses the procedure used for this measurement in Figure 4, but it also provides results in Figure 7, demonstrating that expression of Factor C in HEK293 cells resulted in the claimed residual activity of 10% or higher in the presence of 21 mM sodium citrate (column 5) and 15% or higher in the presence of 16 mM magnesium sulfate (column 2). Ex. 1006 at 96-99, 119.

Paper No. 26, 13-14

PO's Reply to Opposition to RMTA

The '767 Application provides the necessary disclosure. It includes a section titled "***Modes for Carrying Out the Invention***" that states the "Factor C of the present invention is a ***Factor C derived from a horseshoe crab***" and specifically identifies ***L. polyphemus as a known species***. Ex. 1006 at 40[2]. It includes a specific example titled "Expression of Factor C in mammalian cells (HEK293)," describing steps to produce Factor C in HEK293 cells. *Id.* at 87-88. It also states the "present invention encompasses the following modes" and then lists the following: (i) residual activity in the presence of 21 mM sodium citrate at a minimum of 10%, *id.* at 17, and (ii) residual activity in the presence of 16 mM magnesium sulfate at a minimum of 15%. *Id.* at 18.

The '767 Application also explains that the "Factor C of the present invention may have low susceptibility to inhibition of activity by an ion," noting that this "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***," identifying the control Factor C as including ***recombinant Factor C expressed in insect cell Sf9 and a native Factor C***. Ex. 1006 at 50. It provides specific examples of the invention, including (i) higher residual activity in the presence of 21 mM sodium citrate ***as compared with control Factor C***; (ii) higher residual activity in the presence of 16 mM magnesium sulfate ***as compared with control Factor C***; (iii) residual activity of ***10% or higher*** or 20% or higher in the presence of 21 mM sodium citrate; and (iv) residual activity of ***15% or higher*** or 25% or higher in the presence of 16 mM magnesium sulfate. *Id.* at 41-42 [0035]. The '767 Application

Paper No. 44, 2-3

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

United States Patent and Trademark Office

Before the Patent Trial and Appeal Board

Charles River Laboratories, Inc.,

(Petitioner)

v.

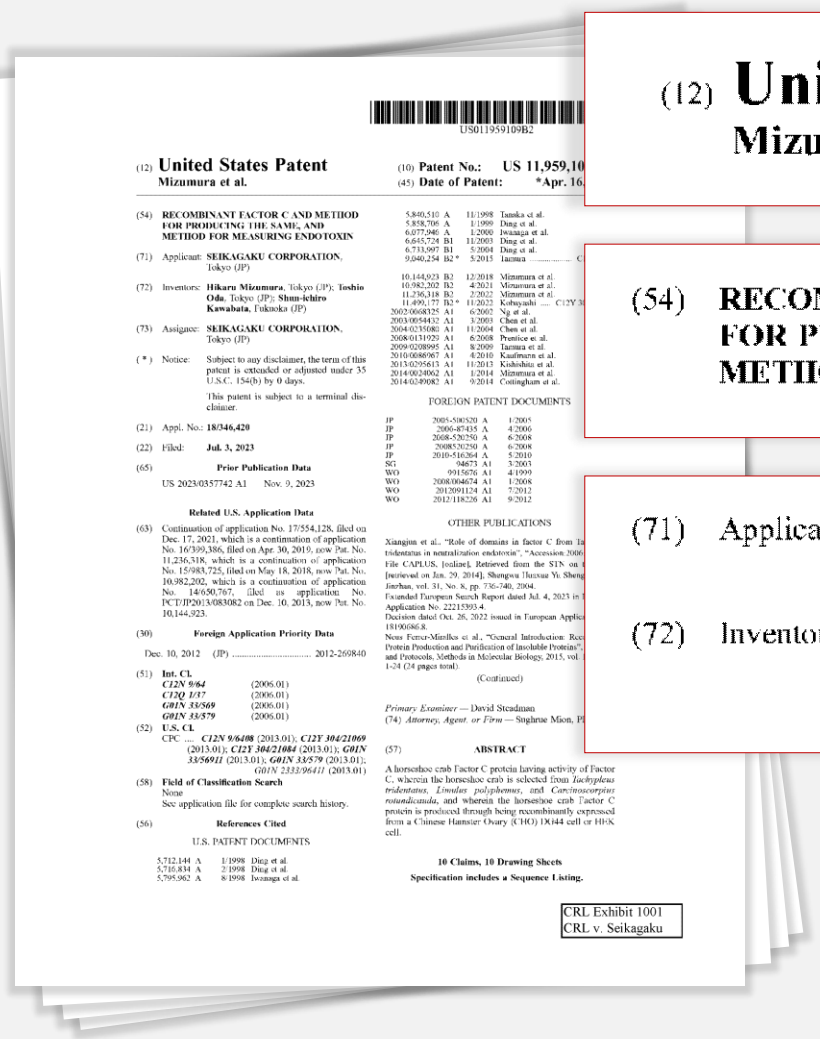
Seikagaku Corporation

(Patent Owner)

PGR2025-00023 | **Patent No. 11,959,109**

Challenged Patent – U.S. Patent No. 11,959,109

EX1001



US011959109B2

(12) **United States Patent**
Mizumura et al.

(10) **Patent No.: US 11,959,10**
(45) **Date of Patent: *Apr. 16,**

(54) **RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN**

(71) Applicant: **SEIKAGAKU CORPORATION**, Tokyo (JP)

(72) Inventors: **Hikaru Mizumura**, Tokyo (JP); **Toshio Oda**, Tokyo (JP); **Shun-ichiro Kawabata**, Fukuoka (JP)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **18/346,420**

(22) Filed: **Jul. 3, 2023**

(65) **Prior Publication Data**

US 2023/0357742 A1 Nov. 9, 2023

Related U.S. Application Data

(63) Continuation of application No. 17/554,128, filed on Dec. 17, 2021, which is a continuation of application No. 16/399,386, filed on Apr. 30, 2019, now Pat. No. 11,236,318, which is a continuation of application No. 15/983,725, filed on May 18, 2018, now Pat. No. 10,982,202, which is a continuation of application No. 14/650,767, filed as application No. PCT/JP2013/083082 on Dec. 10, 2013, now Pat. No. 10,144,923.

(30) **Foreign Application Priority Data**

Dec. 10, 2012 (JP) 2012-269840

(51) **Int. Cl.**
C12N 9/64 (2006.01)
C12Q 1/37 (2006.01)
G01N 33/569 (2006.01)
G01N 33/579 (2006.01)

(52) **U.S. Cl.**
CPC ***C12N 9/6408*** (2013.01); ***C12Y 304/21069*** (2013.01); ***C12Y 304/21084*** (2013.01); ***G01N 33/56911*** (2013.01); ***G01N 33/579*** (2013.01); *G01N 2333/96411* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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Primary Examiner — David Steadman
(74) *Attorney, Agent, or Firm* — Singhrae Mion, P...

(57) **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.

10 Claims, 10 Drawing Sheets
Specification includes a Sequence Listing.

CRL Exhibit 1001
CRL v. Seikagaku

(12) **United States Patent**
Mizumura et al.

(10) **Patent No.: US 11,959,109 B2**
(45) **Date of Patent: *Apr. 16, 2024**

(54) **RECOMBINANT FACTOR C AND METHOD FOR PRODUCING THE SAME, AND METHOD FOR MEASURING ENDOTOXIN**

(71) Applicant: **SEIKAGAKU CORPORATION**, Tokyo (JP)

(72) Inventors: **Hikaru Mizumura**, Tokyo (JP); **Toshio Oda**, Tokyo (JP); **Shun-ichiro Kawabata**, Fukuoka (JP)

EX1001

(21) Appl. No.: **18/346,420**

(22) Filed: **Jul. 3, 2023**

(65) **Prior Publication Data**

US 2023/0357742 A1 Nov. 9, 2023

Related U.S. Application Data

(63) Continuation of application No. 17/554,128, filed on Dec. 17, 2021, which is a continuation of application No. 16/399,386, filed on Apr. 30, 2019, now Pat. No. 11,236,318, which is a continuation of application No. 15/983,725, filed on May 18, 2018, now Pat. No. 10,982,202, which is a continuation of application No. 14/650,767, filed as application No. PCT/JP2013/083082 on Dec. 10, 2013, now Pat. No. 10,144,923.

(30) **Foreign Application Priority Data**

Dec. 10, 2012 (JP) 2012-269840

Overview of Grounds

Petition at 9-12

	Ground Number and Statute	Claims
1	35 U.S.C § 112 (written description) (AIA-law)	1-9
2	35 U.S.C § 112 (enablement) (AIA-law)	1-9
3	35 U.S.C § 102 (a)(1) (AIA-law)	1-9

No dispute that post-AIA law applies to patentability of claims 1-9. Pet., 9-11, n. 2 (explaining why challenged claims are PGR eligible)

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Challenged Patent – U.S. Patent No. 11,959,109

EX1001

’109 Patent

1. A method for producing an endotoxin assay agent comprising a horseshoe crab Factor C, a horseshoe crab Factor B, and a horseshoe crab Pro-clotting enzyme, the method comprising:

expressing the horseshoe crab Factor C in a Chinese Hamster Ovary cell or a Human Embryonic Kidney cell as a host cell, secreting the horseshoe crab Factor C into an extracellular culture supernatant, and recovering the horseshoe crab Factor C in the culture supernatant, wherein the recovered horseshoe Factor C has activity of Factor C, and wherein the horseshoe crab Factor C is a *Tachypleus tridentatus* Factor C or a *Limulus polyphemus* Factor C,

expressing the horseshoe crab Factor B having activity of Factor B in a mammalian cell as a host cell,

expressing the horseshoe crab Pro-clotting enzyme in a mammalian cell as a host cell, and

producing an endotoxin assay agent comprising the horseshoe crab Factor C, the horseshoe crab Factor B, and the horseshoe crab Pro-clotting enzyme,

wherein the host cell for expressing the horseshoe crab Factor C, the host cell for expressing the horseshoe crab Factor B, and the host cell for expressing the horseshoe crab Pro-clotting enzyme may be the same or different.

2. The method according to claim 1, wherein the mammalian cell expressing the Factor B is a Chinese Hamster Ovary cell or a Human Embryonic Kidney cell.

3. The method according to claim 1, wherein the mammalian cell expressing the Pro-clotting enzyme is a Chinese Hamster Ovary cell or a Human Embryonic Kidney cell.

4. The method according to claim 1, wherein the horseshoe crab for the Factor B and/or the horseshoe crab for the Pro-clotting enzyme is selected from the group consisting of *Tachypleus tridentatus* and *Limulus polyphemus*.

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

6. The method according to claim 1, wherein the horseshoe crab Factor C has a molecular weight of 115 kDa to 130 kDa, as measured through SDS-PAGE under a nonreducing condition.

7. The method according to claim 1, further comprising filtering the culture supernatant comprising the horseshoe crab Factor C.

8. The method according to claim 1, wherein the endotoxin assay agent is formulated as a solid, a liquid, or a gel.

9. The method according to claim 1, wherein the horseshoe crab Factor C, the horseshoe crab Factor B, and the horseshoe crab Pro-clotting enzyme are separately expressed in individually established host cells.

EX1001, 47:17-48:29

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Challenged Claim 1

EX1001

’109 Patent

1. A method for producing an endotoxin assay agent comprising a horseshoe crab Factor C, a horseshoe crab Factor B, and a horseshoe crab Pro-clotting enzyme, the method comprising:

expressing the horseshoe crab Factor C in a Chinese Hamster Ovary cell or a Human Embryonic Kidney cell as a host cell, secreting the horseshoe crab Factor C into an extracellular culture supernatant, and recovering the horseshoe crab Factor C in the culture supernatant, wherein the recovered horseshoe Factor C has activity of Factor C, and wherein the horseshoe crab Factor C is a *Tachypleus tridentatus* Factor C or a *Limulus polyphemus* Factor C,

expressing the horseshoe crab Factor B having activity of Factor B in a mammalian cell as a host cell,

expressing the horseshoe crab Pro-clotting enzyme in a mammalian cell as a host cell, and

producing an endotoxin assay agent comprising the horseshoe crab Factor C, the horseshoe crab Factor B, and the horseshoe crab Pro-clotting enzyme,

wherein the host cell for expressing the horseshoe crab Factor C, the host cell for expressing the horseshoe crab Factor B, and the host cell for expressing the horseshoe crab Pro-clotting enzyme may be the same or different.

EX1001, 47:17-40

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Challenged Claim 1

EX1001, Petition at 16

’109 Patent

1. A method for producing an endotoxin assay agent comprising a horseshoe crab Factor C, a horseshoe crab Factor B, and a horseshoe crab Pro-clotting enzyme, the method comprising:

expressing the horseshoe crab Factor C in a Chinese Hamster Ovary cell or a Human Embryonic Kidney cell as a host cell, secreting the horseshoe crab Factor C into an extracellular culture supernatant, and recovering the horseshoe crab Factor C in the culture supernatant, wherein the recovered horseshoe Factor C has activity of Factor C, and wherein the horseshoe crab Factor C is a *Tachypleus tridentatus* Factor C or a *Limulus polyphemus* Factor C,

expressing the horseshoe crab Factor B having activity of Factor B in a mammalian cell as a host cell,

expressing the horseshoe crab Pro-clotting enzyme in a mammalian cell as a host cell, and

producing an endotoxin assay agent comprising the horseshoe crab Factor C, the horseshoe crab Factor B, and the horseshoe crab Pro-clotting enzyme,

wherein the host cell for expressing the horseshoe crab Factor C, the host cell for expressing the horseshoe crab Factor B, and the host cell for expressing the horseshoe crab Pro-clotting enzyme may be the same or different.

EX1001, 47:17-40

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 11,959,109 B2
APPLICATION NO. : 18/346420
DATED : April 16, 2024
INVENTOR(S) : Hikaru Mizumura, Toshio Oda and Shun-ichiro Kawabata

Page 1 of 1

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

Page 2, Under item (56) OTHER PUBLICATIONS, left Column, Line 36, please delete “Gycobiol.” and insert --Glycobiol.-- therefor.

Page 3, Under item (56) OTHER PUBLICATIONS, left Column, Line 1, please delete “Ganssia” and insert --Gaussia-- therefor.

Page 3, Under item (56) OTHER PUBLICATIONS, right Column, Line 12, please delete “espress” and insert --express-- therefor.

In the Specification

Column 14, Line 53, please delete “(MAN)” and insert --(MAM)-- therefor.

Column 30, Line 17, please delete “Celfectin” and insert --Cellfectin-- therefor.

Column 38, Line 54, please delete “(MAN)” and insert --(MAM)-- therefor.

In the Claims

Claim 1, Line 32, please delete “Pro-clotting enzyme in a” and insert --Pro-clotting enzyme having activity of Pro-clotting enzyme in a-- therefor.

Claim 10, Line 43, please delete “Pro-clotting enzyme in a” and insert --Pro-clotting enzyme having activity of Pro-clotting enzyme in a-- therefor.

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

"Activity of"

Reply at 8-10

'767 Patent Application

[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.

[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.

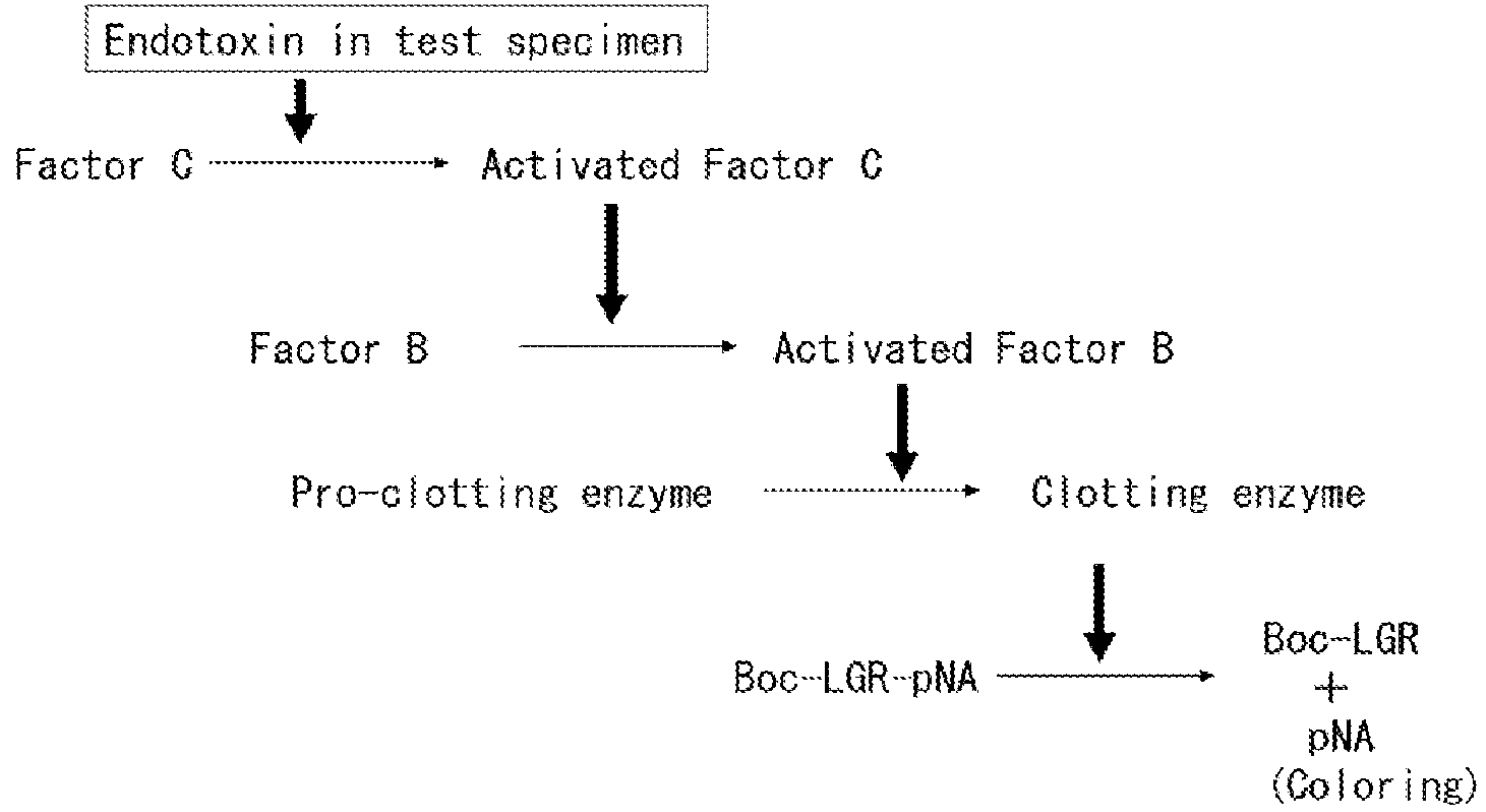
[0051]

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

EX1017, ¶¶ 28, 49, 51 (pp. 46, 59, 60)

’767 Application

[Fig. 1]



EX1017, 118, FIG. 1

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Challenged Claim 1

Petition at 29-33, Reply at 11-13

’109 Patent

1. A method for producing an endotoxin assay agent comprising a horseshoe crab Factor C, a horseshoe crab Factor B, and a horseshoe crab Pro-clotting enzyme, the method comprising:

expressing the horseshoe crab Factor C in a Chinese Hamster Ovary cell or a Human Embryonic Kidney cell as a host cell, secreting the horseshoe crab Factor C into an extracellular culture supernatant, and recovering the horseshoe crab Factor C in the culture supernatant, wherein the recovered horseshoe Factor C has activity of Factor C, and wherein the horseshoe crab Factor C is a *Tachypleus tridentatus* Factor C or a *Limulus polyphemus* Factor C,

expressing the horseshoe crab Factor B having activity of Factor B in a mammalian cell as a host cell,

expressing the horseshoe crab Pro-clotting enzyme in a mammalian cell as a host cell, and

producing an endotoxin assay agent comprising the horseshoe crab Factor C, the horseshoe crab Factor B, and the horseshoe crab Pro-clotting enzyme,

wherein the host cell for expressing the horseshoe crab Factor C, the host cell for expressing the horseshoe crab Factor B, and the host cell for expressing the horseshoe crab Pro-clotting enzyme may be the same or different.

Only recombinant Fc from *Tachypleus tridentatus*

No example

No example

No example

No example

EX1001, 47:17-40

’767 Application

’767 Patent Application

[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.

EX1017, ¶11 (p. 14)

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Prosecution of ’109 Patent

U.S. Application No. 18/346,420

Before the priority date of the persent application, it was widely known to those skilled in the art that if mammalian cells are used as a host, Factor C is not secreted extracellularly but remains intracellularly. Specifically, the inventors (Jeak Ling Ding; Bow Ho) of Ding-1 and Ding-2 stated as follows after filing Ding-1 and Ding-2.

"*The rFC generated from COS-1 cells was associated with the cellular debris.*" (page 360, right column, lines 4-5 of Roopashree et al, Biotechnology Letters Vol 19, No 4, 1997;

EX1002, 125

"*Earlier attempts in our lab to express rFC in E. coli, yeast and mammalian cells ... may not have met the requirements for Factor C folding and modification, thus rFCs expressed were sequestered inside these host cells, and only showed weak LPS-binding affinity.*" (emphasis added.) (page 76, left column, lines 8-14 of Wang et al., Biotechnology Letters 23, 71-76, 2001)

On the other hand, contrary to the above common knowledge, the inventors of the present application have discovered that Factor C is secreted extracellularly when CHO cell or HEK cell are used among mammalian cells, as shown in the Examples of the present application.

EX1002, 126

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

’498 PCT / ’629 Publication

Petition at 62, 65-68; Reply at 4, 6

’498 PCT (EX1005)

[0180] As a result, there was success for the first time in expressing and obtaining a protein from Limulus polyphemus that may have factor C activity (sequence number 2), a protein that may have factor B activity (sequence number 6), and a protein that may have pro-clotting enzyme activity (sequence number 14). The activity analysis of these proteins will be described later.

EX1005, ¶0180

U.S. Patent App. Pub. No. 2019/0241629

[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.

EX1015, ¶0355

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Jain Declaration (EX1003)

71. While claims 1 and 10 require that all three zymogen proteins are expressed in mammalian cells, the '767 Application provides no common structural feature present in all such proteins that would, for example, allow them to be combined in a functioning endotoxin detection assay in the scenario where each protein were expressed in a ***different*** mammalian cell and/or from ***different*** species of horseshoe crabs. Moreover, it was well understood by 2013 that the four existing species of horseshoe crabs fell into two distinct taxonomic families (Limulidae and Tachypleinae), with different physiological and genetic features based on geographical separation. Ex. 1038 (Sekiguchi), 6-7, 10-15 (discussing the discreteness of Family Limulidae and Family Tachypleinae). Given these known differences, a POSA would not have understood the '109 patent inventors to be in possession of a method of producing an endotoxin assay agent comprised of all three zymogen proteins expressed from different species of horseshoe crabs and certainly no group had reported the accomplishment of that feat by 2013.

EX1003, ¶71

72. Additionally, since protein function is defined by their structure, it may be expected that, for instance, activation of *Limulus polyphemus* Factor B by *Limulus polyphemus* Factor C has a different reaction kinetics from *Limulus polyphemus* Factor B activated by *Tachypleus tridentatus* Factor C which may be expected to have different reaction kinetics from *Tachypleus tridentatus* Factor B activated by *Tachypleus tridentatus* Factor C. This difference in reaction kinetics could require either an adjustment in the concentration of each protein, change in the sensitivity to endotoxin concentration or a different reaction duration that would require a POSA to (i) express the proteins in desired combination and (ii) evaluate and optimize the endotoxin agent assay, each of which could require significant experimentation. This is further highlighted by a low degree of sequence homology between *Tachypleus tridentatus* and *Limulus polyphemus* Factor B and Pro-clotting Enzymes that have 84% for both proteins (using sequences from the '109 patent and '629 Publication, respectively).

EX1003, ¶72

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Dr. Siddhartha Jain, Ph.D., PMP

Petition at 42-43

Jain Declaration (EX1003)

89. Likewise, while recombinant technology was not new as of 2013, a significant amount of research and experimentation was still required to generate a particular protein, in a functional state, across a number of different expression systems, through screening of expression systems and optimization of cell culture conditions (including cell culture media composition). Ex. 1031, 15-27 ("Even when hundreds of proteins are produced at commercial scale, the production of recombinant proteins still constitutes a challenge in many cases… A necessary condition for adequate recombinant protein production is the efficient expression of the gene of interest. However, expression can be lost due to structural changes in the recombinant gene or disappearance of the gene from host cells."); Ex. 1030, Abstract ("PTMs directly influence the 2-DE pattern, and knowledge of the rules

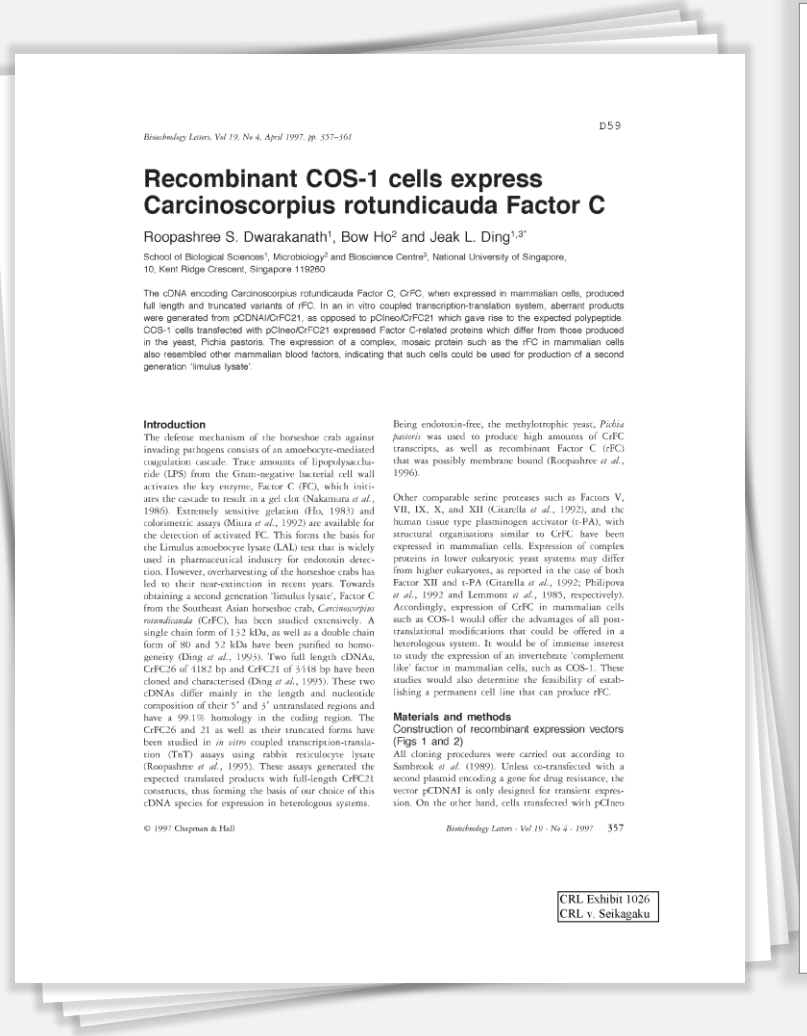
of protein separation is required in order to understand the protein distribution in a 2-DE gel. Glycosylation is the most common PTM and can modify both the molecular weight and the pI of a protein. In particular, the effect of charged monosaccharides (mainly sialic acids, SAs) on the 2-DE pattern of a protein is of major interest since changes in sialylation are regularly observed in comparative studies. Little is known about the pI shift of a glycoprotein induced by the presence of SAs, or whether this shift is the same for all glycoproteins."). Ex. 1039, 936 ("There have been numerous reports on the effects of cell type, culture process, and culture media toward the resulting glycoform profile.")

EX1003, ¶89

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Dwarakanath et al.

Dwarakanath et al. (1997)



Biotechnology Letters, Vol 19, No 4, April 1997, pp. 357–361

Recombinant COS-1 cells express Carcinoscorpius rotundicauda Factor C

Roopashree S. Dwarakanath[1], Bow Ho[2] and Jeak L. Ding[1,3*]

School of Biological Sciences[1], Microbiology[2] and Bioscience Centre[3], National University of Singapore, 10, Kent Ridge Crescent, Singapore 119260

The cDNA encoding Carcinoscorpius rotundicauda Factor C, CrFC, when expressed in mammalian cells, produced full length and truncated variants of rFC. In an in vitro coupled transcription-translation system, aberrant products were generated from pCDNAI/CrFC21, as opposed to pCIneo/CrFC21 which gave rise to the expected polypeptide. COS-1 cells transfected with pCIneo/CrFC21 expressed Factor C-related proteins which differ from those produced in the yeast, Pichia pastoris. The expression of a complex, mosaic protein such as the rFC in mammalian cells also resembled other mammalian blood factors, indicating that such cells could be used for production of a second generation 'limulus lysate'.

Introduction

The defense mechanism of the horseshoe crab against invading pathogens consists of an amoebocyte-mediated coagulation cascade. Trace amounts of lipopolysaccharide (LPS) from the Gram-negative bacterial cell wall activates the key enzyme, Factor C (FC), which initiates the cascade to result in a gel clot (Nakamura *et al.*, 1986). Extremely sensitive gelation (Ho, 1983) and colorimetric assays (Miura *et al.*, 1992) are available for the detection of activated FC. This forms the basis for the Limulus amoebocyte lysate (LAL) test that is widely used in pharmaceutical industry for endotoxin detection. However, overharvesting of the horseshoe crabs has led to their near-extinction in recent years. Towards obtaining a second generation 'limulus lysate', Factor C from the Southeast Asian horseshoe crab, *Carcinoscorpius rotundicauda* (CrFC), has been studied extensively. A single chain form of 132 kDa, as well as a double chain form of 80 and 52 kDa have been purified to homogeneity (Ding *et al.*, 1995). Two full length cDNAs, CrFC26 of 4182 bp and CrFC21 of 3448 bp have been cloned and characterised (Ding *et al.*, 1995). These two cDNAs differ mainly in the length and nucleotide composition of their 5′ and 3′ untranslated regions and have a 99.1% homology in the coding region. The CrFC26 and 21 as well as their truncated forms have been studied in *in vitro* coupled transcripiton-translation (TnT) assays using rabbit reticulocyte lysate (Roopashree *et al.*, 1995). These assays generated the expected translated products with full-length CrFC21 constructs, thus forming the basis of our choice of this cDNA species for expression in heterologous systems.

Being endotoxin-free, the methylotrophic yeast, *Pichia pastoris* was used to produce high amounts of CrFC transcripts, as well as recombinant Factor C (rFC) that was possibly membrane bound (Roopashree *et al.*, 1996).

Other comparable serine proteases such as Factors V, VII, IX, X, and XII (Citarella *et al.*, 1992), and the human tissue type plasminogen activator (t-PA), with structural organisations similar to CrFC have been expressed in mammalian cells. Expression of complex proteins in lower eukaryotic yeast systems may differ from higher eukaryotes, as reported in the case of both Factor XII and t-PA (Citarella *et al.*, 1992; Philipova *et al.*, 1992 and Lemmont *et al.*, 1985, respectively). Accordingly, expression of CrFC in mammalian cells such as COS-1 would offer the advantages of all post-translational modifications that could be offered in a heterologous system. It would be of immense interest to study the expression of an invertebrate 'complement like' factor in mammalian cells, such as COS-1. These studies would also determine the feasibility of establishing a permanent cell line that can produce rFC.

Materials and methods

Construction of recombinant expression vectors (Figs 1 and 2)

All cloning procedures were carried out according to Sambrook *et al.* (1989). Unless co-transfected with a second plasmid encoding a gene for drug resistance, the vector pCDNAI is only designed for transient expression. On the other hand, cells transfected with pCIneo

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CRL v. Seikagaku

pCIneo/CrFC21-transfected COS-1 cells expressed full length and truncated variants of rFC

COS-1 cells were transfected with recombinant vectors pCIneo/CrFC21, pCIneo/CrFC213′-5′ (reverse orientation of insert) and pCDNAI/CrFC21. Transfectants were analysed for expression of rFC at 48 and 72 h post transfection. Cells transfected with pCDNAI/CrFC21 failed to generate rFC. This observation concurred with the earlier TnT assay which generated a major aberrant product of 32 kDa with this construct (Fig. 3).

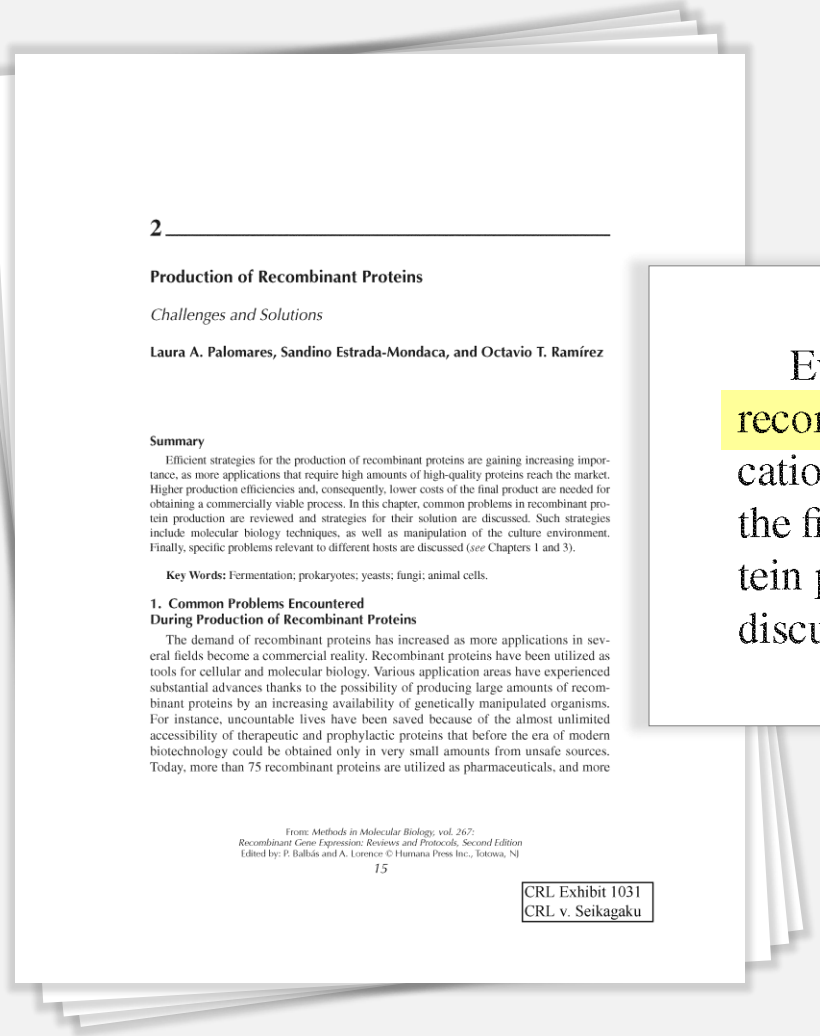
EX1026, 359

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Palomares et al.

Petition at 43-44

Palomares et al. 2004



2

Production of Recombinant Proteins

Challenges and Solutions

Laura A. Palomares, Sandino Estrada-Mondaca, and Octavio T. Ramírez

Summary

Efficient strategies for the production of recombinant proteins are gaining increasing importance, as more applications that require high amounts of high-quality proteins reach the market. Higher production efficiencies and, consequently, lower costs of the final product are needed for obtaining a commercially viable process. In this chapter, common problems in recombinant protein production are reviewed and strategies for their solution are discussed. Such strategies include molecular biology techniques, as well as manipulation of the culture environment. Finally, specific problems relevant to different hosts are discussed (*see* Chapters 1 and 3).

Key Words: Fermentation; prokaryotes; yeasts; fungi; animal cells.

1. Common Problems Encountered During Production of Recombinant Proteins

The demand of recombinant proteins has increased as more applications in several fields become a commercial reality. Recombinant proteins have been utilized as tools for cellular and molecular biology. Various application areas have experienced substantial advances thanks to the possibility of producing large amounts of recombinant proteins by an increasing availability of genetically manipulated organisms. For instance, uncountable lives have been saved because of the almost unlimited accessibility of therapeutic and prophylactic proteins that before the era of modern biotechnology could be obtained only in very small amounts from unsafe sources. Today, more than 75 recombinant proteins are utilized as pharmaceuticals, and more

From: *Methods in Molecular Biology, vol. 267: Recombinant Gene Expression: Reviews and Protocols, Second Edition*
Edited by: P. Balbás and A. Lorence © Humana Press Inc., Totowa, NJ

CRL Exhibit 1031
CRL v. Seikagaku

Even when hundreds of proteins are produced at commercial scale, the production of recombinant proteins still constitutes a challenge in many cases. Moreover, many applications would benefit with higher production efficiencies and consequent lower costs of the final product. In this chapter, typical problems encountered during recombinant protein production are reviewed and strategies to solve them and increase productivity are discussed.

EX1031, 16

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Hossler et al.

Hossler et al. 2009

Glycobiology vol. 19 no. 9 pp. 936–949, 2009
doi:10.1093/glycob/cwp079
Advance Access publication on June 3, 2009

REVIEW

Optimal and consistent protein glycosylation in mammalian cell culture

Patrick Hossler[1,4,2], Sarwat F Khattak[3,4], and Zheng Jian Li[1,4]

[4]Process Sciences Upstream, Technical Operations, Bristol-Myers Squibb Company, East Syracuse, NY 13057, USA

Received on April 6, 2009; revised on June 1, 2009; accepted on June 1, 2009

In the biopharmaceutical industry, mammalian cell culture systems, especially Chinese hamster ovary (CHO) cells, are predominantly used for the production of therapeutic glycoproteins. Glycosylation is a critical protein quality attribute that can modulate the efficacy of a commercial therapeutic glycoprotein. Obtaining a consistent glycoform profile in production is desired due to regulatory concerns because a molecule can be defined by its carbohydrate structures. An optimal profile may involve a spectrum of product glycans that confers a desired therapeutic efficacy, or a homogeneous glycoform profile that can be systemically screened for. Studies have shown some degree of protein glycosylation control in mammalian cell culture, through cellular, media, and process effects. Studies upon our own bioprocesses to produce fusion proteins and monoclonal antibodies have shown an intricate relationship between these variables and the resulting protein quality. Glycosylation optimization will improve therapeutic efficacy and is an ongoing goal for researchers in academia and industry alike. This review will focus on the advancements made in glycosylation control in a manufacturing process, as well as the next steps in understanding and controlling protein glycosylation.

Keywords: bioprocessing/mammalian cell culture/protein glycosylation/protein therapeutics/protein quality

Introduction

Protein glycosylation is of paramount importance to the efficacy and manufacturing of therapeutic glycoproteins. Mammalian cell expression systems are the preferred method for the commercial production of these glycoproteins because their innate protein processing machinery, including that of protein glycosylation, closely resembles that in human.

Glycosylation of proteins takes on the form of oligosaccharides attached to either the side chain of asparagine (*N*-linked) or serine/threonine (*O*-linked) with the former being the most prominent (Warren 1993; Helenius and Aebi 2001; Sinclair and Elliott 2005). Glycans have a very prominent role toward affecting therapeutic efficacy and determining the in vivo half-life (Elliott et al. 2003). It is due to both of these features that the glycoform profile of a therapeutic glycoprotein must be extensively characterized in order to meet regulatory agency demands (FDA 1996).

In this review, we summarize the salient features of protein glycosylation control from a bioprocess perspective. Through our own process development and characterization efforts with fusion proteins such as Orencia[TM] and Belatacept and monoclonal antibody Ipilimumab, we have gained an appreciation over the past, present, and future state of the control of this very important metabolic pathway. Table I lists glycosylated therapeutics currently on the market, which is the impetus for understanding their metabolic control. Although CHO cells are the most prevalent for producing glycoprotein therapeutics, other cell lines such as human embryonic kidney cells (HEK) (Durocher et al. 2007), baby hamster kidney (BHK), mouse myeloma (NS0), and human retinal cells (PERC.6) (Jones et al. 2003; Wurm 2004; Petricciani and Sheets 2008) are being developed as high producing cell lines.

There have been numerous reports on the effects of cell type, culture process, and culture media toward the resulting glycoform profile. This review will summarize these efforts, as well as new attempts toward achieving uniform, consistent, and/or optimal glycosylation. These efforts share a common need for a more fundamental understanding of the metabolic pathway, which is the next logical progression from the current paradigm of glycosylation maintenance. The ultimate goal would be to take the lessons learned from previous studies, combined with new technologies, to transform a well-characterized cell line toward one that can control the glycosylation profile. In order to truly incorporate process quality by design (QbD) into the final glycoprotein product (Rathore and Winkle 2009), more fundamental studies on the dependence of glycosylation on process conditions and cellular physiology will be required to gain physiological insight. The information gathered from such research together with clinical studies will continue to enhance our capability toward generating highly efficacious glycoprotein drugs.

Protein glycosylation and product quality

Metabolic pathway

The addition of oligosaccharides onto a glycoprotein is a complex metabolic pathway, characterized by the en bloc transfer of polysaccharide chains, as well as the step-wise addition and removal of individual monosaccharides. The number of pathways traversed is dependent on reaction site accessibility, the

[1]To whom correspondence should be addressed: Tel: +1-315-432-2131; e-mail: zhengjian.li@bms.com
[2]Present address: Abbott Bioresearch Center, Abbot Laboratories, Worcester, MA 01605, USA.
[3]These authors contributed equally to this work.

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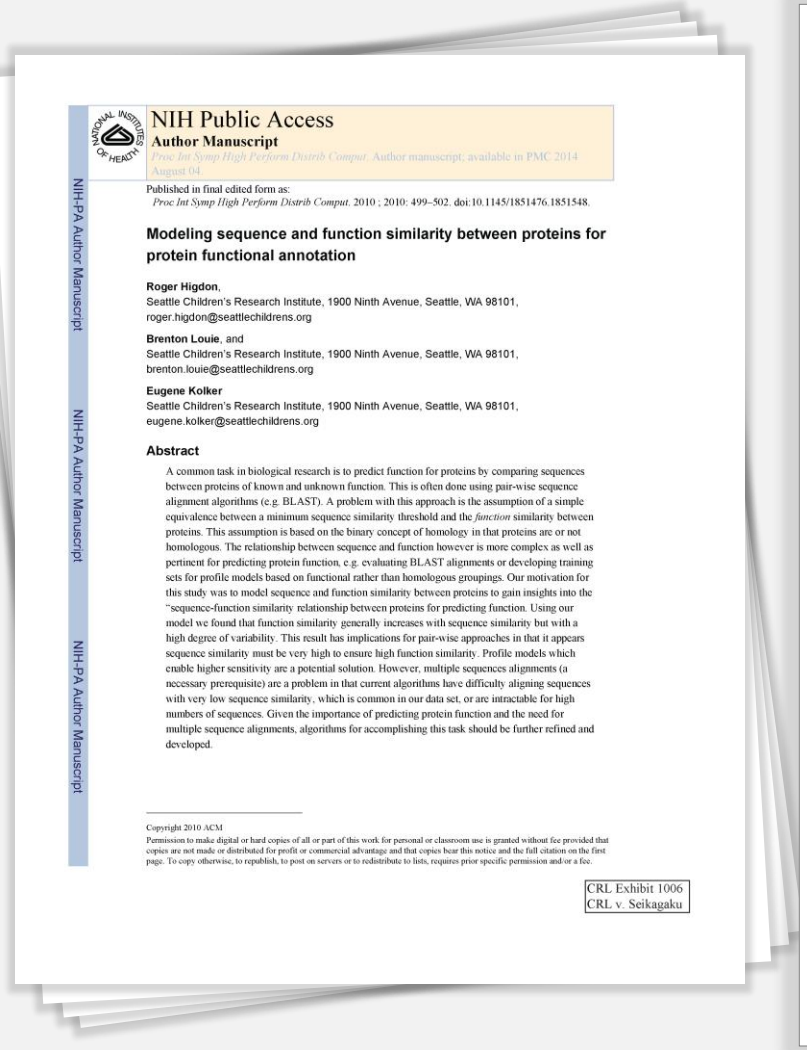
CRL Exhibit 1039
CRL v. Seikagaku

There have been numerous reports on the effects of cell type, culture process, and culture media toward the resulting glycoform profile. This review will summarize these efforts, as well as new attempts toward achieving uniform, consistent, and/or optimal glycosylation. These efforts share a common need for a more fundamental understanding of the metabolic pathway, which is the next logical progression from the current paradigm of glycosylation maintenance. The ultimate goal would be to take the lessons learned from previous studies, combined with new technologies, to transform a well-characterized cell line toward one that can control the glycosylation profile. In order to truly incorporate process quality by design (QbD) into the final glycoprotein product (Rathore and Winkle 2009), more fundamental studies on the dependence of glycosylation on process conditions and cellular physiology will be required to gain physiological insight. The information gathered from such research

EX1039, 936

Higdon et al.

Higdon et al. 2010



NIH Public Access
Author Manuscript
Proc Int Symp High Perform Distrib Comput. Author manuscript; available in PMC 2014 August 04.

Published in final edited form as:
Proc Int Symp High Perform Distrib Comput. 2010 ; 2010: 499–502. doi:10.1145/1851476.1851548.

Modeling sequence and function similarity between proteins for protein functional annotation

Roger Higdon,
Seattle Children's Research Institute, 1900 Ninth Avenue, Seattle, WA 98101, roger.higdon@seattlechildrens.org

Brenton Louie, and
Seattle Children's Research Institute, 1900 Ninth Avenue, Seattle, WA 98101, brenton.louie@seattlechildrens.org

Eugene Kolker
Seattle Children's Research Institute, 1900 Ninth Avenue, Seattle, WA 98101, eugene.kolker@seattlechildrens.org

Abstract

A common task in biological research is to predict function for proteins by comparing sequences between proteins of known and unknown function. This is often done using pair-wise sequence alignment algorithms (e.g. BLAST). A problem with this approach is the assumption of a simple equivalence between a minimum sequence similarity threshold and the *function* similarity between proteins. This assumption is based on the binary concept of homology in that proteins are or not homologous. The relationship between sequence and function however is more complex as well as pertinent for predicting protein function, e.g. evaluating BLAST alignments or developing training sets for profile models based on functional rather than homologous groupings. Our motivation for this study was to model sequence and function similarity between proteins to gain insights into the "sequence-function similarity relationship between proteins for predicting function. Using our model we found that function similarity generally increases with sequence similarity but with a high degree of variability. This result has implications for pair-wise approaches in that it appears sequence similarity must be very high to ensure high function similarity. Profile models which enable higher sensitivity are a potential solution. However, multiple sequences alignments (a necessary prerequisite) are a problem in that current algorithms have difficulty aligning sequences with very low sequence similarity, which is common in our data set, or are intractable for high numbers of sequences. Given the importance of predicting protein function and the need for multiple sequence alignments, algorithms for accomplishing this task should be further refined and developed.

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CRL Exhibit 1006
CRL v. Seikagaku

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Abstract

A common task in biological research is to predict function for proteins by comparing sequences between proteins of known and unknown function. This is often done using pair-wise sequence alignment algorithms (e.g. BLAST). A problem with this approach is the assumption of a simple equivalence between a minimum sequence similarity threshold and the *function* similarity between proteins. This assumption is based on the binary concept of homology in that proteins are or not homologous. The relationship between sequence and function however is more complex as well as pertinent for predicting protein function, e.g. evaluating BLAST alignments or developing training sets for profile models based on functional rather than homologous groupings. Our motivation for this study was to model sequence and function similarity between proteins to gain insights into the "sequence-function similarity relationship between proteins for predicting function. Using our model we found that function similarity generally increases with sequence similarity but with a high degree of variability. This result has implications for pair-wise approaches in that it appears sequence similarity must be very high to ensure high function similarity. Profile models which enable higher sensitivity are a potential solution. However, multiple sequences alignments (a necessary prerequisite) are a problem in that current algorithms have difficulty aligning sequences with very low sequence similarity, which is common in our data set, or are intractable for high numbers of sequences. Given the importance of predicting protein function and the need for multiple sequence alignments, algorithms for accomplishing this task should be further refined and developed.

EX1006, 1

Demain et al.

Demain et al. 2009

Biotechnology Advances 27 (2009) 297–306

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Production of recombinant proteins by microbes and higher organisms

Arnold L. Demain [a,*], Preeti Vaishnav [b]

[a] Research Institute for Scientists Emeriti (R.I.S.E.), Drew University, Madison, NJ 07940, USA
[b] 206 Akshardeep Apts., Near New Jain Temple, GIDC, Ankleshwar 393002, Gujarat, India

ARTICLE INFO

Article history:
Received 26 September 2008
Received in revised form 14 January 2009
Accepted 21 January 2009
Available online 31 January 2009

Keywords:
recombinant proteins
enzymes
bacteria
yeasts
filamentous fungi
insect cells
mammalian cells
transgenic animals
transgenic plants

ABSTRACT

Large proteins are usually expressed in a eukaryotic system while smaller ones are expressed in prokaryotic systems. For proteins that require glycosylation, mammalian cells, fungi or the baculovirus system is chosen. The least expensive, easiest and quickest expression of proteins can be carried out in *Escherichia coli*. However, this bacterium cannot express very large proteins. Also, for S–S rich proteins, and proteins that require post-translational modifications, *E. coli* is not the system of choice. The two most utilized yeasts are *Saccharomyces cerevisiae* and *Pichia pastoris*. Yeasts can produce high yields of proteins at low cost, proteins larger than 50 kD can be produced, signal sequences can be removed, and glycosylation can be carried out. The baculoviral system can carry out more complex post-translational modifications of proteins. The most popular system for producing recombinant mammalian glycosylated proteins is that of mammalian cells. Genetically modified animals secrete recombinant proteins in their milk, blood or urine. Similarly, transgenic plants such as *Arabidopsis thaliana* and others can generate many recombinant proteins.

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1. Introduction

Proteins, the building blocks of life, are synthesized by all living forms as part of their natural metabolism. Some proteins, such as enzymes, serve as biocatalysts and increase the rate of metabolic reactions, while others form the cytoskeleton. Proteins play a significant role in cell signaling, immune responses, cell adhesion,

* Corresponding author. Drew University, R.I.S.E., HS-330, Madison, NJ 007940, USA. Tel.: +1 973 408 3937; fax: +1 973 408 3504.
E-mail address: ademain@drew.edu (A.L. Demain).

0734-9750/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.biotechadv.2009.01.008

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Mammalian systems do have some drawbacks as follows. (i) Poor secretion. Production of secreted foreign proteins by mammalian cells in the 1990s amounted to 1 to 10 mg/L with specific productivities of 0.1 to 1 pg/cell/day (Wurm and Bernard, 1999). The process duration was 5 to 10 days. Although higher titers have been reached, acceptable levels were 10–20 mg/L. (ii) Mammalian processes are expensive. The selling prices (per gram) of recombinant proteins were $375 for human insulin, $23,000 for tPA, $35,000 for human growth hormone, $384,000 for GM-CSF, $450,000 for G-CSF, and $840,000 for EPO. All

EX2110, 7

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Dr. Siddhartha Jain's Qualifications

Reply at 7-8, EX1003

- Extensive work in biological engineering and development of industrial scale processes for protein production inherently requiring strong technical expertise in biochemistry and molecular biology.
- Over 18 years of experience in expressing and characterizing recombinant proteins with significant experience in different formats of endotoxin assays.

EX1003, ¶2 and EX1065, 55:22-56:1, 57:6-8, 58:11-13

- Investigated the use of mammalian cells for recombinant protein expression and experienced firsthand the "challenges of…consistently producing cellulated [sialylated] proteins at certain specifications"

EX1065, 59:2-4; EX1003

- In the course of developing recombinant protein drugs, Dr. Jain has studied the function of homologous proteins across different species and also worked with others to understand the molecular underpinnings of differences in protein activity at the sequence level.

EX1065, 49:20-50:9, 50:12-19

- Not only has Dr. Jain's education in Biochemical Engineering and Bioengineering trained him on the concepts of enzymology but he has also worked on manufacturing enzymes.

EX1065, 49:4-6, 63:14-20

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE