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(54) **PRO-CLOTTING ENZYME, AND METHOD FOR DETECTION OF ENDOTOXIN OR (1-3)-BETA-D-GLUCAN USING THE SAME**

(75) Inventors: **Hiroshi Tamura**, Tokyo (JP); **Shoji Takahashi**, Tokyo (JP)

Correspondence Address:
SUGHRUE MION, PLLC
2100 PENNSYLVANIA AVENUE, N.W., SUITE 800
WASHINGTON, DC 20037 (US)

(73) Assignee: **SEIKAGAKU CORPORATION**, Chiyoda-ku, Tokyo (JP)

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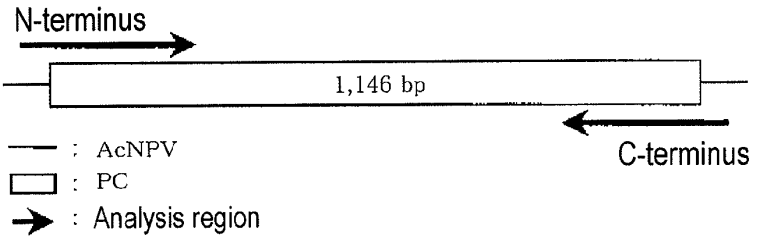
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(57) **ABSTRACT**

Objects of the present invention are to provide a DNA fragment encoding a *limulus*-derived pro-clotting enzyme, a virus harboring the DNA fragment, a cell harboring the virus, a method of producing the pro-clotting enzyme by use of the cell, and means for assaying an endotoxin or (1→3)-β-D-glucan employing the enzyme, wherein these elements are capable of producing an endotoxin or (1→3)-β-D-glucan assay reagent of satisfactory quality, steadily, at low cost, and on a large scale. In the present invention, for example, a DNA fragment encoding a protein having an amino acid sequence defined by SEQ ID NO: 4 is selected as a nucleic acid fragment encoding a *limulus*-derived pro-clotting enzyme, and the corresponding recombinant pro-clotting enzyme. Use of the enzyme can provide a high-sensitivity method and kit for detecting (1→3)-β-D-glucan and an endotoxin, utilizing a cascade reaction system in a horseshoe crab lysate.

[Fig. 1]



· N-terminus

```
TAATCGCGAT CTAGAATGTT GGTGAATAAC GTGTTTTCAC TACTGTGTTT CCCACTCTTG
           ***** ********** ********** ********** **********
           ATGTT GGTGAATAAC GTGTTTTCAC TACTGTGTTT CCCACTCTTG

ATGTCTGTGG TTAGATGCAG TACTCTCAGC AGACAGCGTA GACAGTTTGT TTTCCCTGAC
********** ********** ********** ********** ********** **********
ATGTCTGTGG TTAGATGCAG TACTCTCAGC AGACAGCGTA GACAGTTTGT TTTCCCTGAC

GAGGAAGAAC TTTGCTCAAA CCGATTTACT GAAGAAGGAA CATGCAAAAA TGTCTTGGAT
********** ********** ********** ********** ********** **********
GAGGAAGAAC TTTGCTCAAA CCGATTTACT GAAGAAGGAA CATGCAAAAA TGTCTTGGAT

TGTAGAATAC TTTTACAAAA AAATGATTAT AATTTACTCA AAGAATCAAT ATGCGGCTTT
********** ********** ********** ********** ********** **********
TGTAGAATAC TTTTACAAAA AAATGATTAT AATTTACTCA AAGAATCAAT ATGCGGCTTT

GAAGGCATAA CACCCAAAGT TTGTTGTCCG AAATCAAGCC ATGTAATTTC AAGTACACAG
********** ********** ********** ********** ********** **********
GAAGGCATAA CACCCAAAGT TTGTTGTCCG AAATCAAGCC ATGTAATTTC AAGTACACAG
```

· C-terminus

```
CNCNTNACAA GCGGTCTTAT TATTAAATCT AGTGATGGTG ATGGTGATGC ACCATATGTT
                              ** ********** ********** **********
                              CT AGTGATGGTG ATGGTGATGC ACCATATGTT

CTGCAATCCA ATCTAAAAAC TCTGTCACTT TTGTGTAAAC CCCAGGAAAT CCAGGCAATG
********** ********** ********** ********** ********** **********
CTGCAATCCA ATCTAAAAAC TCTGTCACTT TTGTGTAAAC CCCAGGAAAT CCAGGCAATG

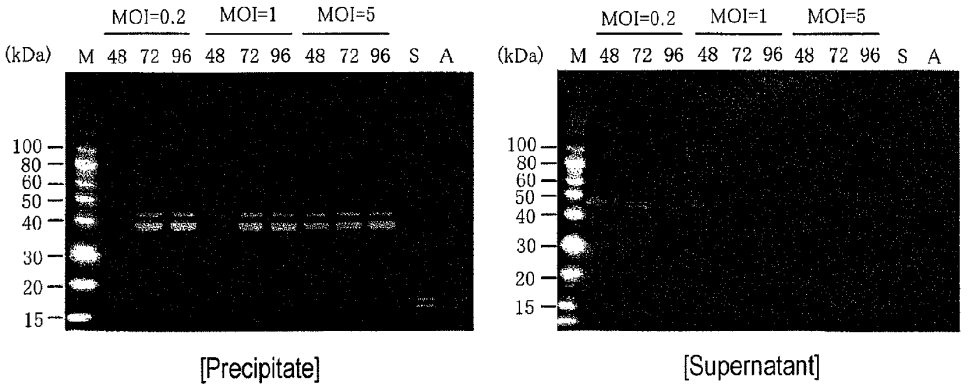
CGCATTTCTT TCCGAAAGAC ACAATTCCAA TGAGATAAAA CTCTCCGGTT TTAACAGGCA
********** ********** ********** ********** ********** **********
CGCATTTCTT TCCGAAAGAC ACAATTCCAA TGAGATAAAA CTCTCCGGTT TTAACAGGCA

ACATCATTGG ACCTCCAGAA TCACCCTGGC AAGCATCCTT CCCGCCATCT GCAAAGCCAG
********** ********** ********** ********** ********** **********
ACATCATTGG ACCTCCAGAA TCACCCTGGC AAGCATCCTT CCCGCCATCT GCAAAGCCAG

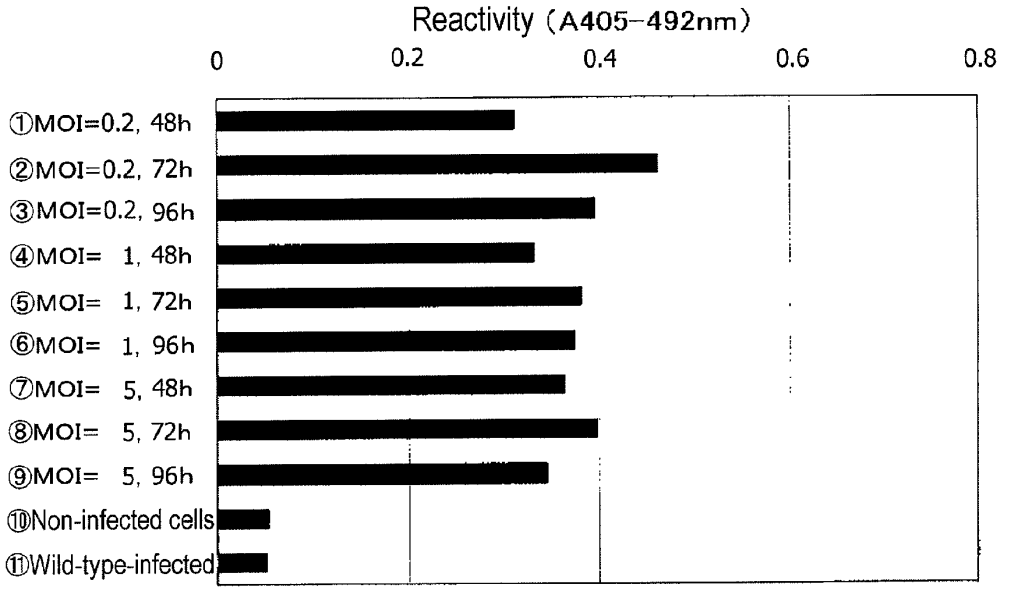
CACACATATA CACGTTTGTA ATATTTAAAT CCTTCTCGTA GGCCTGTCTA CAGGCCTCGT
********** ********** ********** ********** ********** **********
CACACATATA CACGTTTGTA ATATTTAAAT CCTTCTCGTA GGCCTGTCTA CAGGCCTCGT

GTTCCCATAT TGGTAACTGT ACTTCTCTCA ACACTGCACT AGATGGGCCG TTAAATGCTG
********** ********** ********** ********** ********** **********
GTTCCCATAT TGGTAACTGT ACTTCTCTCA ACACTGCACT AGATGGGCCG TTAAATGCTG
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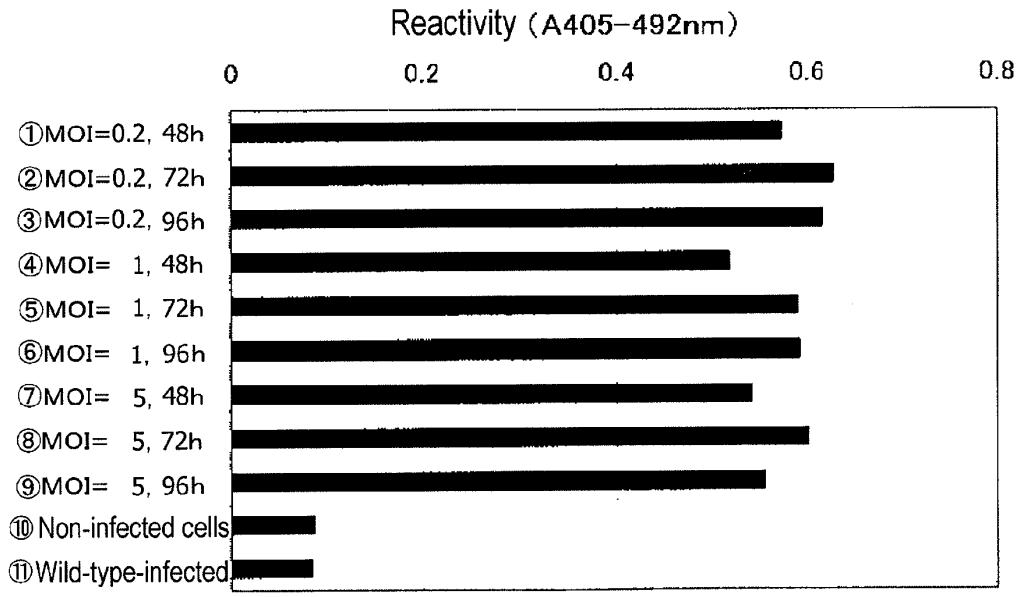
[Fig. 2]


MOI=0.2
MOI=1
MOI=5
(kDa)
M 48 72 96 48 72 96 48 72 96 S A
100
80
60
50
40
30
20
15
[Precipitate]
[Supernatant]

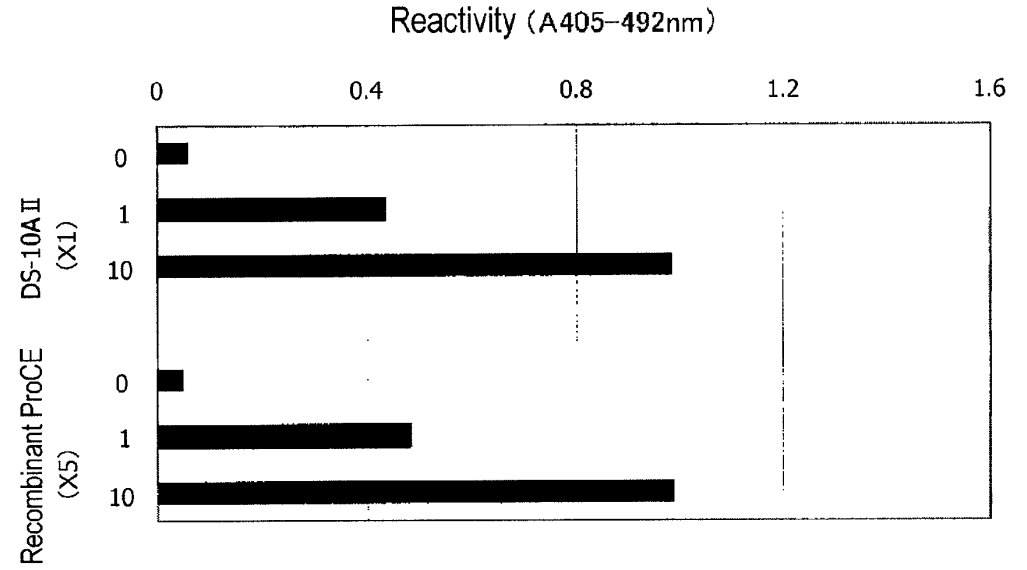
[Fig. 3]


Reactivity (A405-492nm)
0
0.2
0.4
0.6
0.8
①MOI=0.2, 48h
②MOI=0.2, 72h
③MOI=0.2, 96h
④MOI= 1, 48h
⑤MOI= 1, 72h
⑥MOI= 1, 96h
⑦MOI= 5, 48h
⑧MOI= 5, 72h
⑨MOI= 5, 96h
⑩Non-infected cells
⑪Wild-type-infected

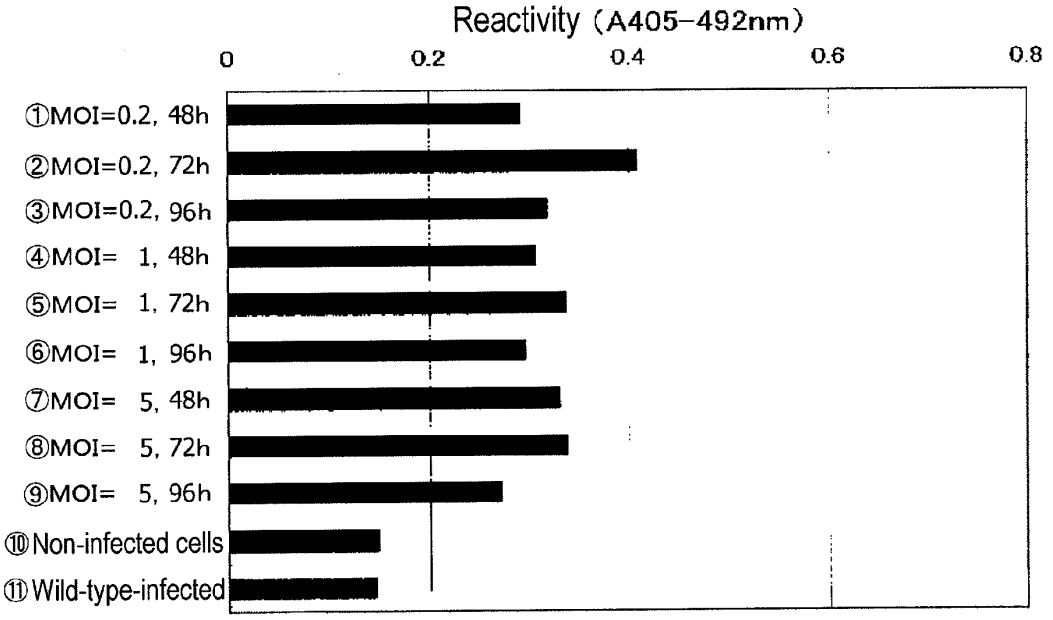
[Fig. 4]


Reactivity (A405-492nm)
0
0.2
0.4
0.6
0.8
①MOI=0.2, 48h
②MOI=0.2, 72h
③MOI=0.2, 96h
④MOI= 1, 48h
⑤MOI= 1, 72h
⑥MOI= 1, 96h
⑦MOI= 5, 48h
⑧MOI= 5, 72h
⑨MOI= 5, 96h
⑩Non-infected cells
⑪Wild-type-infected

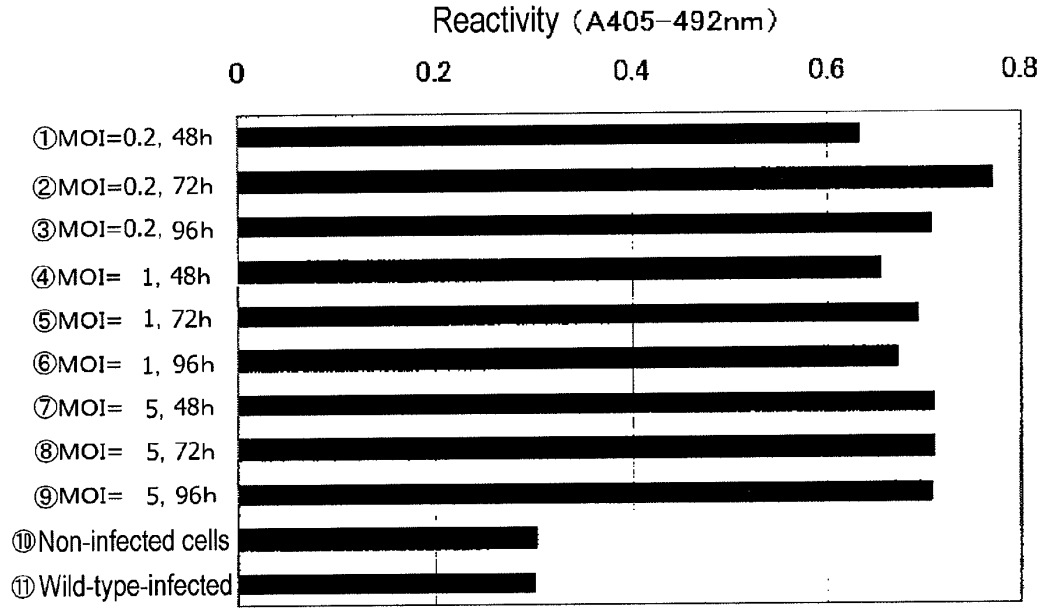
[Fig. 5]


Reactivity (A405-492nm)
0
0.4
0.8
1.2
1.6
DS-10A II (X1)
0
1
10
Recombinant ProCE (X5)
0
1
10

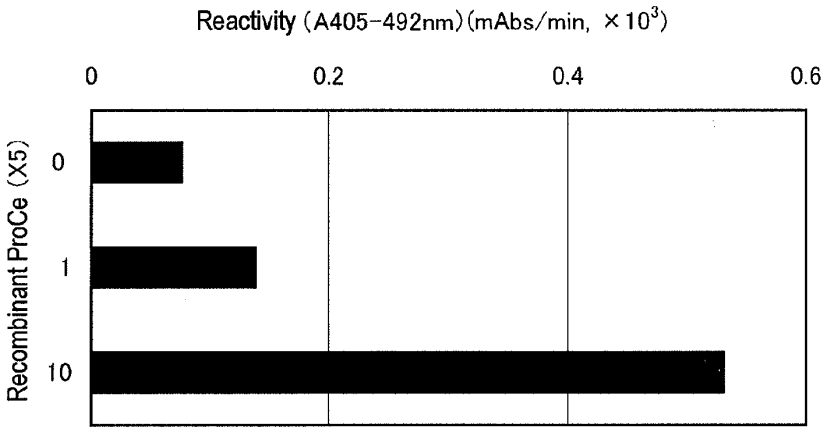
[Fig. 6]


Reactivity (A405-492nm)
0
0.2
0.4
0.6
0.8
①MOI=0.2, 48h
②MOI=0.2, 72h
③MOI=0.2, 96h
④MOI= 1, 48h
⑤MOI= 1, 72h
⑥MOI= 1, 96h
⑦MOI= 5, 48h
⑧MOI= 5, 72h
⑨MOI= 5, 96h
⑩Non-infected cells
⑪Wild-type-infected

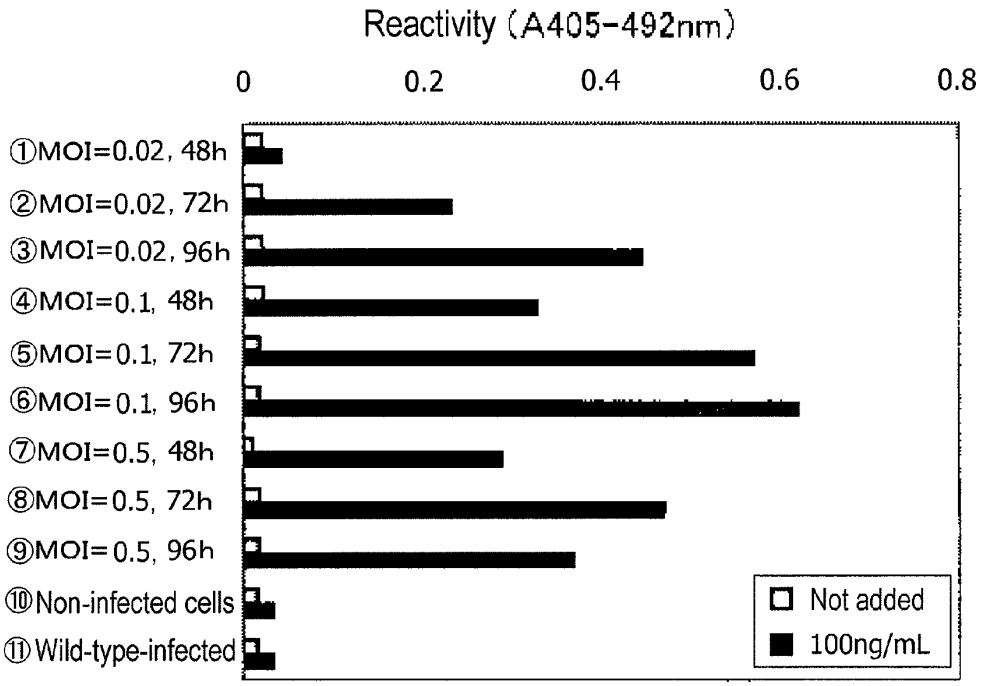
[Fig. 7]


Reactivity (A405-492nm)
0
0.2
0.4
0.6
0.8
①MOI=0.2, 48h
②MOI=0.2, 72h
③MOI=0.2, 96h
④MOI= 1, 48h
⑤MOI= 1, 72h
⑥MOI= 1, 96h
⑦MOI= 5, 48h
⑧MOI= 5, 72h
⑨MOI= 5, 96h
⑩Non-infected cells
⑪Wild-type-infected

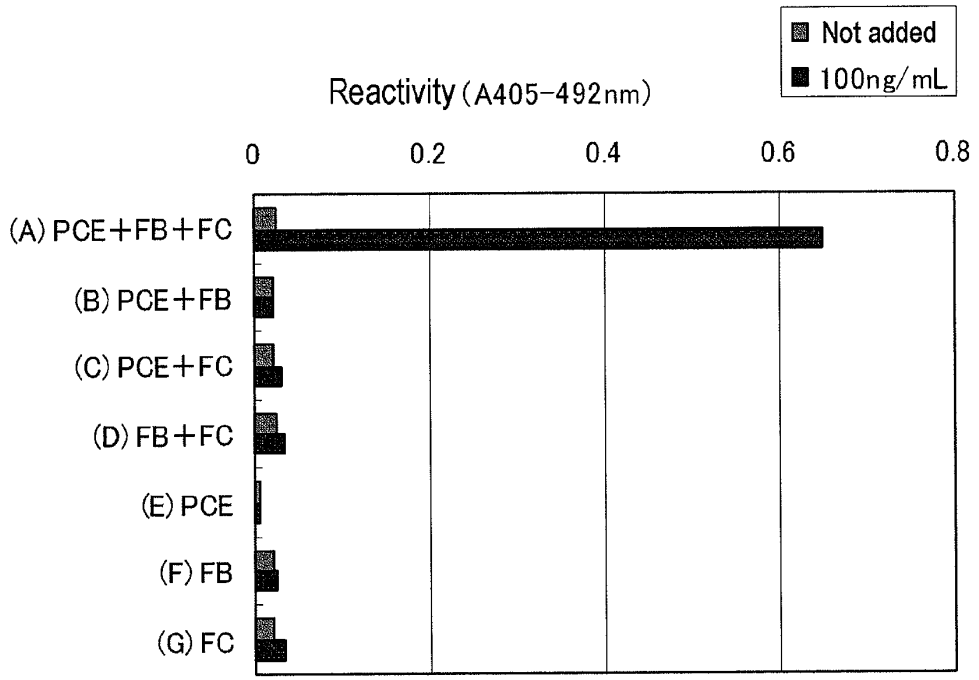
[Fig. 8]


Reactivity (A405-492nm) (mAbs/min, ×10³)
0
0.2
0.4
0.6
Recombinant ProCe (X5)
0
1
10

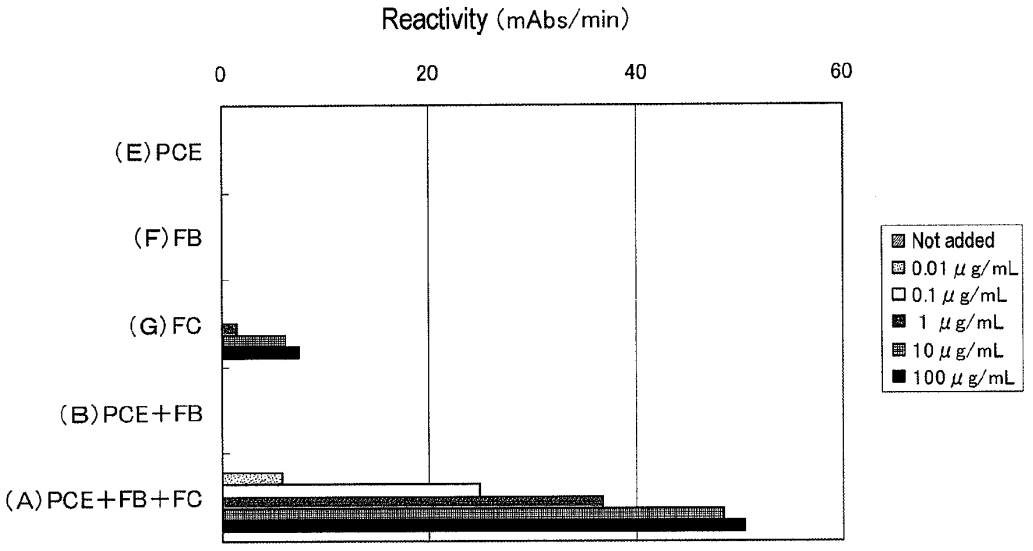
[Fig. 9]


Reactivity (A405-492nm)
0
0.2
0.4
0.6
0.8
①MOI=0.02, 48h
②MOI=0.02, 72h
③MOI=0.02, 96h
④MOI=0.1, 48h
⑤MOI=0.1, 72h
⑥MOI=0.1, 96h
⑦MOI=0.5, 48h
⑧MOI=0.5, 72h
⑨MOI=0.5, 96h
⑩Non-infected cells
⑪Wild-type-infected
Not added
100ng/mL

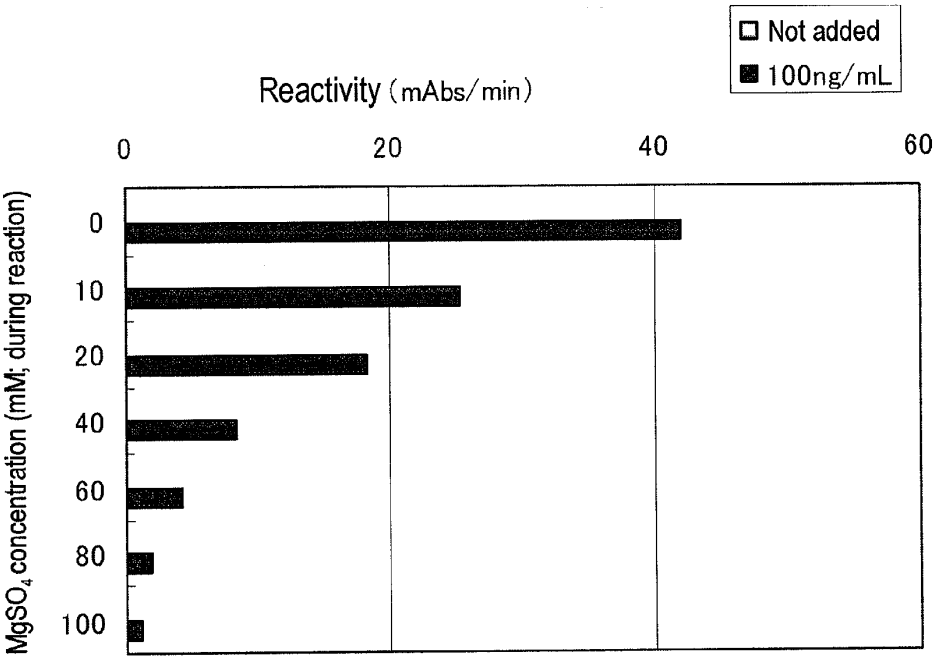
[Fig. 10]


Not added
100ng/mL
Reactivity (A405-492nm)
0
0.2
0.4
0.6
0.8
(A) PCE+FB+FC
(B) PCE+FB
(C) PCE+FC
(D) FB+FC
(E) PCE
(F) FB
(G) FC

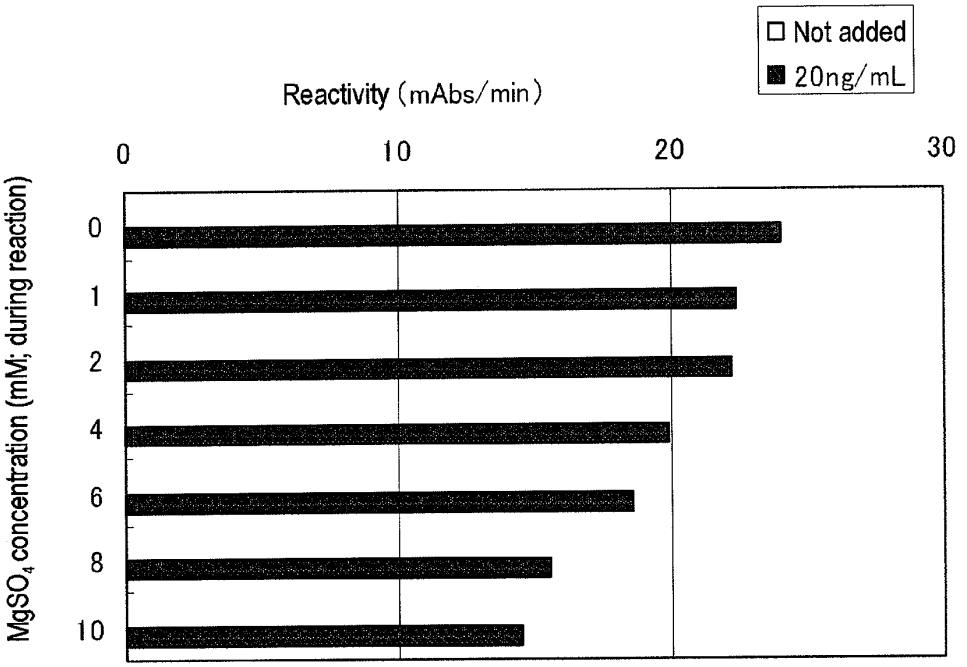
[Fig. 11]


Reactivity (mAbs/min)
0
20
40
60
(E) PCE
(F) FB
(G) FC
(B) PCE+FB
(A) PCE+FB+FC
Not added
0.01 μg/mL
0.1 μg/mL
1 μg/mL
10 μg/mL
100 μg/mL

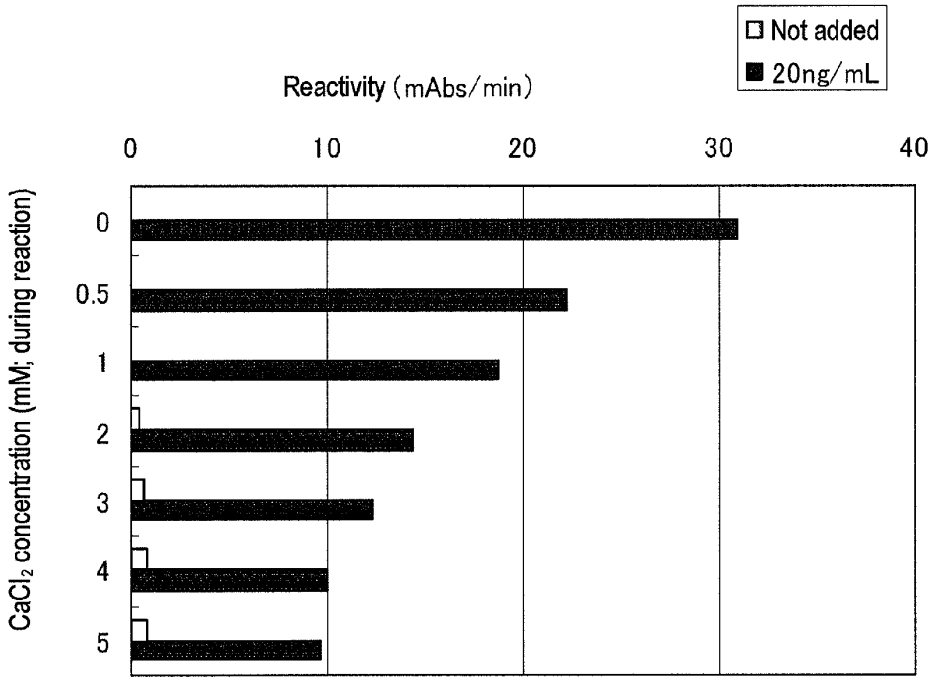
[Fig. 12]


Not added
100ng/mL
Reactivity (mAbs/min)
0
20
40
60
MgSO4 concentration (mM; during reaction)
0
10
20
40
60
80
100

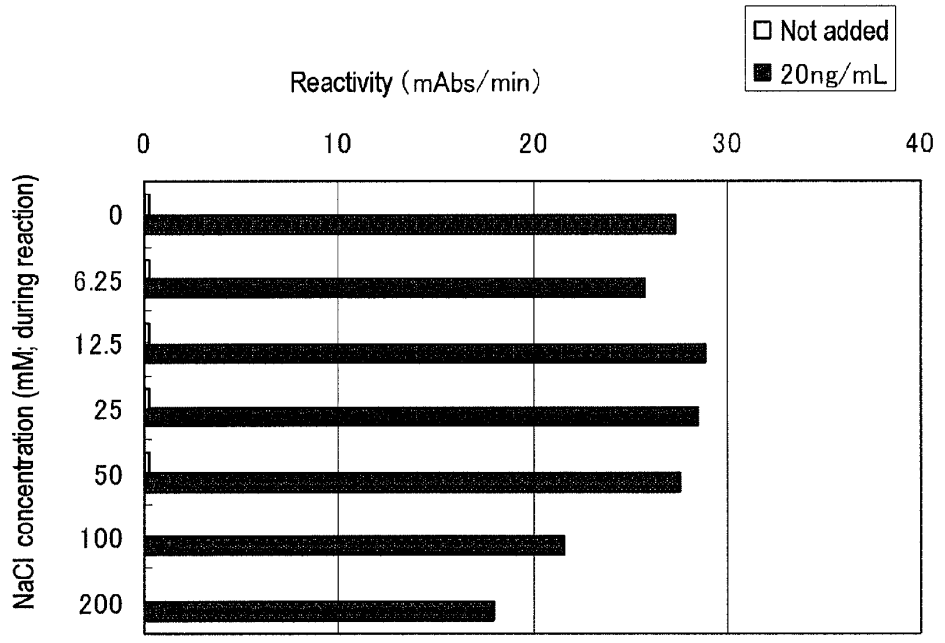
[Fig. 13]


Not added
20ng/mL
Reactivity (mAbs/min)
0
10
20
30
MgSO4 concentration (mM; during reaction)
0
1
2
4
6
8
10

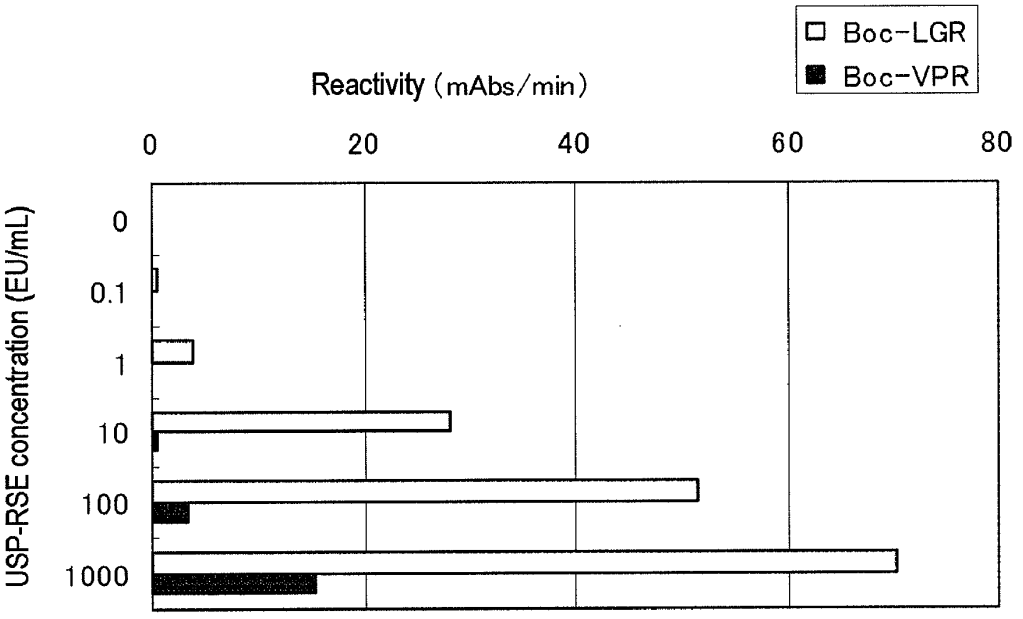
[Fig. 14]


Not added
20ng/mL
Reactivity (mAbs/min)
0
10
20
30
40
CaCl2 concentration (mM; during reaction)
0
0.5
1
2
3
4
5

[Fig. 15]


Not added
20ng/mL
Reactivity (mAbs/min)
0
10
20
30
40
NaCl concentration (mM; during reaction)
0
6.25
12.5
25
50
100
200

[Fig. 16]


Boc-LGR
Boc-VPR
Reactivity (mAbs/min)
0
20
40
60
80
USP-RSE concentration (EU/mL)
0
0.1
1
10
100
1000

PRO-CLOTTING ENZYME, AND METHOD FOR DETECTION OF ENDOTOXIN OR (1-3)-BETA-D-GLUCAN USING THE SAME

TECHNICAL FIELD

[0001] The present invention relates to a nucleic acid fragment encoding a pro-clotting enzyme derived from a horseshoe crab (hereinafter may be referred to as a *limulus*-derived pro-clotting enzyme), to a virus harboring the nucleic acid fragment, to a cell harboring the virus, to a method of producing the pro-clotting enzyme by use of the cell, and to a method and a kit for detecting Et or BG employing the pro-clotting enzyme produced through the production method.

BACKGROUND ART

[0002] There are disclosed methods for determining Et or BG by use of an amebocyte lysate of a horseshoe crab (i.e., a horseshoe crab hematocyte extract, hereinafter referred to simply as a lysate). These methods are based on coagulation of the lysate by Et or BG. The coagulation reaction occurs through cascade reaction of several coagulation factors (Patent Document 1 and Non-Patent Document 1).

[0003] For example, when BG is brought into contact with the lysate, factor G contained in the lysate is activated, to thereby form activated factor G. The activated factor G activates Pro-CE present in the lysate, to thereby form CE. CE hydrolyzes a specific site of a coagulogen molecule present in the lysate, thereby forming coaguline gel, leading to coagulation of the lysate. CE also acts on a synthetic substrate (e.g., t-butoxycarbonyl-leucyl-glycyl-arginine-pNA (Boc-Leu-Gly-Arg-pNA)), to thereby hydrolyze the amino bonds, whereby pNA is released. Thus, BG can be determined through measuring absorbance of the formed coloring substance (pNA) (Patent Document 1).

[0004] Although Pro-CE was previously cloned (Non-Patent Document 2), a protein (Pro-CE) maintaining an enzymatic activity is difficult to develop when the corresponding nucleic acid fragment is employed.

[0005] In other words, although Pro-CE was previously cloned, the technique and the resultant protein disclosed in Non-Patent Document 2 are merely included in a standard technique for producing a target clone of a cDNA fragment encoding a protein of interest. More specifically, the standard technique is limited to selection of 23 clones from a λgt11 cDNA library (1,500,000 clones) by use of an anti-CE antibody, subcloning to a pUC118/119 vector, and determination of the nucleotide sequence. In fact, these working steps are considerably heavy, and there have not yet been performed development of an enzymatic activity of Pro-CE, which is a precursor of serine protease [protease (amidase) activity of CE], and activation of Pro-CE by activated factor B or quantitative determination (including reproduction test) of the enzymatic activity of Pro-CE in the co-presence of activated factor B or C. Completely differing from determination of the nucleotide sequence of a specific protein, yielding the specific protein as a recombinant and establishing a specific assay system employing the recombinant protein can be attained only by highly advanced creation of technical ideas.

Patent Document 1: Japanese Patent Application laid-Open (kokai) No. 08-122334

Patent Document 2: Japanese Patent Application laid-Open (kokai) No. 2006-271384

Non-Patent document 1: J. Protein Chem., 5, p. 255-268 (1986)

Non-Patent document 2: J. Biol. Chem., 265(36), p. 22426-22433 (1990)

DISCLOSURE OF THE INVENTION

Problems to be Solved by the Invention

[0006] Thus, objects of the present invention are to provide a nucleic acid fragment encoding a *limulus*-derived Pro-CE, a virus harboring the nucleic acid fragment, a cell harboring the virus, a method of producing the Pro-CE by use of the cell, and a method and a kit for detecting Et or BG employing the Pro-CE produced through the production method, wherein these elements are capable of mass-producing an Et or BG assay reagent of satisfactory quality, steadily, at low cost, and on a large scale.

[0007] The present inventors have conducted extensive studies in order to attain the aforementioned objects, and have found that a protein having Pro-CE activity can be produced by use of a cell harboring a virus containing a DNA fragment encoding Pro-CE, whereby an Et or BG assay reagent of satisfactory quality can be mass-produced steadily, at low cost, and on a large scale. The present invention has been accomplished on the basis of this finding.

[0008] Abbreviations used in the present invention (including the aforementioned "Background") are as follows.

Pro-CE: pro-clotting enzyme
CE: clotting enzyme
AcNPV: nuclear polyhedrosis virus of *Autographa californica*

BG: (1→3)-β-D-glucan

[0009] Et: endotoxin (also referred to as lipopolysaccharide)
HEPES: 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
HRP: horseradish peroxidase
MOI: multiplicity of infection
NPV: nuclear polyhedrosis virus
PBS: phosphate buffered saline
PCR: polymerase chain reaction
pNA: p-nitroaniline
PVDF: polyvinylidene difluoride
SDS: sodium dodecyl sulfate
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
LAL: *limulus* amebocyte lysate

[0010] Accordingly, the present invention provides a nucleic acid fragment encoding a *limulus*-derived Pro-CE (hereinafter the nucleic acid fragment may be referred to as a "nucleic acid fragment of the present invention").

[0011] The horseshoe crab (*limulus*) employed in the present invention is selected from among the following four species: *Tachypleus tridentatus, Limulus polyphemus, Tachypleus gigas*, and *Tachypleus rotundicauda* (*Carcinoscorpius rotundicauda*).

[0012] The "nucleic acid fragment encoding a *limulus*-derived Pro-CE" employed in the invention is preferably selected from the following nucleic acid fragments (A) to (C):

[0013] (A) a DNA fragment encoding a protein having an amino acid sequence defined by SEQ ID NO: 4,

[0014] (B) a DNA fragment encoding a protein having an amino acid sequence defined by SEQ ID NO: 4 in which one

or several amino acid residues are deleted, substituted, inserted, or translocated, and having activity of a *limulus*-derived Pro-CE, and

[0015] (C) an RNA fragment produced through transcription of the DNA fragment (A) or (B).

[0016] The "nucleic acid fragment encoding a *limulus*-derived Pro-CE" employed in the invention is preferably selected from the following nucleic acid fragments (a) to (c):

[0017] (a) a DNA fragment having a nucleotide sequence defined by nucleotides 1 to 1143 in SEQ ID NO: 3,

[0018] (b) a DNA fragment having a nucleotide mutation in a nucleotide sequence containing a nucleotide sequence defined by nucleotides 1 to 1143 in SEQ ID NO: 3, wherein the mutation causes deletion, substitution, insertion, or translocation of one or several amino acid residues in an amino acid sequence of a protein encoded by the nucleotide sequence defined by nucleotides 1 to 1143 in SEQ ID NO: 3, and a protein expressed by the DNA fragment having a nucleotide mutation has activity of a *limulus*-derived Pro-CE, and

[0019] (c) an RNA fragment produced through transcription of the DNA fragment (a) or (b).

[0020] The present invention also provides a virus harboring the nucleic acid fragment of the present invention (hereinafter referred to as a "virus of the present invention").

[0021] The "virus" employed in the invention is preferably a baculovirus. Among baculoviruses, NPV is preferred, with AcNPV being more preferred.

[0022] The present invention also provides a cell harboring the virus of the present invention (hereinafter referred to as a "cell of the present invention").

[0023] No particular limitation is imposed on the "cell," and the cell is freely selected in consideration of, for example, matching with the virus of the present invention. Examples of the cell include cells derived from *E. coli*, bacteria, yeasts, and insects. As mentioned above, the virus of the present invention is preferably a baculovirus. When a baculovirus is employed, an insect-derived cell is preferably selected.

[0024] The present invention also provides a method of producing a *limulus*-derived Pro-CE, the method comprising the steps of growing the cell of the present invention, and preparing a *limulus*-derived Pro-CE from the growth product (hereinafter referred to as a "production method of the present invention").

[0025] The present invention also provides a *limulus*-derived Pro-CE produced through the production method of the present invention (hereinafter referred to as an "enzyme of the present invention").

[0026] The present invention also provides a method of detecting Et, wherein the method comprises:

[0027] causing "a serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with Et" to be co-present with the enzyme of the present invention in a test sample in which the Et possibly present therein is to be detected (hereinafter may be referred to as an "Et-detection sample"); and

[0028] detecting Et present in the sample through employing, as an index, enzymatic activity in conversion of Pro-CE to CE (hereinafter referred to as a "method 1 of the present invention").

[0029] In the method 1 of the present invention, the "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with the Et" preferably comprises a "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with activated factor C", and "*limulus*-derived factor C and/or recombinant factor C."

[0030] The "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with activated factor C" is preferably *limulus*-derived factor B and/or recombinant factor B.

[0031] The aforementioned factors C and B are preferably recombinants. In one best mode of the method 1 of the present invention, the enzyme of the present invention is caused to be co-present with factors C and B in an Et-detection test sample, and the employed factors C and B are recombinants. Carrying out the method 1 of the present invention by use of *limulus*-derived native factors C and B are considerably preferred from the viewpoint of effectiveness of method, such as detection sensitivity. However, in order to collect the hemolymph of horseshoe crab serving as a raw material for native factors C and B, horseshoe crabs must be captured, and their blood must be collected in such an amount that continual growth thereof is not impaired. In addition, a certain level of stress is unavoidably imposed on horseshoe crabs upon capturing and blood collection, and some people point out a decrease in the number of horseshoe crabs caused by environmental destruction, etc. Thus, it is deeply significant that recombinant proteins are employed as all proteins involved in cascade reaction for carrying out the method 1 of the present invention, particularly from the viewpoint of protection of precious biological resources. Use of recombinant proteins greatly contributes to the protection of lifeforms and provision of animal alternatives.

[0032] In the case where recombinant factors C and B are employed as essential elements of the Et-detection system, no particular limitation is imposed on the selection of a vector into which a gene encoding the corresponding factor is introduced and on the host to which the vector is transduced. However, similar to the case of the aforementioned recombinant Pro-CE, the virus serving as a vector is preferably a baculovirus. Among baculoviruses, NPV is preferred, with AcNPV being more preferred. Examples of the host include cells derived from *E. coli*, bacteria, yeasts, and insects. As mentioned above, the virus of the present invention is preferably a baculovirus. When a baculovirus is employed, an insect-derived cell is preferably selected.

[0033] The concept "employing, as an index, an enzymatic activity in conversion of the enzyme of the present invention to CE" means that the enzymatic activity induced through conversion of Pro-CE (the enzyme of the present invention) to CE is detected quantitatively or qualitatively, whereby the detected enzymatic activity is employed as an index for the amount or presence of Et in the test sample. Examples of the "phenomenon that exhibits enzymatic activity induced through conversion of Pro-CE to CE" include coagulation of horseshoe crab amebocyte lysate through formation of coagulin gel in the presence of CE (which can be detected through observation of gelation, change in turbidity, etc.) and developing color following amide bond cleavage of a synthetic chromogenic substrate. Particularly when the synthetic chromogenic substrate is employed, an assay system of high sensitivity and good reproducibility can be established. Such an assay system, which does not necessarily employ a *limulus*-derived lysate, is very advantageous from the viewpoint of protection of precious biological resources.

[0034] Examples of the synthetic chromogenic substrate include X-A-Y (wherein X represents a protective group, Y

represents a coloring dye, and A represents a tripeptide). The A-Y bond is cleaved in the presence of CE, to thereby cause the coloring dye Y to develop color, which serves as an index for quantitatively or qualitatively detect an Et. Examples of the protective group X include known peptide protective groups such as a t-butoxycarbonyl group and a benzoyl group. Examples of the coloring dye Y include pNA, MCA (7-methoxycoumarin-4-acetic acid), DNP (2,4-dinitroaniline), and Dansyl dye. Examples of the tripeptide include Leu-Gly-Arg (LGR), Ile-Glu-Gly-Arg (IEGR), and Val-Pro-Arg (VPR).

[0035] For quantitatively carrying out the method 1 of the present invention, a correlation (typically a calibration curve) between the Et levels determined by use of a standard Et and the corresponding index intensities (absorbance (optical density) for coloring dye Y, turbidity attributed to coagulation of lysate, etc.) is established in advance. The Et level of a test sample can be quantitatively determined from an actually detected index intensity on the basis of the established correlation.

[0036] No particular limitation is imposed on the test sample, and examples of the sample include injection water, pharmaceuticals, infusions, blood preparations, medical apparatus (medical tools), quasi-drugs, cosmetics, foods, beverages, environmental samples (air, river water, soil, etc.), native proteins, genetically modified proteins, nucleic acids, enzymes, saccharides, electrolytes, and bio-samples (blood, body fluid, tissue, etc.).

[0037] The present invention also provides an Et-detection kit for carrying out the method 1 of the present invention (hereinafter referred to as a "kit 1 of the present invention"), wherein the kit comprises, as essential components, the enzyme of the present invention and a "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with Et."

[0038] In the kit 1 of the present invention, the "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with Et" is preferably a "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with activated factor C", and "*limulus*-derived factor C and/or recombinant factor C."

[0039] The "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with activated factor C" is preferably *limulus*-derived factor B and/or recombinant factor B.

[0040] In the kit 1 of the present invention, the aforementioned factors C and B are preferably recombinants. In one best mode of the kit 1 of the present invention, the employed factors C and B are recombinants. In other words, the proteins contained in the kit 1 of the present invention and involved in cascade reaction more preferably include only the enzyme of the present invention and recombinant factors C and B.

[0041] The kit 1 of the present invention may also include reagents for carrying out the method 1 of the present invention; e.g., the aforementioned synthetic chromogenic substrate (X-A-Y), buffer, diluent, salt, and *limulus*-derived amebocyte lysate, selected in accordance with the mode of the method 1 of the present invention employing the kit 1 of the present invention.

[0042] The present invention also provides a method of detecting BG, wherein the method comprises:

[0043] causing a "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with BG" to be co-present with the enzyme of the present invention in a test sample in which BG possibly present therein is to be detected (hereinafter may be referred to as a "BG-detection sample"); and

[0044] detecting BG present in the sample through employing, as an index, enzymatic activity in conversion of Pro-CE to CE (hereinafter the method referred to as a "method 2 of the present invention").

[0045] In the method 2 of the present invention, the "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with BG" is preferably *limulus*-derived factor G and/or recombinant factor G.

[0046] The aforementioned factor G is preferably recombinant. In one best mode of the method 2 of the present invention, the enzyme of the present invention is caused to be co-present with factor G in a BG-detection sample, and the factor G is recombinant.

[0047] The reason why recombinant factor G is preferred in the method 2 of the present invention is the same as mentioned in relation to that recombinant factors C and B are preferred in the method 1 of the present invention. For producing recombinant factor G, the virus serving as a vector is preferably a baculovirus. Among baculoviruses, NPV is preferred, with AcNPV being more preferred. This feature is the same as described in relation to factors C and B. Examples of the host include cells derived from *E. coli*, bacteria, yeasts, and insects. As mentioned above, the virus of the present invention is preferably a baculovirus. When a baculovirus is employed, an insect-derived cell is preferably selected. This feature is also the same as described in relation to factors C and B.

[0048] Similar to the method 1 of the present invention, the concept "employing, as an index, an enzymatic activity in conversion of the enzyme of the present invention to CE" means that the phenomenon of conversion of Pro-CE (the enzyme of the present invention) to CE is quantitatively or qualitatively detected, whereby the detected enzymatic activity is employed as an index for the amount or presence of BG in the test sample. Examples of the phenomenon that exhibits enzymatic activity induced through conversion of Pro-CE to CE are the same as disclosed in relation to the method 1 of the present invention. The synthetic coloring substrate (X-A-Y) may also be employed in the method 2 of the present invention.

[0049] For quantitatively carrying out the method 2 of the present invention, a correlation (typically a calibration curve) between the BG levels determined by use of standard BG and the corresponding index intensities (absorbance (optical density) for coloring dye Y, turbidity attributed to coagulation of lysate, etc.) is established in advance. The BG level of a test sample can be quantitatively determined from an actually detected index intensity on the basis of the established correlation.

[0050] No particular limitation is imposed on the test sample, and examples of the sample include injection water, pharmaceuticals, infusions, blood preparations, medical apparatus (medical tools), quasi-drugs, cosmetics, foods, beverages, environmental samples (air, river water, soil, etc.), native proteins, genetically modified proteins, nucleic acids, enzymes, saccharides, electrolytes, and bio-samples (blood, body fluid, tissue, etc.).

[0051] The present invention also provides a BG-detection kit for carrying out the method 2 of the present invention (hereinafter referred to as a "kit 2 of the present invention"), wherein the kit comprises, as essential components, the

enzyme of the present invention and a "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with BG."

[0052] In the kit 2 of the present invention, the "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with BG" is preferably *limulus*-derived factor G and/or recombinant factor G.

[0053] In the kit 2 of the present invention, the aforementioned factor G is preferably a recombinant. In one best mode of the kit 2 of the present invention, the employed recombinant factor G includes entire factor G molecular components (α- and β-subunits). In other words, the proteins contained in the kit 2 of the present invention and involved in cascade reaction preferably include exclusively the enzyme of the present invention and recombinant factor G.

[0054] The kit 2 of the present invention may also include reagents for carrying out the method 2 of the present invention; e.g., the aforementioned synthetic chromogenic substrate (X-A-Y), buffer, diluent, salt, and *limulus*-derived lysate, selected in accordance with the mode of the method 2 of the present invention employing the kit 2 of the present invention.

EFFECTS OF THE INVENTION

[0055] The nucleic acid fragment of the present invention is very useful, since the virus of the present invention, which is useful for steady, low-cost, and large-scale production of Pro-CE of satisfactory quality, can be provided through employment of the nucleic acid fragment. The virus of the present invention is very useful, since the cell of the present invention, which is useful for steady, low-cost, and large-scale production of Pro-CE of satisfactory quality, can be provided through employment of the virus. The cell of the present invention is very useful, since the cell can produce a protein maintaining Pro-CE activity and satisfactory quality, steadily, at low cost, and on a large scale and can also provide the method and kit of the present invention. The method and kit of the present invention realize detection and measurement of Et and BG without preparing a lysate from horseshoe crabs, which are precious bio-resources, and can provide a very useful technique in terms of protection of lifeforms, provision of animal alternatives, cost, precision, reproducibility, etc.

BRIEF DESCRIPTION OF THE DRAWINGS

[0056] [FIG. **1**] Results of nucleotide sequence analysis of the N-terminus and C-terminus regions of a target sequence of Pro-CE recombinant virus

[0057] [FIG. **2**] Results of western blotting showing Pro-CE expressed products.

[0058] [FIG. **3**] A graph showing reactivity value of supernatant fractions at a BG concentration of 1 ng/mL.

[0059] [FIG. **4**] A graph showing reactivity value of supernatant fractions at a BG concentration of 10 ng/mL.

[0060] [FIG. **5**] Reactivity value of BG in DS-3GII fraction.

[0061] [FIG. **6**] A graph showing reactivity value of supernatant fractions at an Et concentration of 10 ng/mL.

[0062] [FIG. **7**] A graph showing reactivity value of supernatant fractions at an Et concentration of 100 ng/mL.

[0063] [FIG. **8**] Reactivity value of BG in recombinant factor G (5-fold diluted).

[0064] [FIG. **9**] A graph showing the investigation results of recombinant factor B to be employed in Et reaction.

[0065] [FIG. **10**] A graph showing Et reactivity value of samples.

[0066] [FIG. **11**] A graph showing hydrolysis performance of factors to a Boc-LGR-pNA substrate in the presence of Et at high concentration.

[0067] [FIG. **12**] A graph showing the effect of magnesium sulfate concentration (0 to 100 mM) on Et reaction in a complete reconstitution system.

[0068] [FIG. **13**] A graph showing the effect of magnesium sulfate concentration (0 to 10 mM) on Et reaction in a complete reconstitution system.

[0069] [FIG. **14**] A graph showing the effect of calcium chloride concentration (0 to 5 mM) on Et reaction in a complete reconstitution system.

[0070] [FIG. **15**] A graph showing the effect of sodium chloride concentration (0 to 2.5 M) on Et reaction in a complete reconstitution system.

[0071] [FIG. **16**] A graph showing difference in Et reactivity value in a complete reconstitution system investigated through a test employing Boc-VPR-pNA or Boc-LGR-pNA as a substrate.

BEST MODES FOR CARRYING OUT THE INVENTION

[0072] Best modes for carrying out the present invention will next be described.

<1> The Nucleic Acid Fragment of the Present Invention

[0073] The nucleic acid fragment of the present invention is a nucleic acid fragment encoding a *limulus*-derived Pro-CE. No particular limitation is imposed on the "nucleic acid fragment encoding a *limulus*-derived Pro-CE" (i.e., the nucleic acid fragment of the present invention), so long as the nucleic acid fragment encodes a *limulus*-derived Pro-CE. For example, the nucleic acid fragment may be a nucleic acid fragment having a nucleotide sequence defined by SEQ ID NO: 1 (SEQ ID NO: 2 shows only the amino acid sequence corresponding to the nucleotide sequence). It will be readily appreciated by those skilled in the art that the nucleic acid fragment of the present invention also encompasses nucleic acid fragments having different nucleotide sequences attributed to degeneration of genetic codes.

[0074] The "nucleic acid fragment" employed in the present invention may be a DNA fragment or an RNA fragment, which may be selected by those skilled in the art in consideration of the use of the nucleic acid fragment. For example, when stability is emphasized, a DNA fragment may be selected.

[0075] The nucleic acid fragment of the present invention may be, for example, a nucleic acid fragment encoding Pro-CE derived from any of the following horseshoe crabs: *Tachypleus tridentatus, Limulus polyphemus, Tachypleus gigas*, and *Tachypleus rotundicauda*.

[0076] Of these, a nucleic acid fragment encoding Pro-CE derived from *Tachypleus tridentatus* or *Limulus polyphemus* is preferred, with a nucleic acid fragment encoding Pro-CE derived from *Tachypleus tridentatus* being more preferred.

[0077] The nucleic acid fragment of the present invention may be chemically synthesized or may be produced through a genetic engineering technique. For production through a genetic engineering technique, for example, by use of an artificially prepared primer having a nucleotide sequence defined by SEQ ID NO: 5 or 6, a target DNA fragment is

amplified, through PCR technique, from a cDNA library prepared through a customary method from hemocytes (amebocytes) of a horseshoe crab such as *Limulus polyphemus, Tachypleus tridentatus, Tachypleus gigas*, or *Tachypleus rotundicauda*. The resultant PCR product may be readily isolated through molecular-weight-based separation means (e.g., gel electrophoresis).

[0078] In the present invention, the "nucleic acid fragment encoding *limulus*-derived Pro-CE" is more preferably any of the following nucleic acid fragments (A) to (C):

[0079] (A) a DNA fragment encoding a protein having the amino acid sequence defined by SEQ ID NO: 4,

[0080] (B) a DNA fragment encoding a "protein having an amino acid sequence defined by SEQ ID NO: 4 in which one or several amino acid residues are deleted, substituted, inserted, or translocated, and having activity of a *limulus*-derived Pro-CE," and

[0081] (C) an RNA fragment obtained through transcription of the aforementioned DNA fragment (A) or (B).

[0082] As used herein, the "DNA fragment encoding a protein having an amino acid sequence defined by SEQ ID NO: 4" is a DNA fragment encoding a Pro-CE derived from *Tachypleus tridentatus*.

[0083] A naturally occurring protein may include a mutation in the amino acid sequence (e.g., substitution, deletion, insertion, or translocation of amino acid residues) caused by the polymorphism or mutation of the DNA fragment encoding the protein. Meanwhile, a produced protein may include a post-translational modification (e.g., phosphorylation, glycosylation, or lipidation of amino acid residues, or hydroxylation of proline) caused by intracellular modification during purification. Although having such a mutation, some proteins are known to exhibit physiological and biological activities substantially the same as those of the protein having none of the aforementioned mutations. Thus, the "protein encoded by the DNA fragment (B)," which slightly differs from the "protein encoded by the DNA fragment (A)" in structure but has no great difference in function, can be regarded as substantially equivalent to the "protein encoded by the DNA fragment (A)." A similar logic is also applied to the case where the aforementioned mutations are intentionally introduced into an amino acid sequence of protein. In this case, a wider range of variants can be prepared. For example, as has been known, a polypeptide engineered from human interleukin 2 (IL-2) so that a certain cysteine residue in the amino acid sequence of IL-2 is substituted by serine maintains human interleukin 2 activity (Science, 224, 1431 (1984)). Such a "mutated protein" may be prepared through a known technique; for example, "site-specific mutagenesis." Also, a certain protein is known to have a peptide region that is not essential in terms of activity. Examples of such a protein include a signal peptide present in an extracellularly secreted protein, and a prosequence observed in a protease precursor or a similar substance. Most of these peptide regions are removed after translation or during conversion to the corresponding activated proteins. Although such proteins have a primary structure different from that of the "protein encoded by the DNA fragment (A)," the proteins have a function substantially the same as that of the "protein encoded by the DNA fragment (A)." Therefore, the "protein encoded by the DNA fragment (B)" represents these proteins.

[0084] As used herein, the term "several amino acid residues" refers to amino acid residues which are allowed to have mutations without impairing the protein activity. For example, when a protein includes 600 amino acid residues, the number of such amino acid residues is about 2 to about 30, preferably 2 to 15, more preferably 2 to 8.

[0085] The protein encoded by the DNA fragment (B) has an activity of *limulus*-derived Pro-CE. Activity of Pro-CE may be determined by causing Pro-CE to be co-present with a synthetic substrate (e.g., t-butoxycarbonyl-leucyl-glycyl-arginine-pNA (Boc-Leu-Gly-Arg-pNA)), Et, factor C, and factor B, and examining the reactivity of Pro-CE. Specifically, when Pro-CE has activity, pNA is released by causing Pro-CE to be co-present with the synthetic substrate, Et, and factor C. The amount of thus-produced pNA can be determined through measurement of absorbance. A specific determination method will be described in Example 2.

[0086] In the present invention, no particular limitation is imposed on the state of "co-presence," so long as co-present elements are freely allowed to be in contact with one another. Specifically, the state of "co-presence" refers to the state where Pro-CE, a synthetic substrate, Et, factor C, and factor B are freely allowed to be in contact with one another, or the state where Pro-CE, a synthetic substrate, BG, and factor G are freely allowed to be in contact with one another.

[0087] As used herein, the term "reaction" refers to a reaction in which the Pro-CE is converted into CE by causing Pro-CE to be co-present with the synthetic substrate, and CE acts on the synthetic substrate, to thereby hydrolyze the amino bonds thereof, whereby pNA is released.

[0088] The DNA fragment (A) encoding a "protein having an amino acid sequence defined by SEQ ID NO: 4" may be, for example, a DNA fragment having a nucleotide sequence of nucleotides 1 to 1,143 in SEQ ID NO: 3. Alternatively, a DNA fragment deposited in GenBank with an accession No. D161657 may also be employed.

[0089] The DNA fragment (B) encoding a "protein having an amino acid sequence defined by SEQ ID NO: 4 in which one or several amino acid residues are deleted, substituted, inserted, or translocated, and having activity of *limulus*-derived Pro-CE" may be, for example, the aforementioned DNA fragment (A), a DNA fragment complementary thereto, or a DNA fragment which hybridizes with any of these DNA fragments under stringent conditions.

[0090] As used herein, the term "stringent conditions" refers to conditions which allow formation of a so-called specific hybrid but do not allow formation of a non-specific hybrid (see, for example, Sambrook, J., et al., Molecular Cloning A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)). Specific examples of the "stringent conditions" include performing hybridization at 42° C. in a solution containing 50% formamide, 4×SSC, 50 mM HEPES (pH 7.0), 10×Denhardt's solution, and 100 μg/mL salmon sperm DNA, and washing at room temperature with 2×SSC and a 0.1% SDS solution and at 50° C. with 0.1×SSC and a 0.1% SDS solution.

[0091] Preferably, the nucleic acid fragment of the present invention is further linked to a DNA fragment encoding, for example, a marker peptide. Examples of the marker peptide include protein A, an insulin signal sequence, His-tag, FLAG, CBP (calmodulin-binding protein), and GST (glutathione S-transferase). The nucleic acid fragment of the present invention also encompasses an RNA fragment obtained through transcription of the aforementioned DNA fragment (A) or (B).

[0092] The nucleic acid fragment of the present invention may be employed for production of "the virus of the present

invention" described hereinbelow, and thus for production of, for example, the cell of the present invention.

<2> The Virus of the Present Invention

[0093] The virus of the present invention harbors the nucleic acid fragment of the present invention.

[0094] The nucleic acid fragment of the present invention is the same as mentioned above.

[0095] The state "harboring a nucleic acid fragment" in the virus of the present invention does not exclude the state in which the virus harbors other nucleotides or nucleic acid fragments, so long as the relevant nucleic acid fragment is harbored. Thus, in addition to the relevant nucleic acid fragment, for example, other nucleic acid fragments encoding a marker peptide or a similar peptide may be harbored.

[0096] For example, the virus of the present invention also encompasses a vector harboring a linked DNA fragment formed of "the aforementioned DNA fragment (A) or (B) (i.e., the nucleic acid fragment of the present invention)" and a "DNA fragment encoding, for example, a marker peptide." When the nucleic acid fragment harbored is designed in the above manner, a protein fused with, for example, a marker peptide may be expressed. The thus-expressed protein is advantageous in that the purification, detection, analysis, etc. thereof can be facilitated. Examples of the marker peptide include protein A, an insulin signal sequence, His-tag, FLAG, CBP (calmodulin-binding protein), and GST (glutathione S-transferase). For example, a protein fused with protein A may be purified in a simple manner through affinity chromatography employing an IgG-bound solid phase. Similarly, a His-tag-fused protein may be purified with a magnetic nickel-bound solid phase, whereas a FLAG-fused protein may be purified with an anti-FLAG antibody-bound solid phase. Since a protein fused with an insulin signal sequence is extracellularly secreted (e.g., secreted in a culture medium), an extraction process including crushing of cells may be eliminated.

[0097] No particular limitation is imposed on the production method for the virus of the present invention. One exemplary method for producing the virus of the present invention will be described as follows. More specific procedure thereof will be described in the Examples.

[0098] Firstly, a nucleic acid fragment encoding *limulus*-derived Pro-CE is provided. In the case where the aforementioned DNA fragment (A) is employed as the nucleic acid fragment, a DNA fragment encoding a "protein having an amino acid sequence defined by SEQ ID NO: 4" is provided. In the case where the aforementioned DNA fragment (B) is employed as the nucleic acid fragment, there is provided a DNA fragment encoding a "protein having an amino acid sequence defined by SEQ ID NO: 4 in which one or several amino acid residues are deleted, substituted, inserted, or translocate (transposed), and having an activity of *limulus*-derived Pro-CE." No particular limitation is imposed on the type of the DNA fragment employed, so long as the DNA fragment encodes the corresponding protein. The DNA fragment includes those having a variety of nucleotide sequences attributed to degeneration of genetic codes. However, any of these DNA fragments having a specific nucleotide sequence may be employed.

[0099] The virus of the present invention can be produced through introduction of such a nucleic acid fragment into a virus.

[0100] No particular limitation is imposed on the virus into which such a nucleic acid fragment is introduced, so long as the virus can be employed for gene transfection. Particularly, a baculovirus (in particular, an NPV) is preferably employed. No particular limitation is imposed on the species of the NPV employed, so long as the NPV is a virus belonging to NPVs. For example, AcNPV or Bombyx mori NPV (BmNPV) may be employed. of these, AcNPV is preferred.

[0101] Introduction of a nucleic acid fragment into a virus may be carried out through homologous recombination by use of a transfer vector. No particular limitation is imposed on the type of the transfer vector employed. For example, pPSC8 (Protein Science), pFastBac (Invitrogen), or pVL1393 (Pharmingen) may be employed. Of these, pPSC8 is preferred. These transfer vectors may be commercially available ones.

[0102] No particular limitation is imposed on the method of homologous recombination by use of a transfer vector. A specific example thereof will be described in the Examples.

[0103] Whether or not the produced virus harbors the aforementioned DNA fragment (A) or (B) may be determined by, for example, any of the following procedures: checking that the produced virus harbors a DNA fragment encoding a *limulus*-derived Pro-CE through analysis of the nucleotide sequence of the virus; checking that a protein expressed by the produced virus has the amino acid sequence of *limulus*-derived Pro-CE; and checking that a protein expressed by the produced virus has a Pro-CE activity.

[0104] The virus of the present invention may be employed for production of "the cell of the present invention" described below, and thus employed, for example, in the method of the present invention.

<3> The Cell of the Present Invention

[0105] The cell of the present invention harbors the virus of the present invention.

[0106] "The virus of the present invention" is the same as mentioned above.

[0107] No particular limitation is imposed on the "cell" employed in the present invention, so long as the cell allows infection with the virus of the present invention, and can express the nucleic acid fragment encoding a *limulus*-derived Pro-CE that is harbored by the virus of the present invention. Examples of the cell include insect-derived cells. Specific examples of the insect-derived cells include an Sf9 cell.

[0108] No particular limitation is imposed on the method for causing the cell to harbor the virus of the present invention. For example, when the virus of the present invention is a NPV, the cell can be infected with the virus only by bringing the cell into contact with the virus, whereby the cell can harbor the virus. A specific method therefor will be described in the Examples hereinbelow.

[0109] Since the cell of the present invention can produce a *limulus*-derived Pro-CE, the cell of the present invention may be selected on the basis of the production performance as an index.

[0110] The cell of the present invention may be employed in, for example, the below-described production method of the present invention.

<4> The Production Method of the Present Invention

[0111] The production method of the present invention for producing *limulus*-derived Pro-CE includes at least the steps

of growing the cell of the present invention, and preparing *limulus*-derived Pro-CE from the growth product.

[0112] "The cell of the present invention" is the same as mentioned above.

[0113] As used herein, the term "grow" refers to a concept including proliferation of cells which are transformants and growing an organism (e.g., animal or insect) into which transformant cells have been incorporated. As used herein, the term "growth product" is a concept including, for example, a culture medium (supernatant of the culture liquid) after completion of growth of transformants, cultured cells themselves, and matter secreted or discharged from an organism (e.g., animal or insect) into which the cells have been incorporated.

[0114] No particular limitation is imposed on the growth conditions (e.g., culture medium and culture conditions), so long as the cell of the present invention can grow and produce *limulus*-derived Pro-CE. The growth conditions may be appropriately determined in consideration of, for example, the type of a vector or cell employed. For example, culturing may be carried out at a temperature of about 20 to about 40° C.

[0115] The growth period of the cell of the present invention may also be appropriately regulated in consideration of, for example, the amount of the cell of the present invention employed, the amount of a desired Pro-CE produced, or other growth conditions.

[0116] Those skilled in the art may appropriately select the method for preparing a *limulus*-derived Pro-CE from the growth product from generally employed methods in consideration of the type of the growth product.

[0117] For example, in the case where Pro-CE is produced in a soluble form secreted into a culture medium (culture supernatant), the culture medium is collected and may be employed as is. In the case where Pro-CE is produced in a soluble form secreted in cytoplasm, or produced in an insoluble (membrane-bound) form, the Pro-CE may be extracted through, for example, any of the following treatments: extraction with cell crushing such as a method employing a nitrogen cavitation apparatus, homogenizing, glass beads milling, sonication, the hypotonic extraction, or freeze-thawing; extraction with a surfactant; or a combination thereof. The resultant extract may be employed, as is, as the Pro-CE.

[0118] The production method of the present invention may further include other steps, so long as the method includes at least a "step of growing the cell of the present invention, and preparing *limulus*-derived Pro-CE from the growth product." For example, the method may further include the step of purifying the thus-prepared Pro-CE. The purification may be incomplete (partial) purification or complete purification, and may be appropriately selected in consideration of, for example, the use purpose of the Pro-CE.

[0119] Specific examples of the purification method include salting out by the mediation of a salt such as ammonium sulfate or sodium sulfate, centrifugation, dialysis, ultrafiltration, adsorption chromatography, ion-exchange chromatography, hydrophobic chromatography, reversed-phase chromatography, gel filtration, gel permeation chromatography, affinity chromatography, electrophoresis, and combinations thereof.

[0120] Whether or not, for example, the thus-produced protein is formed of Pro-CE, or the protein maintains an activity of *limulus*-derived Pro-CE may be determined through analysis of the collected protein, in terms of, for example, amino acid sequence, molecular weight, electrophoresis features, or Western blotting employing an antibody reacting specifically to the Pro-CE.

[0121] The method of the present invention realizes very effective production of a protein which maintains Pro-CE activity.

<5> The Enzyme of the Present Invention

[0122] The enzyme of the present invention is Pro-CE produced through the production method of the present invention.

[0123] "The production method of the present invention" is the same as mentioned above.

[0124] The enzyme of the present invention may be employed in, for example, the method 1 of the present invention described below.

<6> The Method 1 of the Present Invention

[0125] The method 1 of the present invention is a method for sensitively detecting Et. A characteristic feature of the method 1 of the present invention resides in that the enzyme of the present invention is caused to be co-present with a "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with Et" in an Et-detection sample, and the Et present in the sample is detected by employing, as an index, enzymatic activity in conversion of the enzyme of the present invention to CE.

[0126] "The enzyme of the present invention" is the same as mentioned above.

[0127] In the present invention, no particular limitation is imposed on the state of "co-presence," so long as co-present elements are allowed to be in contact with one another.

[0128] For example, no particular limitation is imposed on the state of "co-presence," so long as the enzyme of the present invention, an Et-detection sample, and a "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with Et" are allowed to be in contact with one another. These elements may be caused to be co-present with one another in a solution. Alternatively, Pro-CE or a "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with Et" may be immobilized on a solid phase, and thus-immobilized Pro-CE or serine protease precursor may be respectively brought into contact with a "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with Et" or Pro-CE.

[0129] In the method 1 of the present invention, preferably, the "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with Et" is a "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with activated factor C", and "*limulus*-derived factor C and/or recombinant factor C."

[0130] The "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with activated factor C" is preferably *limulus*-derived factor B and/or recombinant factor B.

[0131] No particular limitation is imposed on the "factor C" or "factor B" employed in the method 1 of the present invention, so long as the factor maintains its function. For example, the factor C or B employed may be naturally occurring factor C or B fraction prepared through purification (e.g., chromatography) of amebocyte lysate derived from any of the four

horseshoe crabs: *Tachypleus tridentatus, Limulus polyphemus, Tachypleus gigas*, and *Tachypleus rotundicauda*. Alternatively, the factor C or B employed may be recombinant factor C or B. The naturally occurring factor C or B fraction may be prepared through treatment of the aforementioned lysate with, for example, a carrier to which dextran sulfate, a sulfopropyl group, or the like is bound, or a specific adsorption carrier. The recombinant factor C may be appropriately prepared, for example, on the basis of the known amino acid sequence of naturally occurring factor C derived from *Tachypleus tridentatus* or *Tachypleus rotundicauda*. No particular limitation is imposed on the method for preparing the recombinant factor C. For example, the recombinant factor C may be prepared through the following procedure: a target nucleotide sequence for factor C having a His-tag at the C terminus is synthesized and introduced into a transfer vector (e.g., pPSC8, product of Takara Bio Inc.); Sf9 cells are co-transfected with the resultant expression vector (Factor C/pPSC8) DNA fragment and a baculovirus (AcNPV) DNA fragment; and the virus fluid obtained from the resultant culture supernatant is purified, followed by amplification. The recombinant factor B may also be prepared in a manner similar to that described above. The amino acid sequence of factor C or factor B and the gene coding therefor have already been known. Factor C is commercially available, and the commercial product will be described in the Examples hereinbelow. The nucleotide sequence of the gene for factor B is shown in SEQ ID NO: 15, and the amino acid sequence of factor B is shown in SEQ ID NO: 16. The recombinant of such a factor may be produced on the basis of the corresponding sequence data through a process substantially the same as that for the aforementioned enzyme of the present invention. It will be readily appreciated by those skilled in the art that a protein produced on the basis of a nucleotide sequence which, as a result of degeneration of genetic codes, differs from the known nucleotide sequence of factor C or the nucleotide sequence defined by SEQ ID NO: 15 may be employed in the present invention as factor C or B, so long as the protein has an effect intrinsic to factor C or B.

[0132] Operation of the reconstruction system does not require provision of insect cells as a reaction site. For facilitation of at least triggering of cascade reaction, sequential activation of serine protease precursors, and reaction of CE in a cell-free system, preferably, conditions for the operation include, for example, constant heating and co-presence of ions of a metal such as an alkaline earth metal (e.g., calcium, strontium, barium, beryllium, or magnesium) or an alkali metal (e.g., lithium, sodium, or potassium).

[0133] In the method 1 of the present invention, more preferably, the enzyme of the present invention, recombinant factor C, and recombinant factor B are exclusively caused to be co-present as cascade reaction proteins.

[0134] As used herein, the term "cascade reaction" refers to the following reaction "1." and/or reaction "2.":

[0135] 1. a series of reactions in which factor C (Et-sensitive factor, molecular weight: 123,000) present in amebocyte lysate is activated through addition of Et to the lysate, to thereby form activated factor C; the activated factor C hydrolyzes a specific site of factor B (molecular weight: 64,000) to thereby form activated factor B; the activated factor B activates Pro-CE (molecular weight: 54,000) to thereby convert it into CE; and the CE hydrolyzes specific sites in a loop cross-linked by disulfide bonds of coagulogen (coagulated protein, molecular weight: 19,723); i.e., hydrolyzes the bond between Arg^{18} and Thr^{19} and the bond between Arg^{46} and Gly^{47}, to thereby release peptide C (28 amino acid residues) represented by H-Thr^{19} . . . Arg^{46}-OH and to convert the remaining portion into coagulin gel; and

[0136] 2. a series of reactions in which factor G (BG-sensitive factor) present in amebocyte lysate is activated through addition of BG to the lysate; the activated factor G activates Pro-CE to thereby convert it into CE; and the CE hydrolyzes specific sites in a loop cross-linked by disulfide bonds of coagulogen, to thereby form coagulin gel.

[0137] The term "cascade reaction protein" refers to a protein involved in "cascade reaction"; i.e., a serine protease precursor (factor C, factor B, factor G, or Pro-CE). Specifically, the cascade reaction proteins in the aforementioned cascade reaction "1." are factor C, factor B, and Pro-CE, and the cascade reaction proteins in the cascade reaction "2." are factor G and Pro-CE.

[0138] As shown in the Examples hereinbelow, the origins of the genetically engineered coagulation factors employed in the method 1 of the present invention may be different from one another. For example, the Et detection method may include the step of causing the enzyme of the present invention to be co-present with factor C derived from *Tachypleus rotundicauda* and factor B derived from *Limulus polyphemus*.

[0139] The method 1 of the present invention can be readily carried out by means of the below-described kit 1 of the present invention.

<7> The Kit 1 of the Present Invention

[0140] The kit 1 of the present invention is an Et detection kit for carrying out the method 1 of the present invention. A characteristic feature of the kit 1 of the present invention resides in that the kit includes at least the enzyme of the present invention and a "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with Et."

[0141] "The enzyme of the present invention" is the same as mentioned above.

[0142] The "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with activated factor C" is preferably *limulus*-derived factor B and/or recombinant factor B.

[0143] Preferably, the kit 1 of the present invention includes, as cascade reaction proteins, exclusively the enzyme of the present invention, recombinant factor C, and recombinant factor B.

[0144] Those skilled in the art may appropriately employ the kit 1 of the present invention on the basis of the method 1 of the present invention.

[0145] The terms used in the kit 1 of the present invention such as "factor C," "factor B," and "cascade reaction" have the same meanings as defined above in the method 1 of the present invention. As described above, reagents, etc. employed for carrying out the method 1 of the present invention (e.g., the aforementioned synthetic chromogenic substrate (X-A-Y), buffer, diluent, salt, and *limulus*-derived amebocyte lysate) may be included in the kit 1 of the present invention, which may be selected in consideration of the mode of the method 1 of the present invention performed by means of the kit.

<8> The Method 2 of the Present Invention

[0146] The method 2 of the present invention is a method for detecting BG. A characteristic feature of the method 2 of

the present invention resides in that the enzyme of the present invention is caused to be co-present with a "serine protease precursor which expresses an activity of converting pro-clotting enzyme to clotting enzyme upon contact with BG" in a BG-detection sample, and BG present in the sample is detected by employing, as an index, enzymatic activity induced through conversion of the enzyme of the present invention to clotting enzyme.

[0147] "The enzyme of the present invention" is the same as mentioned above.

[0148] In the method 2 of the present invention, preferably, the "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with BG" is *limulus*-derived factor G and/or recombinant factor G.

[0149] No particular limitation is imposed on the "factor G" employed in the method 2 of the present invention, so long as the factor maintains its function. For example, the factor G employed may be a naturally occurring factor G fraction prepared through purification (e.g., chromatography) of amebocyte lysate derived from any of the aforementioned four horseshoe crabs. Alternatively, the factor G employed may be recombinant factor G. Recombinant factor G is a protein formed of subunits α and β, and the respective subunits may be produced through the following procedures.

[0150] Firstly, a DNA fragment encoding subunit α of *limulus*-derived factor G is provided. The DNA fragment may be, for example, a DNA fragment deposited in GenBank with an accession No. 16622 (SEQ ID NO: 17, the amino acid sequence corresponding to the DNA fragment is shown in SEQ ID NO: 18). The DNA fragment is treated with BamHI/Hind III, and DNA fragments having a target gene sequence are collected. The sample is blunt-ended and then ligated through mixing with Nru I-treated pPSC8 (transfer vector). Subsequently, *E. coli* JM109 is transformed with the ligation product, to thereby yield a transformant. Plasmid in which fragments having a target size have been determined is purified. Sf9 cells are co-transfected with the thus-selected expression vector (Factor G-α/pPSC8) DNA fragment and a baculovirus (AcNPV) DNA fragment. Thereafter, the virus fluid obtained from the resultant culture supernatant is purified, followed by amplification. Then, expres SF+ cells are infected with the virus fluid, and the resultant culture liquid is centrifuged, to thereby yield a supernatant fraction and a precipitate fraction. Subunit α of factor G may be prepared from these fractions. Subunit β may be prepared by performing the same procedure as for subunit α, except that the subunit-α-encoding DNA fragment is replaced with a DNA fragment encoding subunit β of *limulus*-derived factor G. The subunit-β-encoding DNA fragment may be, for example, a nucleotide sequence deposited in GenBank with an accession No. 16623 (SEQ ID NO: 19) (the amino acid sequence corresponding thereto is shown in SEQ ID NO: 20). It will be readily appreciated by those skilled in the art that a protein produced on the basis of nucleotide sequences which, as a result of degeneration of genetic codes, differ from the nucleotide sequences of SEQ ID NOs: 16 and 17 may be employed in the present invention as factor G, so long as the protein has an effect intrinsic to factor G.

[0151] Operation of the reconstruction system does not require provision of a certain type of cells (e.g., insect cells) as a reaction site. For facilitation of at least triggering of cascade reaction, sequential activation of serine protease precursors, and reaction of CE in a cell-free system, preferably, conditions for the operation include, for example, constant heating and co-presence of ions of a metal such as an alkaline earth metal or an alkali metal.

[0152] In the method 2 of the present invention, more preferably, the enzyme of the present invention and recombinant factor G are exclusively caused to be co-present as cascade reaction proteins. Similar to the case of the method 1 of the present invention, the enzyme of the present invention and recombinant factor G may be derived from the same origin or different origins.

[0153] The terms used in the method 2 of the present invention such as "co-present," "cascade reaction," and "cascade reaction protein" have the same meanings as defined above in the method 1 of the present invention.

[0154] The method 2 of the present invention may be employed in the below-described kit 2 of the present invention.

<9> The Kit 2 of the Present Invention

[0155] The kit 2 of the present invention is a BG detection kit for carrying out the method 2 of the present invention. A characteristic feature of the kit 2 of the present invention resides in that the kit includes at least the enzyme of the present invention and a "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with BG."

[0156] "The enzyme of the present invention" is the same as mentioned above.

[0157] In the kit 2 of the present invention, preferably, the "serine protease precursor which expresses an activity of converting Pro-CE to CE upon contact with BG" is *limulus*-derived factor G and/or recombinant factor G.

[0158] More preferably, factor G employed in the kit is in a recombinant form. In a most preferred mode of the kit 2 of the present invention, all the factors G molecules included in the kit are in a recombinant form. That is, more preferably, the kit 2 of the present invention includes, as cascade reaction proteins, exclusively the enzyme of the present invention and recombinant factor G.

[0159] Those skilled in the art may appropriately employ the kit 2 of the present invention on the basis of the method 2 of the present invention.

[0160] The term "factor G" used in the kit 2 of the present invention has the same meaning as described in the method 2 of the present invention. The terms "cascade reaction" and "cascade reaction protein" have the same meanings as defined above in the method 1 of the present invention. As described above, reagents, etc. employed for carrying out the method 2 of the present invention (e.g., the aforementioned synthetic chromogenic substrate (X-A-Y), buffer, diluent, salt, and *limulus*-derived amebocyte lysate) may be included in the kit 2 of the present invention, which may be selected in consideration of the mode of the method 2 of the present invention performed by means of the kit.

EXAMPLES

[0161] The present invention will next be described in more detail by way of examples.

Example 1

Expression of Pro-CE by Use of Insect Cells

[0162] A cDNA fragment represented by SEQ ID NO: 3 (having a nucleotide sequence of nucleotides 1 to 1125 in

SEQ ID NO: 3, a His-Tag sequence being attached to the C-terminus) was kindly offered by Dr. Tatsushi MUTA (Department of Molecular and Cellular Biochemistry, Kyushu University Graduate School of Medical Science; currently, Department of Bio-Science, Tohoku University Graduate School). The cDNA fragment had been prepared through a method disclosed in Non-Patent Document 2. The cDNA fragment was introduced into a transfer vector (pPSC8), and a clone having a predetermined nucleotide sequence was selected. The thus-selected expression vector (PC/pPSC8) DNA fragment and a baculovirus (AcNPV) DNA fragment were co-transfected into Sf9 cells. The virus fluid obtained from the culture supernatant was purified and amplified. The viral DNA fragment was extracted from the cells infected with the baculovirus, and sequenced. Insect cells (expresSF+, registered trademark, product of protein Science) were infected with the thus-obtained virus fluid, and the expression product was analyzed through Western blotting. Details of these steps will next be described.

1. Construction of Expression Vector

[0163] PC/pUC118 (20 ng/μg) (1 μL), 2.5 mM dNTP (12 μL), KOD buffer # (15 μL), 25 mM magnesium chloride solution (2 μL), PC-F and PC-R (4 pmol/μL) (each 2.5 μL), KOD DNA polymerase (product of Toyobo) (1 μL), and sterilized pure water (24 μL) were added to a 0.2-mL sample tube under stirring, and the mixture was subjected to PCR. The presence of a target gene in the PCR product was confirmed, and the product was diluted with TE buffer to the total volume of 16 μL. Then, 100 mM ATP (1 μL), 10× Buffer (1 μL), and T4Polynucleotide Kinase (product of Takara Bio) were added to the obtained PCR product, and the mixture was incubated at 37° C. for 30 minutes. The PCR product was purified and subjected to ligation through mixing with Sma I-treated pPSC12. *E. coli* JM109 was transformed with the ligation product, to thereby form a transformant (PC/pPSC12). PC/pPSC12 was digested with Xba I/Bgl II, and a fragment containing the target gene was recovered. The thus-produced fragment was mixed with pPSC8 which had been treated with the same enzyme, and the resultant mixture was subjected to ligation. *E. coli* JM109 was transformed with the ligation product, to thereby form a transformant. Plasmids in which fragments of the target size had been confirmed were purified, and sequenced. The sequencing was performed by use of the below-described primers and ABI Prism Big Dye Terminator Cycle Sequencing Kit Ver. 3 (Applied Biosystems). The analysis was performed by means of an automated sequencer ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequences of the primers are shown in the following sequence list by SEQ ID NOs: 5 to 10.

SEQ ID NO: 5: PC-F

SEQ ID NO: 6: PC-R

SEQ ID NO: 7: PSC2

SEQ ID NO: 8: PSC4

SEQ ID NO: 9: PC 453/472-F

SEQ ID NO: 10: PC 683/664-R

[0164] A clone in which insertion of a target gene had been confirmed was inoculated to an LB medium (100 mL) containing 50 μg/mL ampicillin, and cultivated at 30° C. for one night. Proliferated cells were collected, and plasmids were purified in accordance with an instruction manual of Plasmid Midi Kit (QIAGEN).

2. Co-Transfection

[0165] To Sf9 cells (1.0×10^6) plated in a 25-cm^2 flask was added a serum-free Sf-900 II medium (product of Invitrogen) (200 μL) containing an expression vector harboring a cDNA fragment encoding Pro-CE (2.3 μg), a linear AcNPV DNA (85 ng), and LIPOFECTIN Reagent (product of Invitrogen) (5 μL). After the culture had been allowed to stand at 28° C. for six hours, a serum-free Sf-900 II medium was further added so as to adjust the volume of the culture liquid to 5 mL. The culture was further cultivated at 28° C. for five days, and the culture supernatant was collected. The supernatant was employed as a co-transfection solution.

3. Purification of Recombinant Virus

[0166] The recombinant virus was purified through the plaque assay method. The specific procedure is as follows.

[0167] Sf9 cells (2.0×10^6) were plated onto a plate (diameter: 60 mm) and allowed to stand at 28° C. for one hour, whereby the cells were adhered to the bottom surface. The aforementioned co-transfection solution was diluted with a serum-free Sf-900 II medium at dilution factors of 10^4, 10^5, 10^6, and 10^7. An aliquot (1 mL) of each of these diluted solutions was added to the cells, followed by gentle shaking at room temperature for one hour. After removal of the plate supernatant (virus fluid), a serum-free Sf-900 II medium (4 mL) containing 0.5% SeaKemGTG agarose (product of BMA) was added to the plate, and stationary culture was performed at 28° C. for eight days. From each culture medium, six plaques of infected insect cells including no polyhedra were collected. The plaques of each medium were suspended in a serum-free Sf-900 II medium (1 mL), to thereby obtain a virus fluid.

4. Amplification of Recombinant Virus

[0168] Next, amplification of the recombinant virus (preparation of recombinant virus fluid) was performed. The specific procedure is as follows.

[0169] To Sf9 cells (2.0×10^6) plated in a 25-cm^2 flask was added each (0.5 mL) of the aforementioned virus fluids, followed by stationary cultivation at 28° C. for one hour. A serum-free Sf-900 II medium was added to the culture so as to adjust the volume of the culture liquid to 5 mL, and the culture was further stationary-cultivated for three days, to thereby yield a first-generation virus fluid.

[0170] To Sf9 cells (6.0×10^6) plated in a 75-cm^2 flask was added the entirety of the aforementioned first-generation virus fluid, followed by stationary cultivation at 28° C. for one hour. Subsequently, a serum-free Sf-900 II medium (10 mL) was added to the culture, followed by stationary cultivation for three days. After completion of cultivation, the culture supernatant was recovered and centrifuged at 3,000×g and 4° C. for 15 minutes, to thereby fractionate into the supernatant and the precipitate. The culture supernatant was recovered and employed as a second-generation virus fluid.

5. Production of Recombinant Virus Fluid

[0171] Insect cells (expresSF+) which were in the logarithmic growth phase during cultivation were diluted with a serum-free Sf-900 II medium so as to adjust the concentration

to 1.5×10^6 cells/mL, and the diluted product (50 mL) was placed in a 125-mL Erlenmeyer flask. The aforementioned second-generation virus fluid (0.5 mL) was added thereto, and the mixture was subjected to shake cultivation at 130 rpm and 28° C. for three days. After completion of cultivation, the culture liquid was centrifuged at 10,000×g and 4° C. for 30 minutes, to thereby fractionate into the supernatant and the precipitate. The culture supernatant was recovered and employed as a third-generation virus fluid.

6. Confirmation of Gene Insertion

[0172] Subsequently, insertion of a DNA fragment into a cell was confirmed through the following procedure.

[0173] The thus-recovered third-generation virus fluid (0.7 mL) was placed in a 1.5-mL microtube, and an equiamount of 20% polyethylene glycol and 1M sodium chloride solution were added to the microtube, followed by sufficiently mixing. The mixture was allowed to stand for one hour. Thereafter, the culture liquid was centrifuged at 10,000×g for 10 minutes, to thereby fractionate into the supernatant and the precipitate. The precipitate was recovered and, in accordance with a manual of QIAamp DNA Miki Kit (QIAGEN), dissolved in Buffer ALT (180 μL), whereby a viral DNA fragment was extracted. PCR was performed in the following manner by use of the thus-extracted viral DNA fragment as a template and the following primers.

SEQ ID NO: 11: PSC N3F

SEQ ID NO: 12: PSC N3R

[0174] To a 0.2-mL sample tube, the aforementioned viral DNA fragment (1 μL), 10×PCR buffer for KOD -Plus- (5 μL), 2 mM dNTPs (5 μL), 25 mM magnesium sulfate solution (2 μL), primers PSC N3F and PSC N3R (4 pmol/mL, 3.75 μL, each), KOD -Plus-DNA polymerase (product of TOYOBO) (1 μL), and sterilized pure water (19.5 μL) were added, and the mixture was sufficiently stirred. The mixture was subjected to PCR for 30 cycles, each cycle consisting of 94° C. for one minute, 58° C. for one minute, and 72° C. for four minutes.

[0175] The PCR product (10 μL) was subjected to electrophoresis on agarose gel, and the length of the amplified fragments was determined. A PCR product of a fragment having a target length was purified, and the sequences of the N-terminus and the C-terminus were determined, through use of the same reagents and apparatuses as employed in the aforementioned "1. Construction of expression vector." The following primers represented by SEQ ID NOs: 13 and 14 were employed.

SEQ ID NO: 13: PSC NF2

SEQ ID NO: 14: PSC 3

7. Titer Determination

[0176] Sf9 cells (2.0×10^6) were plated onto a plate (diameter: 60 mm) and allowed to stand at 28° C. for one hour, whereby the cells were adhered to the bottom surface. Subsequently, the culture liquid was removed. Separately, the third-generation virus fluid was diluted with a serum-free Sf-900 II medium at dilution factors of 10^5, 10^6, 10^7, and 10^8. An aliquot (1 mL) of each of these solutions was added to the plate, followed by gentle shaking at room temperature for one hour. After removal of the plate supernatant (virus fluid), a serum-free Sf-900 II medium (4 mL) containing 0.5% SeaKemGTG agarose (product of BMA) was added to the plate, and stationary culture was performed at 28° C. for seven days. In each culture medium, the number of observed plaques was counted, thereby determining the titer.

8. Expression Test

[0177] Insect cells (expresSF+) were diluted with a serum-free Sf-900 II medium so as to adjust the concentration to 1.5×10^6 cells/mL, and the diluted product (100 mL/per flask) was placed in nine 250-mL Erlenmeyer flasks. The aforementioned third-generation virus fluid was added thereto so as to attain MOIs of 0.2, 1, and 5 (3 flasks each), respectively. Each mixture was subjected to shake cultivation at 130 rpm and 28° C. for 48, 72, and 96 hours. After completion of cultivation, each culture liquid was centrifuged at 3,000×g and 4° C. for 20 minutes, to thereby fractionate into the supernatant and the precipitate.

9. Detection of Expression Product

[0178] Each of the samples collected in "8. Expression test" above was subjected to SDS-PAGE through a routine method. A protein was transferred to a blotting membrane through the semi-dry blotting method, and western blotting was performed under the following conditions. Note that the "DNA fragment encoding Pro-CE" incorporated into the virus was designed so as to express a His-Tag-bound protein.

Sample Treatment: the Supernatant was Mixed with Laemmli Sample Buffer (product of BIO-RAD), and the mixture was heated at 99° C. for three minutes. The precipitate (200 μL) was mixed with PBS (400 μL), to thereby form an aqueous suspension. Laemmli Sample Buffer was added to the suspension, and the mixture was heated at 99° C. for three minutes.

Amount of applied sample: 20 μL/lane

SDS-PAGE gel concentration: 10 to 20% gel (product of BIO-RAD)

Voltage application in SDS-PAGE: 150V, CV

Blotting membrane: PVDF

Voltage application in blotting: 15V, CV, 30 minutes

Primary antibody: Penta•His Antibody (product of QIAGEN)

Secondary antibody: Goat Anti-Mouse IgG(H+L)-HRP Conjugate (product of BIO-RAD)

Detection: Immobilon Western Chemiluminescent HRP Substrate (product of Milipore)

10. Results

[0179] FIG. **1** shows the results of sequence analysis of the target sequence of the recombinant virus. The upper column shows the analysis results, and the lower columns show the sequence of Pro-CE. As is clear from FIG. **1**, the recombinant virus was found to have the same N-terminal sequence and C-terminal sequence as those of Pro-CE. Thus, the presence of a DNA fragment having a nucleotide sequence encoding Pro-CE was confirmed in the recombinant virus.

[0180] The titer was 0.7×10^8 pfu/mL.

[0181] In the results of "9. Detection of expressed product" above, a band attributed to reaction with an anti-His-Tag antibody was observed at a target position (about 40 kDa) (FIG. **2**). In FIG. **2**, M represents a molecular weight marker, 48, 72, and 96 represent 48-hour-infection-lane, 72-hour-infection-lane, and 96-hour-infection-lane, respectively, S

represents a non-virus-infected lane, and A represents a wild-type-virus-infected lane. As is clear from FIG. **2**, expression of Pro-CE was verified.

Example 2

Detection of Recombinant Pro-CE Activity

[0182] Insect cells (expresSF+) were diluted with a serum-free Sf-900 II medium so as to adjust the concentration to 1.5×10^6 cells/mL, and the diluted product (100 mL/per flask) was placed in nine 250-mL Erlenmeyer flasks. The aforementioned third-generation virus fluid was added thereto so as to attain MOIs of 0.2, 1, and 5 (3 flasks each), respectively. Each mixture was subjected to shake cultivation at 130 rpm and 28° C. for 48, 72, and 96 hours. After completion of cultivation, each culture liquid was centrifuged at 3,000×g and 4° C. for 20 minutes, to thereby fractionate into the supernatant and the precipitate. The supernatant was preserved in a frozen state.

Sample 1: MOI=0.2, 48 hours
Sample 2: MOI=0.2, 72 hours
Sample 3: MOI=0.2, 96 hours
Sample 4: MOI=1, 48 hours
Sample 5: MOI=1, 72 hours
Sample 6: MOI=1, 96 hours
Sample 7: MOI=5, 48 hours
Sample 8: MOI=5, 72 hours
Sample 9: MOI=5, 96 hours
Sample 10: non-virus-infected cells
Sample 11: wild-type-virus-infected cells

Reagents

[0183] DS-3GII fraction: Factor G derived from a horseshoe crab hemocyte extract [product fractionated/purified with dextran sulfate Sepharose CL-6B (hereinafter referred to as DS-Sepharose)]

DS-10AII fraction: Pro-CE derived from a horseshoe crab hemocyte extract (product purified with DS-Sepharose)

BG: CSBG (1,495 ng/vial) dissolved in distilled water (1.495 mL), followed by ×10-stepwise dilution

1. Reactivity of Supernatant Fractions Employing Factor G at a BG Concentration of 1 to 100 ng/mL

[0184] Firstly, there was investigated whether or not the expressed protein maintained Pro-CE activity in each of the supernatants of the aforementioned nine samples.

[0185] Each supernatant fraction recovered after culture was 10-fold diluted with 50 mL Tris-HCl buffer (pH: 7.5) containing 150 mM NaCl. To each diluted product (25 μL), there were added DS-3GII fraction (25 μL), dextran (final concentration: 2.4%), Tris-HCl buffer (pH: 8.0) (final concentration: 80 mM), $MgSO_4$ (final concentration: 64 mM), $CaCl_2$ (final concentration: 0.4 mM), Na_2SO_4 (final concentration: 8 mM), distilled water for injection (15 μL), Boc-Leu-Gly-Arg-pNA substrate (see the aforementioned Patent Document 1) (final concentration: 0.24 mM), and a BG solution (0, 1, 10, or 100 ng/mL) (25 μL). The total volume of the sample was adjusted to 125 μL, and the sample was transferred to a Wellreader SK603, where it was allowed to react at 37° C. for two hours. The absorbance of the sample was automatically determined at a measurement wavelength of 405 nm (control wavelength: 492 nm). As a positive control, a DS-10AII fraction was employed. The measurement was carried out twice, and the average absorbance was calculated. The results are shown in FIGS. **3** and **4** and Table 1.

TABLE 1

[Endpoint Assay]

	Sample	Sample No.	Reactivity (A 405 to 492 nm)		Average
CSBG (0 ng/mL)	Recombinant Pro-CE (×10 diluted)	1) MOI = 0.2, 48 h	0.029	0.029	0.029
		2) MOI = 0.2, 72 h	0.029	0.030	0.030
		3) MOI = 0.2, 96 h	0.031	0.031	0.031
		4) MOI = 1, 48 h	0.030	0.032	0.031
		5) MOI = 1, 72 h	0.030	(0.041)	0.030
		6) MOI = 1, 96 h	0.031	0.030	0.031
		7) MOI = 5, 48 h	0.030	0.030	0.030
		8) MOI = 5, 72 h	0.030	0.030	0.030
		9) MOI = 5, 96 h	0.031	0.031	0.031
	(×10 diluted)	10) Non-infected cells	0.029	0.030	0.030
		11) Wild-type-infected	0.030	0.029	0.030
	DS-10AII fraction (control)		0.040	0.041	0.041
CSBG (1 ng/mL)	Recombinant Pro-CE (×10 diluted)	1) MOI = 0.2, 48 h	0.308	0.317	0.313
		2) MOI = 0.2, 72 h	0.462	0.461	0.462
		3) MOI = 0.2, 96 h	0.396	0.397	0.397
		4) MOI = 1, 48 h	0.332	0.331	0.332
		5) MOI = 1, 72 h	0.382	0.380	0.381
		6) MOI = 1, 96 h	0.376	0.370	0.373
		7) MOI = 5, 48 h	0.359	0.365	0.362
		8) MOI = 5, 72 h	0.401	0.396	0.399
		9) MOI = 5, 96 h	0.344	0.344	0.344
	(×10 diluted)	10) Non-infected cells	0.052	0.053	0.053
		11) Wild-type-infected	0.051	0.052	0.052
	DS-10AII fraction (control)		0.510	0.481	0.496
CSBG (10 ng/mL)	Recombinant Pro-CE (×10 diluted)	1) MOI = 0.2, 48 h	0.567	0.578	0.573
		2) MOI = 0.2, 72 h	0.612	0.642	0.627
		3) MOI = 0.2, 96 h	0.606	0.628	0.617
		4) MOI = 1, 48 h	0.516	0.518	0.517
		5) MOI = 1, 72 h	0.589	0.588	0.589
		6) MOI = 1, 96 h	0.595	0.589	0.592
		7) MOI = 5, 48 h	0.527	0.555	0.541
		8) MOI = 5, 72 h	0.605	0.597	0.601
		9) MOI = 5, 96 h	0.552	0.558	0.555
	(×10 diluted)	10) Non-infected cells	0.084	0.084	0.084
		11) Wild-type-infected	0.083	0.083	0.083
	DS-10AII fraction (control)		0.699	0.725	0.712

[0186] As shown in Table 1, the virus-infected cell culture supernatants of samples 1 to 9 each contain the enzyme in almost the same amount, indicating the presence of expression of Pro-CE in all fractions. In particular, sample 2 exhibited strong activity.

[0187] Table 2 and FIG. **5** show the results of comparison of recombinant Pro-CE of sample 2 with DS-10AII fraction in terms of in enzymatic activity.

TABLE 2

[Endpoint assay]

	Sample	CSBG concentration (ng/mL)	Reactivity (A 405 to 492 nm)		Average	ΔA
DS-3GII (X1)	DS-10AII (X1)	0	0.056	0.060	0.058	—
		1	0.440	0.434	0.437	0.379
		10	0.969	1.002	0.986	0.928
	Recombinant ProCE (X5)	0	0.051	0.049	0.050	—
		1	0.480	0.487	0.484	0.434
		10	0.988	0.986	0.987	0.937

[0188] As is clear from Table 2, the recombinant Pro-CE exhibited an enzymatic activity almost equivalent to that of Pro-CE derived from a horseshoe crab hemocyte extract.

2. Reactivity of Supernatant Fractions Employing Factors B and C at an Et Concentration of 1 to 100 ng/mL

[0189] The recombinant Pro-CE or the DS10-AII fraction (positive control, Pro-CE-eluted fraction); a DS-12BCI fraction (factor-B-C-eluted fraction) (25 μL) instead of factors B and C; and Et were added to the diluted products obtained in [1.] above. The Et concentration was adjusted to 0, 1, 10, or 100 ng/mL. The other mixture components were added in amounts and at concentrations equivalent to those employed in [1.]. The total volume of each sample was adjusted to 125 μL, and the sample was transferred to a Wellreader SK603, where it was allowed to react at 37° C. for 30 minutes. The absorbance of the sample was automatically determined at a measurement wavelength of 405 nm (control wavelength: 492 nm). The measurement was carried out twice, and the average absorbance was calculated. The results are shown in FIGS. **6** and **7** and Table 3.

TABLE 3

[Endpoint Assay]

	Sample	Sample No.	Reactivity (A 405 to 492 nm)		Average
Endotoxin (0 ng/mL)	Recombinant Pro-CE (×10 diluted)	1) MOI = 0.2, 48 h	0.036	0.035	0.036
		2) MOI = 0.2, 72 h	0.036	0.037	0.037
		3) MOI = 0.2, 96 h	0.038	0.037	0.038
		4) MOI = 1, 48 h	0.037	0.038	0.038
		5) MOI = 1, 72 h	0.037	0.039	0.038
		6) MOI = 1, 96 h	0.037	0.037	0.037
		7) MOI = 5, 48 h	0.038	0.038	0.038
		8) MOI = 5, 72 h	0.038	0.038	0.038
		9) MOI = 5, 96 h	0.038	0.039	0.039
	(×10 diluted)	10) Non-infected cells	0.036	0.038	0.037
		11) Wild-type-infected	0.039	0.038	0.039
	DS-10AII fraction (control)		0.044	0.044	0.044
Endotoxin (1 ng/mL)	Recombinant Pro-CE (×10 diluted)	1) MOI = 0.2, 48 h	0.066	0.067	0.067
		2) MOI = 0.2, 72 h	0.084	0.083	0.084
		3) MOI = 0.2, 96 h	0.070	0.072	0.071
		4) MOI = 1, 48 h	0.070	0.071	0.071
		5) MOI = 1, 72 h	0.075	0.075	0.075
		6) MOI = 1, 96 h	0.069	0.069	0.069
		7) MOI = 5, 48 h	0.074	0.075	0.075
		8) MOI = 5, 72 h	0.074	0.077	0.076
		9) MOI = 5, 96 h	0.065	0.069	0.067
	(×10 diluted)	10) Non-infected cells	0.049	0.054	0.052
		11) Wild-type-infected	0.051	0.053	0.052
	DS-10AII fraction (control)		0.156	0.143	0.150
Endotoxin (10 ng/mL)	Recombinant Pro-CE (×10 diluted)	1) MOI = 0.2, 48 h	0.306	0.276	0.291
		2) MOI = 0.2, 72 h	0.420	0.395	0.408
		3) MOI = 0.2, 96 h	0.317	0.317	0.317
		4) MOI = 1, 48 h	0.306	0.302	0.304
		5) MOI = 1, 72 h	0.333	0.338	0.336
		6) MOI = 1, 96 h	0.295	0.297	0.296
		7) MOI = 5, 48 h	0.333	0.329	0.331
		8) MOI = 5, 72 h	0.339	0.337	0.338
		9) MOI = 5, 96 h	0.271	0.268	0.270
	(×10 diluted)	10) Non-infected cells	0.146	0.147	0.147
		11) Wild-type-infected	0.146	0.145	0.146
	DS-10AII fraction (control)		0.722	0.724	0.723

TABLE 3-continued

[Endpoint Assay]

	Sample	Sample No.	Reactivity (A 405 to 492 nm)		Average
Endotoxin (100 ng/mL)	Recombinant Pro-CE (×10 diluted)	1) MOI = 0.2, 48 h	0.634	0.634	0.634
		2) MOI = 0.2, 72 h	0.736	0.806	0.771
		3) MOI = 0.2, 96 h	0.727	0.686	0.707
		4) MOI = 1, 48 h	0.662	0.650	0.656
		5) MOI = 1, 72 h	0.707	0.678	0.693
		6) MOI = 1, 96 h	0.677	0.666	0.672
		7) MOI = 5, 48 h	0.729	0.693	0.711
		8) MOI = 5, 72 h	0.720	0.699	0.710
		9) MOI = 5, 96 h	0.710	0.704	0.707
	(×10 diluted)	10) Non-infected cells	0.306	0.301	0.304
		11) Wild-type-infected	0.300	0.302	0.301
	DS-10AII fraction (control)		0.733	0.757	0.745

[0190] As is clear from Table 3, all virus-infected cell culture supernatants of samples each contain Pro-CE. In particular, sample 2 exhibited strong activity.

Example 3

Detection of Expression of Activity in a Complete Reconstitution System Employing Recombinant Pro-CE and Recombinant Factor G

Reagents

[0191] (1) recombinant factor G (culture supernatant; α-subunit:β-subunit=2:1)

(2) recombinant Pro-CE (culture supernatant of sample 2 in Example 2)

(3) BG:CSBG (1,495 ng/vial) dissolved in distilled water (1.495 mL), followed by ×10-stepwise dilution

[0192] Each of the culture supernatants (1) and (2), which had been produced through the method disclosed in Japanese Patent Application Laid-Open (kokai) No. 2006-271384 (Patent Document 2), was five-fold diluted with Tris-HCl buffer (pH: 7.5) (50 mL) containing 150 mM NaCl. In this experiment, a nucleotide sequence represented by SEQ ID NO: 21 was employed as a DNA fragment encoding α-subunit of factor G. To the thus-diluted recombinant factor G (25 μL), recombinant Pro-CE (25 μL), Tris-HCl buffer (pH: 8.0) (final concentration: 80 mM), $MgSO_4$ (final concentration: 64 mm), $CaCl_2$ (final concentration: 0.4 mM), Na_2SO_4 (final concentration: 8 mm), distilled water for injection (15 μL), dextran (final concentration: 2.4%), Boc-Leu-Gly-Arg-pNA substrate (final concentration: 0.24 mm), and a BG solution (0, 1, or 10 ng/mL) (25 μL) were added. The total volume of each sample was adjusted to 125 μL, and the sample was transferred to a Wellreader SK603, where it was allowed to react through a routine procedure. The absorbance of the sample was automatically determined at a measurement wavelength of 405 nm (control wavelength: 492 nm). The measurement was carried out twice, and the average absorbance was calculated. The results are shown in Table 4 and FIG. **8**.

TABLE 4

[Endpoint assay]

	Sample	CSBG concentration (ng/mL)	Reactivity (A 405 to 492 nm) ($\times 10^3$)		Average ($\times 10^3$)	ΔA ($\times 10^3$)
Recombinant Factor G (×5)	Recombinant ProCE (X5)	0	0.079	0.076	0.078	—
		1	0.140	0.138	0.139	0.061
		10	0.543	0.520	0.532	0.454

[0193] As is clear from Table 4 and FIG. **8**, similar to the case of a factor contained in amebocyte lysate (native factor LAL), reaction of BG proceeds in a concentration-dependent manner through employment of recombinant factor G and recombinant Pro-CE in combination. Also, the same enzymatic activity as that of native factor LAL was found to be expressed.

Example 4

Detection of Et Reaction by Use of a Complete Reconstitution System Employing Recombinant Pro-CE, Recombinant Factor B, and Recombinant Factor C

[0194] A nucleotide sequence targeting for expressing factor B in which a His-Tag sequence is attached to the C-terminus was synthesized, and the synthesized nucleotide sequence was introduced into a transfer vector (pPSC8). The thus-obtained expression vector (factor B/pPSC8) DNA fragment and a baculovirus (AcNPV) DNA fragment were co-transfected into Sf9 cells. The virus fluid obtained from the culture supernatant was purified and amplified. The viral DNA fragment was extracted from the cells and sequenced, to thereby determine the sequences of the N- and C-terminuses of the introduced gene fragment. The expresSF+ cells (equivalent to 100 mL of culture liquid) were caused to be infected with the thus-produced recombinant virus so as to attain MOIs of 0.02, 0.1, and 0.5, and culture supernatants and precipitates were recovered at hour 48, hour 72, and hour 96. The recovered expression products were analyzed through western blotting employing an anti-His-Tag antibody for confirming expression. Subsequently, Et (0, 0.01, 0.1, 1, 10, or 100 μg/mL) was added to a reaction system in the presence of this factor, recombinant factor C (product of PyroGene), and recombinant ProCE, and the system was allowed to react at 37° C. for one hour. Through analyzing the ability to hydrolyze a synthetic substrate (Boc-Leu-Gly-Arg-pNA) in the presence of Pro-CE, activation of ProCE (i.e., formation of CE) was determined.

[0195] Through the same experiment as carried out in Example 1, the activity of recombinant factor B was confirmed. In the study of reactivity of Et, the highest Et reactivity was attained in a sample (MOI=0.1, 96 h). Thus, the sample was employed for reconstitution of the factor C-cascade (Table 5 and FIG. **9**).

TABLE 5

[Endpoint Assay]

	Sample	Sample No.	Reactivity (A 405 to 492 nm)		Average
Endotoxin (0 ng/mL)	Recombinant Factor B (×5 diluted)	1) MOI = 0.02, 48 h	0.022	—	0.022
		2) MOI = 0.02, 72 h	0.022	—	0.022
		3) MOI = 0.02, 96 h	0.022	—	0.022
		4) MOI = 0.1, 48 h	0.023	—	0.023
		5) MOI = 0.1, 72 h	0.018	—	0.018
		6) MOI = 0.1, 96 h	0.018	—	0.018
		7) MOI = 0.5, 48 h	0.010	—	0.010
		8) MOI = 0.5, 72 h	0.019	—	0.019
		9) MOI = 0.5, 96 h	0.018	—	0.018
	(×5 diluted)	10) Non-infected cells	0.016	—	0.016
		11) Wild-type-infected	0.016	—	0.016
Endotoxin (10 ng/mL)	Recombinant Factor B (×5 diluted)	1) MOI = 0.02, 48 h	0.044	0.043	0.044
		2) MOI = 0.02, 72 h	0.232	0.230	0.231
		3) MOI = 0.02, 96 h	0.440	0.451	0.446
		4) MOI = 0.1, 48 h	0.340	0.318	0.329
		5) MOI = 0.1, 72 h	0.568	0.572	0.570
		6) MOI = 0.1, 96 h	0.617	0.622	0.620
		7) MOI = 0.5, 48 h	0.300	0.276	0.288
		8) MOI = 0.5, 72 h	0.478	0.462	0.470
		9) MOI = 0.5, 96 h	0.368	0.369	0.369
	(×5 diluted)	10) Non-infected cells	0.034	0.033	0.034
		11) Wild-type-infected	0.035	0.034	0.035

[0196] In order to confirm whether or not cascade reaction proceeds when a different *limulus*-derived factor is employed in combination, recombinant factor C (derived from *Tachypleus rotundicauda*), which is a commercial element of PyroGene (product of Cambrex) was used.

Reagents

[0197] (1) recombinant factor B (culture supernatant, MOI=0.1, 96 h)

(2) recombinant Pro-CE (culture supernatant of sample 2 in Example 2)

(3) recombinant factor C (PyroGene (commercial product), non-diluted)

(4) Et: *E. coli* O111:B4-derived (Product of Sigma) was processed with distilled water to 1 mg/mL, followed by ×10-stepwise dilution

[0198] In order to confirm that the experimental results are attributable to reconstitution of the cascade, the following combination samples were tested.

Sample A: recombinant Pro-CE, recombinant factor B, and recombinant factor C

Sample B: recombinant Pro-CE, recombinant factor B, and distilled water

Sample C: recombinant Pro-CE, recombinant factor C, and distilled water

Sample D: recombinant factor B, recombinant factor C, and distilled water

Sample E: recombinant Pro-CE and distilled water

Sample F: recombinant factor B and distilled water

Sample G: recombinant factor C and distilled water

[0199] The aforementioned culture supernatants (1) and (2) employed in the samples each were diluted in advance five-fold with ice-cooled Tris-HCl buffer (pH: 7.5) (50 mL) containing 150 mM NaCl. The total volume of each sample was adjusted to 60 μL.

[0200] To each sample, there were added Tris-HCl buffer (pH: 8.0) (final concentration: 80 mM), $MgSO_4$ (final concentration: 64 mM), $CaCl_2$ (final concentration: 0.4 mM), Na_2SO_4 (final concentration: 8 mM), dextran (final concentration: 2.4%), distilled water for injection (15 μL), Boc-Leu-Gly-Arg-pNA substrate (final concentration: 0.24 mM), and Et solution (0 or 100 ng/mL) (25 μL). The total volume of the sample was adjusted to 125 μL, and the sample was transferred to a Wellreader SK603, where it was allowed to react at 37° C. for 10 hours. The absorbance of the sample was automatically determined at a measurement wavelength of 405 nm (control wavelength: 492 nm). The measurement was carried out twice, and the average absorbance was calculated. The results are shown in Table 6 and FIG. **10**.

TABLE 6

Et concentration (ng/mL)	Reconstitution system	Reactivity (A405 to 492 nm)		Av.
0	(A) PCE + FB + FC	0.024	0.022	0.023
	(B) PCE + FB	0.023	0.022	0.023
	(C) PCE + FC	0.020	0.020	0.020
	(D) FB + FC	0.024	0.025	0.025
	(E) PCE	0.009	0.006	0.008
	(F) FB	0.022	0.022	0.022
	(G) FC	0.022	0.023	0.023
100	(A) PCE + FB + FC	0.652	0.645	0.649
	(B) PCE + FB	0.022	0.023	0.023
	(C) PCE + FC	0.029	0.030	0.030
	(D) FB + FC	0.032	0.034	0.033
	(E) PCE	0.006	0.006	0.006
	(F) FB	0.024	0.026	0.025
	(G) FC	0.034	0.035	0.035

[0201] The hydrolysis performance of the factors (samples A, B, and E to G) with respect to the Boc-Leu-Gly-Arg-pNA substrate in the presence of Et at high concentration was investigated. As a result, in the samples containing recombinant Pro-CE and/or recombinant factor B, activation attributed to high concentration Et was not observed. In contrast, remarkable hydrolysis activity was observed in a sample containing recombinant factor C and in the complete reconstitution system containing recombinant factors C and B and recombinant Pro-CE. However, the hydrolysis activity induced in the complete reconstitution system containing recombinant Pro-CE, recombinant B, and recombinant C was remarkably high (about some 10^3) as compared with that induced in the sample containing only recombinant factor C (FIG. **11**).

[0202] As is clear from Table 6 and FIGS. **10** and **11**, similar to the case of a native factor contained in amebocyte lysate (native factor LAL), cascade reaction of Et proceeds in a concentration-dependent manner through employment of recombinant factors C and B and recombinant Pro-CE in combination. Also, even when a different *limulus*-derived clotting factor is employed for reconstitution of the cascade, the same enzymatic activity as that of native factor LAL was found to be expressed.

Example 5

Effect of Metal Salt on Et Reaction Activity of a Complete Reconstitution System

[0203] In Et reaction in the complete reconstitution system (sample A) shown in Example 4, effects of variation amount of metal salt on Et reaction activity was investigated.

(A) Effect of Magnesium Sulfate (No Calcium Chloride or Sodium Chloride was Added)

(a) In a Concentration Range of 0 to 100 mM

[0204] In Example 5, the magnesium sulfate concentration in Et reaction in the complete reconstitution system of Example 4, which was 64 mM, was changed within the range of 0 to 100 mM, and the change in reaction activity was observed (during reaction). The results are shown in Table 7 and FIG. **12**. In the reaction, an Et solution of 0 ng/mL (control) and that of 100 ng/mL were employed.

TABLE 7

Et concentration (ng/mL)	NaCl concentration (mM: during reaction)	Reactivity (mAbs/min)		Av.
0	0	0.21	—	0.21
	6.25	0.22	—	0.22
	12.5	0.21	—	0.21
	25	0.21	—	0.21
	50	0.20	—	0.20
	100	0.19	—	0.19
	200	0.14	—	0.14
20	0	27.07	27.50	27.29
	6.25	25.83	25.79	25.81
	12.5	28.30	29.32	28.81
	25	28.18	28.65	28.42
	50	27.27	27.96	27.62
	100	21.79	21.46	21.63
	200	17.66	18.42	18.04

[0205] As is clear from Table 7 and FIG. **12**, in the complete reconstitution system of the method 1 of the present invention, the reactivity value significantly decreased in response to the increase in concentration of magnesium sulfate added to the measurement system.

(b) In a Concentration Range of 0 to 10 mM

[0206] In the reaction system (a) of the Example (Et concentration: 0 ng/mL (control) or 20 ng/mL), the magnesium sulfate concentration was changed to 0 to 10 mM during reaction, and change in activity was observed after reconstitution. The results are shown in Table 8 and FIG. **13**.

TABLE 8

Et concentration (ng/mL)	$MgSO_4$ concentration (mM: during reaction)	Reactivity (mAbs/min)		Av.
0	0	0.11	—	0.11
	1	0.10	—	0.10
	2	0.08	—	0.08
	4	0.08	—	0.08
	6	0.09	—	0.09
	8	0.08	—	0.08
	10	0.09	—	0.09
20	0	24.03	23.96	24.00
	1	22.33	22.42	22.38
	2	21.53	22.88	22.21
	4	20.22	19.59	19.91
	6	18.70	18.40	18.55
	8	15.33	15.78	15.56
	10	14.44	14.57	14.51

[0207] As is clear from Table 8 and FIG. **13**, in the complete reconstitution system of the method 1 of the present invention, even when the magnesium sulfate concentration was 10 mM or lower, Et reaction was suppressed in a concentration-dependent manner.

[0208] Thus, in Et reaction in the measurement system, activity of the factors can be clearly detected and maintained after reconstitution, even when no magnesium sulfate was added.

(B) Effects of Calcium Chloride (No Magnesium Sulfate or Sodium Chloride was Added)

[0209] In Et reaction in the complete reconstitution system of Example 4 (Et concentration: 0 (control) or 20 ng/mL), the calcium chloride concentration was changed to 0 to 5 mM during reaction, and change in activity was observed after reconstitution. The results are shown in Table 9 and FIG. **14**.

TABLE 9

Et concentration (ng/mL)	$CaCl_2$ concentration (mM: during reaction)	Reactivity (mAbs/min)		Av.
0	0	0.06	—	0.06
	0.5	0.03	—	0.03
	1	0.13	—	0.13
	2	0.36	—	0.36
	3	0.64	—	0.64
	4	0.73	—	0.73
	5	0.71	—	0.71
20	0	30.83	31.02	30.93
	0.5	21.66	22.79	22.23
	1	17.53	20.01	18.77
	2	14.09	14.60	14.35
	3	11.78	12.96	12.37
	4	9.88	10.04	9.96
	5	9.15	10.04	9.60

(Slightly turbid at a $CaCl_2$ concentration of 1 to 5 mM)

[0210] As is clear from Table 9 and FIG. **14**, in Et reaction in the complete reconstitution system, activity of the factors can be clearly detected and maintained after reconstitution, even when no calcium chloride was added. The feature is similar to the case of addition of a magnesium salt.

(C) Effects of Sodium Chloride (No Magnesium Sulfate or Calcium Chloride was Added)

[0211] In the experiment system, Cl^- was present at a concentration of 80 mM in reaction buffer (Tris-Cl, pH=8.0) during reaction. However, since Na^+ was absent in the experiment system, the buffer-derived Cl^- was not included in the sodium chloride concentration.

[0212] In Et reaction in the complete reconstitution system of Example 4, Et concentration was 0 (control) or 20 ng/mL). In Example 5, the calcium chloride concentration, which was 150 mm in Example 4, was changed in the range of 0 to 2.5 M (0, 0.078, 0.156, 0.313, 0.625, 1.25, and 2.5 (M)), and change in reactivity value was observed (during reaction). The results are shown in Table 10 and FIG. **15**.

TABLE 10

Et concentration (ng/mL)	NaCl concentration (mM: during reaction)	Reactivity (mAbs/min)		Av.
0	0	0.21	—	0.21
	6.25	0.22	—	0.22
	12.5	0.21	—	0.21
	25	0.21	—	0.21
	50	0.20	—	0.20
	100	0.19	—	0.19
	200	0.14	—	0.14
20	0	27.07	27.50	27.29
	6.25	25.83	25.79	25.81
	12.5	28.30	29.32	28.81
	25	28.18	28.65	28.42
	50	27.27	27.96	27.62
	100	21.79	21.46	21.63
	200	17.66	18.42	18.04

[0213] As is clear from Table 10 and FIG. **15**, when the sodium chloride concentration during reaction was 0 to 50 mm, virtually no effect was observed on activity after reconstitution. However, when the concentration was higher, reaction was significantly suppressed (reduction by about 30% at 200 mM).

Example 6

Substrate Specificity of Et Reaction in the Complete Reconstitution System

[0214] In Example 6, a synthetic chromogenic substrate Boc-Val-Pro-Arg-pNA (Boc-VPR-pNA) (acetate salt) (hereinafter referred to as substrate 1)—similar to a synthetic fluorescent substrate contained in an Et assay reagent (PyroGene, product of Cambrex) containing recombinant factor C as an essential component (Boc-Val-Pro-Arg-MCA)—was employed. Comparison in terms of Et reaction in the complete reconstitution system of Example 4 was made to Boc-Leu-Gly-Arg-pNA (Boc-LGR-pNA) (hereinafter referred to as substrate 2), which is an optimum substrate in conventional LAL reaction.

[0215] The each substrate (1 or 2) concentration in Et reaction was predetermined to 0.3 mM.

[0216] Table 11 and FIG. **16** show the results.

TABLE 11

Substrate	Et concentration (EU/mL)	Reactivity (mAbs/min)		Av.
Boc-LGR-pNA	0	0.10	0.09	0.10
	0.1	0.47	0.45	0.46
	1	3.87	3.76	3.82
	10	28.61	27.90	28.26
	100	50.71	52.37	51.54
	1000	70.48	70.23	70.36
Boc-VPR-pNA	0	0.11	0.09	0.10
	0.1	0.12	0.11	0.12
	1	0.12	0.14	0.13
	10	0.45	0.48	0.47
	100	3.54	3.34	3.44
	1000	15.46	15.30	15.38

[0217] As is clear from Table 11 and FIG. **16**, Et activity in the complete reconstitution system is about 200 times higher in the case of Boc-Leu-Gly-Arg-pNA (substrate 2) than that in the case of Boc-Val-Pro-Arg-pNA (substrate 1).

[0218] In an additional experiment, Et reaction in the system only containing recombinant factor C (substrate concentration: 0.3 mM (substrates 1 and 2) was investigated. As a result, reactivity value when substrate 1 was used was about 1.6 times higher than that when substrate 2 was used.

[0219] Therefore, as compared with use of recombinant factor C alone, a complete reconstitution system exhibits remarkably high reactivity (Et activity). Those skilled in the art readily understand that the same tendency may be observed when Et is changed to (1→3)-β-D-glucan. Thus, according to the present invention, detection and determination of endotoxin and (1→3)-β-D-glucan at high sensitivity and high reproducibility can be realized, even when the assay target is present in a small amount.

INDUSTRIAL APPLICABILITY

[0220] The present invention provides an in vitro tool and a method for genetically mass-producing a *limulus*-derived Pro-CE or for detecting and determining a bacterial component derived from a microorganism at high efficiency and reproducibility. The Pro-CE produced according to the present invention serves as a main factor forming, with another recombinant factor relating to LAL reaction, a reaction system. On the basis of detection and assay of an Et and BG, the method of the present invention finds a wide range of non-temporary uses, safety evaluation of pharmaceutical products and medical tools; serum diagnosis of sepsis, fungal infections, etc.; tools for detecting microorganism contamination in environmental and food hygiene fields; reagents for use in research fields.

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Met Leu Val Asn Asn Val
1               5

ttt tca cta ctg tgt ttc cca ctc ttg atg tct gtg gtt aga tgc agt      282
Phe Ser Leu Leu Cys Phe Pro Leu Leu Met Ser Val Val Arg Cys Ser
10                  15                  20

act ctc agc aga cag cgt aga cag ttt gtt ttc cct gac gag gaa gaa      330
Thr Leu Ser Arg Gln Arg Arg Gln Phe Val Phe Pro Asp Glu Glu Glu
25                  30                  35

ctt tgc tca aac cga ttt act gaa gaa gga aca tgc aaa aat gtc ttg      378
Leu Cys Ser Asn Arg Phe Thr Glu Glu Gly Thr Cys Lys Asn Val Leu
40                  45                  50

gat tgt aga ata ctt tta caa aaa aat gat tat aat tta ctc aaa gaa      426
Asp Cys Arg Ile Leu Leu Gln Lys Asn Asp Tyr Asn Leu Leu Lys Glu
55                  60                  65                  70

tca ata tgc ggc ttt gaa ggc ata aca ccc aaa gtt tgt tgt ccg aaa      474
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Ser Ile Cys Gly Phe Glu Gly Ile Thr Pro Lys Val Cys Cys Pro Lys
75                  80                  85

tca agc cat gta att tca agt aca cag gca cct cca gaa acc act acg      522
Ser Ser His Val Ile Ser Ser Thr Gln Ala Pro Pro Glu Thr Thr Thr
90                  95                  100

act gaa cgc cca cca aaa cag ata cca ccc aat ctt cct gaa gtg tgt      570
Thr Glu Arg Pro Pro Lys Gln Ile Pro Pro Asn Leu Pro Glu Val Cys
105                 110                 115

gga att cac aat act aca act acc agg att att gga ggt cgg gaa gca      618
Gly Ile His Asn Thr Thr Thr Thr Arg Ile Ile Gly Gly Arg Glu Ala
120                 125                 130

cct att gga gcc tgg ccg tgg atg act gct gtc tac ata aaa caa gga      666
Pro Ile Gly Ala Trp Pro Trp Met Thr Ala Val Tyr Ile Lys Gln Gly
135                 140                 145                 150

gga atc aga agt gtt cag tgt ggt ggc gca ctt gtc act aac agg cac      714
Gly Ile Arg Ser Val Gln Cys Gly Gly Ala Leu Val Thr Asn Arg His
155                 160                 165

gtg att aca gct tcg cac tgt gtt gta aac agt gca gga aca gat gtg      762
Val Ile Thr Ala Ser His Cys Val Val Asn Ser Ala Gly Thr Asp Val
170                 175                 180

atg cca gct gat gta ttc tcg gtt cgt ctg ggt gaa cac aat tta tac      810
Met Pro Ala Asp Val Phe Ser Val Arg Leu Gly Glu His Asn Leu Tyr
185                 190                 195

agt acc gat gac gat tcg aat cca ata gat ttt gca gtt acg tcg gtg      858
Ser Thr Asp Asp Asp Ser Asn Pro Ile Asp Phe Ala Val Thr Ser Val
200                 205                 210

aaa cat cac gaa cac ttt gta ctc gcg acg tat ttg aat gac atc gca      906
Lys His His Glu His Phe Val Leu Ala Thr Tyr Leu Asn Asp Ile Ala
215                 220                 225                 230

att cta acg tta aat gac aca gtt acg ttt aca gac aga att cga ccc      954
Ile Leu Thr Leu Asn Asp Thr Val Thr Phe Thr Asp Arg Ile Arg Pro
235                 240                 245

att tgt cta cct tat cgt aag ttg aga tac gat gat cta gca atg aga     1002
Ile Cys Leu Pro Tyr Arg Lys Leu Arg Tyr Asp Asp Leu Ala Met Arg
250                 255                 260

aaa ccg ttt atc act gga tgg gga aca aca gca ttt aac ggc cca tct     1050
Lys Pro Phe Ile Thr Gly Trp Gly Thr Thr Ala Phe Asn Gly Pro Ser
265                 270                 275

agt gca gtg ttg aga gaa gta cag tta cca ata tgg gaa cac gag gcc     1098
Ser Ala Val Leu Arg Glu Val Gln Leu Pro Ile Trp Glu His Glu Ala
280                 285                 290

tgt aga cag gcc tac gag aag gat tta aat att aca aac gtg tat atg     1146
Cys Arg Gln Ala Tyr Glu Lys Asp Leu Asn Ile Thr Asn Val Tyr Met
295                 300                 305                 310

tgt gct ggc ttt gca gat ggc ggg aag gat gct tgc cag ggt gat tct     1194
Cys Ala Gly Phe Ala Asp Gly Gly Lys Asp Ala Cys Gln Gly Asp Ser
315                 320                 325

gga ggt cca atg atg ttg cct gtt aaa acc gga gag ttt tat ctc att     1242
Gly Gly Pro Met Met Leu Pro Val Lys Thr Gly Glu Phe Tyr Leu Ile
330                 335                 340

gga att gtg tct ttc gga aag aaa tgc gca ttg cct gga ttt cct ggg     1290
Gly Ile Val Ser Phe Gly Lys Lys Cys Ala Leu Pro Gly Phe Pro Gly
345                 350                 355

gtt tac aca aaa gtg aca gag ttt tta gat tgg att gca gaa cat atg     1338
Val Tyr Thr Lys Val Thr Glu Phe Leu Asp Trp Ile Ala Glu His Met
360                 365                 370

gtg tag actaaagctg tgaatgactc catttcgaat atttaaccca tactcgcctg      1394
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Val
375

tagcaaaacg actgaagaat caagcagggg acaaaaccaa ctttttttt ttttttcctt 1454

caatagttct accatgtatt gaaaaccaac ataagacaat caaataa 1501

<210> SEQ ID NO 2
<211> LENGTH: 375
<212> TYPE: PRT
<213> ORGANISM: Tachypleus tridentatus

<400> SEQUENCE: 2

Met Leu Val Asn Asn Val Phe Ser Leu Leu Cys Phe Pro Leu Leu Met
1 5 10 15

Ser Val Val Arg Cys Ser Thr Leu Ser Arg Gln Arg Arg Gln Phe Val
20 25 30

Phe Pro Asp Glu Glu Glu Leu Cys Ser Asn Arg Phe Thr Glu Glu Gly
35 40 45

Thr Cys Lys Asn Val Leu Asp Cys Arg Ile Leu Leu Gln Lys Asn Asp
50 55 60

Tyr Asn Leu Leu Lys Glu Ser Ile Cys Gly Phe Glu Gly Ile Thr Pro
65 70 75 80

Lys Val Cys Cys Pro Lys Ser Ser His Val Ile Ser Ser Thr Gln Ala
85 90 95

Pro Pro Glu Thr Thr Thr Thr Glu Arg Pro Pro Lys Gln Ile Pro Pro
100 105 110

Asn Leu Pro Glu Val Cys Gly Ile His Asn Thr Thr Thr Thr Arg Ile
115 120 125

Ile Gly Gly Arg Glu Ala Pro Ile Gly Ala Trp Pro Trp Met Thr Ala
130 135 140

Val Tyr Ile Lys Gln Gly Gly Ile Arg Ser Val Gln Cys Gly Gly Ala
145 150 155 160

Leu Val Thr Asn Arg His Val Ile Thr Ala Ser His Cys Val Val Asn
165 170 175

Ser Ala Gly Thr Asp Val Met Pro Ala Asp Val Phe Ser Val Arg Leu
180 185 190

Gly Glu His Asn Leu Tyr Ser Thr Asp Asp Asp Ser Asn Pro Ile Asp
195 200 205

Phe Ala Val Thr Ser Val Lys His His Glu His Phe Val Leu Ala Thr
210 215 220

Tyr Leu Asn Asp Ile Ala Ile Leu Thr Leu Asn Asp Thr Val Thr Phe
225 230 235 240

Thr Asp Arg Ile Arg Pro Ile Cys Leu Pro Tyr Arg Lys Leu Arg Tyr
245 250 255

Asp Asp Leu Ala Met Arg Lys Pro Phe Ile Thr Gly Trp Gly Thr Thr
260 265 270

Ala Phe Asn Gly Pro Ser Ser Ala Val Leu Arg Glu Val Gln Leu Pro
275 280 285

Ile Trp Glu His Glu Ala Cys Arg Gln Ala Tyr Glu Lys Asp Leu Asn
290 295 300

Ile Thr Asn Val Tyr Met Cys Ala Gly Phe Ala Asp Gly Gly Lys Asp
305 310 315 320

Ala Cys Gln Gly Asp Ser Gly Gly Pro Met Met Leu Pro Val Lys Thr

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325                 330                 335

Gly Glu Phe Tyr Leu Ile Gly Ile Val Ser Phe Gly Lys Lys Cys Ala
340                 345                 350

Leu Pro Gly Phe Pro Gly Val Tyr Thr Lys Val Thr Glu Phe Leu Asp
355                 360                 365

Trp Ile Ala Glu His Met Val
370                 375


<210> SEQ ID NO 3
<211> LENGTH: 1146
<212> TYPE: DNA
<213> ORGANISM: Tachypleus tridentatus
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(1143)

<400> SEQUENCE: 3

atg ttg gtg aat aac gtg ttt tca cta ctg tgt ttc cca ctc ttg atg       48
Met Leu Val Asn Asn Val Phe Ser Leu Leu Cys Phe Pro Leu Leu Met
1               5                   10                  15

tct gtg gtt aga tgc agt act ctc agc aga cag cgt aga cag ttt gtt       96
Ser Val Val Arg Cys Ser Thr Leu Ser Arg Gln Arg Arg Gln Phe Val
20                  25                  30

ttc cct gac gag gaa gaa ctt tgc tca aac cga ttt act gaa gaa gga      144
Phe Pro Asp Glu Glu Glu Leu Cys Ser Asn Arg Phe Thr Glu Glu Gly
35                  40                  45

aca tgc aaa aat gtc ttg gat tgt aga ata ctt tta caa aaa aat gat      192
Thr Cys Lys Asn Val Leu Asp Cys Arg Ile Leu Leu Gln Lys Asn Asp
50                  55                  60

tat aat tta ctc aaa gaa tca ata tgc ggc ttt gaa ggc ata aca ccc      240
Tyr Asn Leu Leu Lys Glu Ser Ile Cys Gly Phe Glu Gly Ile Thr Pro
65                  70                  75                  80

aaa gtt tgt tgt ccg aaa tca agc cat gta att tca agt aca cag gca      288
Lys Val Cys Cys Pro Lys Ser Ser His Val Ile Ser Ser Thr Gln Ala
85                  90                  95

cct cca gaa acc act acg act gaa cgc cca cca aaa cag ata cca ccc      336
Pro Pro Glu Thr Thr Thr Thr Glu Arg Pro Pro Lys Gln Ile Pro Pro
100                 105                 110

aat ctt cct gaa gtg tgt gga att cac aat act aca act acc agg att      384
Asn Leu Pro Glu Val Cys Gly Ile His Asn Thr Thr Thr Thr Arg Ile
115                 120                 125

att gga ggt cgg gaa gca cct att gga gcc tgg ccg tgg atg act gct      432
Ile Gly Gly Arg Glu Ala Pro Ile Gly Ala Trp Pro Trp Met Thr Ala
130                 135                 140

gtc tac ata aaa caa gga gga atc aga agt gtt cag tgt ggt ggc gca      480
Val Tyr Ile Lys Gln Gly Gly Ile Arg Ser Val Gln Cys Gly Gly Ala
145                 150                 155                 160

ctt gtc act aac agg cac gtg att aca gct tcg cac tgt gtt gta aac      528
Leu Val Thr Asn Arg His Val Ile Thr Ala Ser His Cys Val Val Asn
165                 170                 175

agt gca gga aca gat gtg atg cca gct gat gta ttc tcg gtt cgt ctg      576
Ser Ala Gly Thr Asp Val Met Pro Ala Asp Val Phe Ser Val Arg Leu
180                 185                 190

ggt gaa cac aat tta tac agt acc gat gac gat tcg aat cca ata gat      624
Gly Glu His Asn Leu Tyr Ser Thr Asp Asp Asp Ser Asn Pro Ile Asp
195                 200                 205

ttt gca gtt acg tcg gtg aaa cat cac gaa cac ttt gta ctc gcg acg      672
Phe Ala Val Thr Ser Val Lys His His Glu His Phe Val Leu Ala Thr
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210                 215                 220

tat ttg aat gac atc gca att cta acg tta aat gac aca gtt acg ttt      720
Tyr Leu Asn Asp Ile Ala Ile Leu Thr Leu Asn Asp Thr Val Thr Phe
225                 230                 235                 240

aca gac aga att cga ccc att tgt cta cct tat cgt aag ttg aga tac      768
Thr Asp Arg Ile Arg Pro Ile Cys Leu Pro Tyr Arg Lys Leu Arg Tyr
245                 250                 255

gat gat cta gca atg aga aaa ccg ttt atc act gga tgg gga aca aca      816
Asp Asp Leu Ala Met Arg Lys Pro Phe Ile Thr Gly Trp Gly Thr Thr
260                 265                 270

gca ttt aac ggc cca tct agt gca gtg ttg aga gaa gta cag tta cca      864
Ala Phe Asn Gly Pro Ser Ser Ala Val Leu Arg Glu Val Gln Leu Pro
275                 280                 285

ata tgg gaa cac gag gcc tgt aga cag gcc tac gag aag gat tta aat      912
Ile Trp Glu His Glu Ala Cys Arg Gln Ala Tyr Glu Lys Asp Leu Asn
290                 295                 300

att aca aac gtg tat atg tgt gct ggc ttt gca gat ggc ggg aag gat      960
Ile Thr Asn Val Tyr Met Cys Ala Gly Phe Ala Asp Gly Gly Lys Asp
305                 310                 315                 320

gct tgc cag ggt gat tct gga ggt cca atg atg ttg cct gtt aaa acc     1008
Ala Cys Gln Gly Asp Ser Gly Gly Pro Met Met Leu Pro Val Lys Thr
325                 330                 335

gga gag ttt tat ctc att gga att gtg tct ttc gga aag aaa tgc gca     1056
Gly Glu Phe Tyr Leu Ile Gly Ile Val Ser Phe Gly Lys Lys Cys Ala
340                 345                 350

ttg cct gga ttt cct ggg gtt tac aca aaa gtg aca gag ttt tta gat     1104
Leu Pro Gly Phe Pro Gly Val Tyr Thr Lys Val Thr Glu Phe Leu Asp
355                 360                 365

tgg att gca gaa cat atg gtg cat cac cat cac cat cac tag             1146
Trp Ile Ala Glu His Met Val His His His His His His
370                 375                 380
```

<210> SEQ ID NO 4
<211> LENGTH: 381
<212> TYPE: PRT
<213> ORGANISM: Tachypleus tridentatus

<400> SEQUENCE: 4

```
Met Leu Val Asn Asn Val Phe Ser Leu Leu Cys Phe Pro Leu Leu Met
1               5                   10                  15

Ser Val Val Arg Cys Ser Thr Leu Ser Arg Gln Arg Arg Gln Phe Val
20                  25                  30

Phe Pro Asp Glu Glu Glu Leu Cys Ser Asn Arg Phe Thr Glu Glu Gly
35                  40                  45

Thr Cys Lys Asn Val Leu Asp Cys Arg Ile Leu Leu Gln Lys Asn Asp
50                  55                  60

Tyr Asn Leu Leu Lys Glu Ser Ile Cys Gly Phe Glu Gly Ile Thr Pro
65                  70                  75                  80

Lys Val Cys Cys Pro Lys Ser Ser His Val Ile Ser Ser Thr Gln Ala
85                  90                  95

Pro Pro Glu Thr Thr Thr Thr Glu Arg Pro Pro Lys Gln Ile Pro Pro
100                 105                 110

Asn Leu Pro Glu Val Cys Gly Ile His Asn Thr Thr Thr Thr Arg Ile
115                 120                 125

Ile Gly Gly Arg Glu Ala Pro Ile Gly Ala Trp Pro Trp Met Thr Ala
130                 135                 140
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Val Tyr Ile Lys Gln Gly Gly Ile Arg Ser Val Gln Cys Gly Gly Ala
145                 150                 155                 160

Leu Val Thr Asn Arg His Val Ile Thr Ala Ser His Cys Val Val Asn
165                 170                 175

Ser Ala Gly Thr Asp Val Met Pro Ala Asp Val Phe Ser Val Arg Leu
180                 185                 190

Gly Glu His Asn Leu Tyr Ser Thr Asp Asp Asp Ser Asn Pro Ile Asp
195                 200                 205

Phe Ala Val Thr Ser Val Lys His His Glu His Phe Val Leu Ala Thr
210                 215                 220

Tyr Leu Asn Asp Ile Ala Ile Leu Thr Leu Asn Asp Thr Val Thr Phe
225                 230                 235                 240

Thr Asp Arg Ile Arg Pro Ile Cys Leu Pro Tyr Arg Lys Leu Arg Tyr
245                 250                 255

Asp Asp Leu Ala Met Arg Lys Pro Phe Ile Thr Gly Trp Gly Thr Thr
260                 265                 270

Ala Phe Asn Gly Pro Ser Ser Ala Val Leu Arg Glu Val Gln Leu Pro
275                 280                 285

Ile Trp Glu His Glu Ala Cys Arg Gln Ala Tyr Glu Lys Asp Leu Asn
290                 295                 300

Ile Thr Asn Val Tyr Met Cys Ala Gly Phe Ala Asp Gly Gly Lys Asp
305                 310                 315                 320

Ala Cys Gln Gly Asp Ser Gly Gly Pro Met Met Leu Pro Val Lys Thr
325                 330                 335

Gly Glu Phe Tyr Leu Ile Gly Ile Val Ser Phe Gly Lys Lys Cys Ala
340                 345                 350

Leu Pro Gly Phe Pro Gly Val Tyr Thr Lys Val Thr Glu Phe Leu Asp
355                 360                 365

Trp Ile Ala Glu His Met Val His His His His His His
370                 375                 380


<210> SEQ ID NO 5
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Primer for sequencing

<400> SEQUENCE: 5

tctagaatgt tggtgaataa cgtgtt                                        26


<210> SEQ ID NO 6
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Primer for sequencing

<400> SEQUENCE: 6

agatctagtg atggtgatgg tgatgcacca tatgttctgc                          40


<210> SEQ ID NO 7
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
```

-continued

<223> OTHER INFORMATION: Primer for sequencing

<400> SEQUENCE: 7

tcacaaactg gaaatgtc 18

<210> SEQ ID NO 8
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Primer for sequencing

<400> SEQUENCE: 8

ccggaccagt gaacagag 18

<210> SEQ ID NO 9
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Primer for sequencing

<400> SEQUENCE: 9

aatcagaagt gttcagtgtg 20

<210> SEQ ID NO 10
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Primer for sequencing

<400> SEQUENCE: 10

tcattcaaat acgtcgcgag 20

<210> SEQ ID NO 11
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PCR Primer

<400> SEQUENCE: 11

gccattgtaa tgagacgcac a 21

<210> SEQ ID NO 12
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PCR Primer

<400> SEQUENCE: 12

cgtacaacaa ttgtctgtaa atc 23

<210> SEQ ID NO 13
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Primer for sequencing

<400> SEQUENCE: 13

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```
tagttgctga tatcatggag a                                                  21


<210> SEQ ID NO 14
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Primer for sequencing

<400> SEQUENCE: 14

tcacaaactg gaaatgtc                                                      18


<210> SEQ ID NO 15
<211> LENGTH: 1887
<212> TYPE: DNA
<213> ORGANISM: Tachypleus tridentatus
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (143)..(1345)

<400> SEQUENCE: 15

gttgaatctt accggtaaga attagataac aaatttttaa aagttacact aacagaagct     60

ttttgacgtc agtatcttat atgtattacg tattcccaat ttaattggat cgttaacgcc    120

agacaactaa ccattttctg ga atg acg tgg ata tgt gtg ata acg ttg ttt     172
Met Thr Trp Ile Cys Val Ile Thr Leu Phe
1               5                   10

gct ctg gct tct gct acg ttg ggt aac aaa gtt agt aga gtg ggg gtc      220
Ala Leu Ala Ser Ala Thr Leu Gly Asn Lys Val Ser Arg Val Gly Val
15                  20                  25

ctc ttc ccc aag aca cgg aac gac aat gag tgt aca gca aga ggg gga      268
Leu Phe Pro Lys Thr Arg Asn Asp Asn Glu Cys Thr Ala Arg Gly Gly
30                  35                  40

ttg aaa gga tcc tgc aaa tcc ctc ata gac tgt cct agt gtc ttg gct      316
Leu Lys Gly Ser Cys Lys Ser Leu Ile Asp Cys Pro Ser Val Leu Ala
45                  50                  55

acg ttg aag gac agt ttt cct gtc gtt tgc tct tgg aat ggt cga ttt      364
Thr Leu Lys Asp Ser Phe Pro Val Val Cys Ser Trp Asn Gly Arg Phe
60                  65                  70

cag cct att gtc tgc tgt cct gat gca ata gca cca cca cct gta acc      412
Gln Pro Ile Val Cys Cys Pro Asp Ala Ile Ala Pro Pro Pro Val Thr
75                  80                  85                  90

aca aca gct gta act gta ata tct aca aaa gaa cca aag ctt cca aga      460
Thr Thr Ala Val Thr Val Ile Ser Thr Lys Glu Pro Lys Leu Pro Arg
95                  100                 105

tta cat ata tca ggt tgt gga aaa aga aaa gtc aaa ata gat att aca      508
Leu His Ile Ser Gly Cys Gly Lys Arg Lys Val Lys Ile Asp Ile Thr
110                 115                 120

act gtt gga cgc tct gga tca cca ata ctt cct ccg ata tct act cct      556
Thr Val Gly Arg Ser Gly Ser Pro Ile Leu Pro Pro Ile Ser Thr Pro
125                 130                 135

caa aat tca aca ggt ggg aga gga att att gct gga ggc gta gaa gcc      604
Gln Asn Ser Thr Gly Gly Arg Gly Ile Ile Ala Gly Gly Val Glu Ala
140                 145                 150

aaa att ggc gcg tgg cct tgg atg gca gct gtt ttt gtg aaa aac ttt      652
Lys Ile Gly Ala Trp Pro Trp Met Ala Ala Val Phe Val Lys Asn Phe
155                 160                 165                 170

ggc att ggc aga ttc cac tgt gct ggt agc ata atc agt aac aag tac      700
Gly Ile Gly Arg Phe His Cys Ala Gly Ser Ile Ile Ser Asn Lys Tyr
175                 180                 185
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att ttg tca gct gcc cac gcc ttc ctt atc gga ggt cga aag ttg acc      748
Ile Leu Ser Ala Ala His Ala Phe Leu Ile Gly Gly Arg Lys Leu Thr
190                 195                 200

cca act cgc tta gct gtc cgt gtg gga ggc cac tac ata aag agg ggt      796
Pro Thr Arg Leu Ala Val Arg Val Gly Gly His Tyr Ile Lys Arg Gly
205                 210                 215

caa gag tat cca gtg aaa gac gtg att atc cat cct cat tat gta gaa      844
Gln Glu Tyr Pro Val Lys Asp Val Ile Ile His Pro His Tyr Val Glu
220                 225                 230

aag gag aac tac aat gat ata gcc ata atc gag tta aaa gag gaa ctg      892
Lys Glu Asn Tyr Asn Asp Ile Ala Ile Ile Glu Leu Lys Glu Glu Leu
235                 240                 245                 250

aac ttt acg gac ttg gtc aat cct ata tgt ctc cct gat cca gag aca      940
Asn Phe Thr Asp Leu Val Asn Pro Ile Cys Leu Pro Asp Pro Glu Thr
255                 260                 265

gta acg gat cca tta aaa gac aga att gtg act gca gcg gga tgg ggc      988
Val Thr Asp Pro Leu Lys Asp Arg Ile Val Thr Ala Ala Gly Trp Gly
270                 275                 280

gat ctg gat ttc tcc ggt cca cgg agc caa gtt cta cgt gag gta agc     1036
Asp Leu Asp Phe Ser Gly Pro Arg Ser Gln Val Leu Arg Glu Val Ser
285                 290                 295

atc cca gtt gtt cca gtt gat aaa tgt gat caa gcc tat gag aaa ctc     1084
Ile Pro Val Val Pro Val Asp Lys Cys Asp Gln Ala Tyr Glu Lys Leu
300                 305                 310

aac acc cct tca cta aaa aat ggg ata acg aat aac ttc ctt tgc gct     1132
Asn Thr Pro Ser Leu Lys Asn Gly Ile Thr Asn Asn Phe Leu Cys Ala
315                 320                 325                 330

gga ttg gaa gaa gga ggg aaa gac gct tgc caa ggc gat tct ggt gga     1180
Gly Leu Glu Glu Gly Gly Lys Asp Ala Cys Gln Gly Asp Ser Gly Gly
335                 340                 345

ccg ttg atg cta gtg aac aac act agg tgg ata gta gta gga gtt gtg     1228
Pro Leu Met Leu Val Asn Asn Thr Arg Trp Ile Val Val Gly Val Val
350                 355                 360

tcg ttc ggg cac aag tgt gcc gag gaa gga tat cct ggt gtg tac tcg     1276
Ser Phe Gly His Lys Cys Ala Glu Glu Gly Tyr Pro Gly Val Tyr Ser
365                 370                 375

cgc gta gcg agt tac cta gac tgg atc gcg aaa gtt acg aac tcg tta     1324
Arg Val Ala Ser Tyr Leu Asp Trp Ile Ala Lys Val Thr Asn Ser Leu
380                 385                 390

gat cat gcc gtc act aac tga ttgtgcgtta aacaacatat tttgttgtga        1375
Asp His Ala Val Thr Asn
395                 400

agaaatactg tccaaacgta acttttgaac ttgtattatg tattaacgta ttataagttc   1435

tagttttggt ttcaaccact agatgacgca aatgctcgta ttgtccaaca gctttattcc   1495

ttttgaagaa tatttttttcg cacctacaag cttgtgaaac ttgtagataa tagttattcg   1555

taattttctc attgttttta caatttttgt gtgatctgtt tttttatttt aactttgaag   1615

taccggaatg aaaatattta attttatatt tcattaattc aaaacgtttt tgttctgggc   1675

tgccatgtaa attgtataat aagtcgtggt tgtttagtaa gttatattat cggtagagtg   1735

tacgttaaga tctgactgtg aattttgaag aaaaacctta catctaaaca ttttatcttt   1795

actgactgta gttatttatc tctaatgttc agcgtgttta gccagtaatg taaatgtgaa   1855

ttataattga ttaaagtttg attgaaatgt tt                                1887
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<210> SEQ ID NO 16
<211> LENGTH: 400
<212> TYPE: PRT
<213> ORGANISM: Tachypleus tridentatus

<400> SEQUENCE: 16

Met Thr Trp Ile Cys Val Ile Thr Leu Phe Ala Leu Ala Ser Ala Thr
1 5 10 15

Leu Gly Asn Lys Val Ser Arg Val Gly Val Leu Phe Pro Lys Thr Arg
20 25 30

Asn Asp Asn Glu Cys Thr Ala Arg Gly Gly Leu Lys Gly Ser Cys Lys
35 40 45

Ser Leu Ile Asp Cys Pro Ser Val Leu Ala Thr Leu Lys Asp Ser Phe
50 55 60

Pro Val Val Cys Ser Trp Asn Gly Arg Phe Gln Pro Ile Val Cys Cys
65 70 75 80

Pro Asp Ala Ile Ala Pro Pro Pro Val Thr Thr Thr Ala Val Thr Val
85 90 95

Ile Ser Thr Lys Glu Pro Lys Leu Pro Arg Leu His Ile Ser Gly Cys
100 105 110

Gly Lys Arg Lys Val Lys Ile Asp Ile Thr Thr Val Gly Arg Ser Gly
115 120 125

Ser Pro Ile Leu Pro Pro Ile Ser Thr Pro Gln Asn Ser Thr Gly Gly
130 135 140

Arg Gly Ile Ile Ala Gly Gly Val Glu Ala Lys Ile Gly Ala Trp Pro
145 150 155 160

Trp Met Ala Ala Val Phe Val Lys Asn Phe Gly Ile Gly Arg Phe His
165 170 175

Cys Ala Gly Ser Ile Ile Ser Asn Lys Tyr Ile Leu Ser Ala Ala His
180 185 190

Ala Phe Leu Ile Gly Gly Arg Lys Leu Thr Pro Thr Arg Leu Ala Val
195 200 205

Arg Val Gly Gly His Tyr Ile Lys Arg Gly Gln Glu Tyr Pro Val Lys
210 215 220

Asp Val Ile Ile His Pro His Tyr Val Glu Lys Glu Asn Tyr Asn Asp
225 230 235 240

Ile Ala Ile Ile Glu Leu Lys Glu Glu Leu Asn Phe Thr Asp Leu Val
245 250 255

Asn Pro Ile Cys Leu Pro Asp Pro Glu Thr Val Thr Asp Pro Leu Lys
260 265 270

Asp Arg Ile Val Thr Ala Ala Gly Trp Gly Asp Leu Asp Phe Ser Gly
275 280 285

Pro Arg Ser Gln Val Leu Arg Glu Val Ser Ile Pro Val Val Pro Val
290 295 300

Asp Lys Cys Asp Gln Ala Tyr Glu Lys Leu Asn Thr Pro Ser Leu Lys
305 310 315 320

Asn Gly Ile Thr Asn Asn Phe Leu Cys Ala Gly Leu Glu Glu Gly Gly
325 330 335

Lys Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Met Leu Val Asn
340 345 350

Asn Thr Arg Trp Ile Val Val Gly Val Val Ser Phe Gly His Lys Cys
355 360 365

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Ala Glu Glu Gly Tyr Pro Gly Val Tyr Ser Arg Val Ala Ser Tyr Leu
370                 375                 380

Asp Trp Ile Ala Lys Val Thr Asn Ser Leu Asp His Ala Val Thr Asn
385                 390                 395                 400


<210> SEQ ID NO 17
<211> LENGTH: 2394
<212> TYPE: DNA
<213> ORGANISM: Tachypleus tridentatus
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (57)..(2078)

<400> SEQUENCE: 17

gtgggggttt tagttgaaac agtaaatacg ttaactgttt aatcttgtta attgca atg     59
Met
1

ttg gtg ttg ctg tgt tgt gtt gtt ttg cat gtt ggt gtt gca aga att      107
Leu Val Leu Leu Cys Cys Val Val Leu His Val Gly Val Ala Arg Ile
5                   10                  15

tgc tgt agc cac gaa cca aag tgg cag ctc gtc tgg tcg gat gaa ttt      155
Cys Cys Ser His Glu Pro Lys Trp Gln Leu Val Trp Ser Asp Glu Phe
20                  25                  30

acc aat gga ata agt tct gat tgg gaa ttt gaa atg ggc aat ggc ctc      203
Thr Asn Gly Ile Ser Ser Asp Trp Glu Phe Glu Met Gly Asn Gly Leu
35                  40                  45

aat ggt tgg ggt aat aac gaa ctg caa tat tat cgt cgt gaa aat gcc      251
Asn Gly Trp Gly Asn Asn Glu Leu Gln Tyr Tyr Arg Arg Glu Asn Ala
50                  55                  60                  65

caa gtt gag gga ggg aaa ctg gta att act gct aaa aga gaa gac tat      299
Gln Val Glu Gly Gly Lys Leu Val Ile Thr Ala Lys Arg Glu Asp Tyr
70                  75                  80

gat ggc ttc aaa tac act tct gct agg ctg aaa acc cag ttt gat aaa      347
Asp Gly Phe Lys Tyr Thr Ser Ala Arg Leu Lys Thr Gln Phe Asp Lys
85                  90                  95

tct tgg aag tat ggt aaa att gaa gcc aaa atg gcg att cca tca ttt      395
Ser Trp Lys Tyr Gly Lys Ile Glu Ala Lys Met Ala Ile Pro Ser Phe
100                 105                 110

cgg gga gtc tgg gtg atg ttc tgg atg tca gga gac aac act aat tat      443
Arg Gly Val Trp Val Met Phe Trp Met Ser Gly Asp Asn Thr Asn Tyr
115                 120                 125

gtt aga tgg cca tct tct ggt gaa att gac ttt att gaa cat aga aac      491
Val Arg Trp Pro Ser Ser Gly Glu Ile Asp Phe Ile Glu His Arg Asn
130                 135                 140                 145

act aac aat gaa aaa gtc aga gga act att cac tgg tcc act cct gac      539
Thr Asn Asn Glu Lys Val Arg Gly Thr Ile His Trp Ser Thr Pro Asp
150                 155                 160

ggt gct cat gcg cat cat aac aga gaa agt aat aca aat ggg att gat      587
Gly Ala His Ala His His Asn Arg Glu Ser Asn Thr Asn Gly Ile Asp
165                 170                 175

tat cac att tat tct gta gag tgg aat tct tcc att gtt aaa tgg ttt      635
Tyr His Ile Tyr Ser Val Glu Trp Asn Ser Ser Ile Val Lys Trp Phe
180                 185                 190

gtt aat gga aat caa tac ttt gaa gtg aaa att cag gga gga gta aat      683
Val Asn Gly Asn Gln Tyr Phe Glu Val Lys Ile Gln Gly Gly Val Asn
195                 200                 205

ggg aaa agt gca ttt cgt aac aaa gtt ttc gtt att tta aac atg gcg      731
Gly Lys Ser Ala Phe Arg Asn Lys Val Phe Val Ile Leu Asn Met Ala
210                 215                 220                 225
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att ggt gga aac tgg cca gga ttc gat gtt gct gac gag gct ttc cct      779
Ile Gly Gly Asn Trp Pro Gly Phe Asp Val Ala Asp Glu Ala Phe Pro
230                 235                 240

gct aaa atg tac att gat tat gtc cgt gta tac cag gat gcc agt aca      827
Ala Lys Met Tyr Ile Asp Tyr Val Arg Val Tyr Gln Asp Ala Ser Thr
245                 250                 255

tct tct cct gtt ggg gat acc tct tta gat ggt tac tat ttt gtc caa      875
Ser Ser Pro Val Gly Asp Thr Ser Leu Asp Gly Tyr Tyr Phe Val Gln
260                 265                 270

aac agg cac agt gaa ttg tat ctt gat gtc act gat gcc agt aac gaa      923
Asn Arg His Ser Glu Leu Tyr Leu Asp Val Thr Asp Ala Ser Asn Glu
275                 280                 285

gat gga gca ttt ctg caa caa tgg tct tat agt ggt aat gag aac caa      971
Asp Gly Ala Phe Leu Gln Gln Trp Ser Tyr Ser Gly Asn Glu Asn Gln
290                 295                 300                 305

cag ttt gat ttt gag cat ctc gaa aat aat gtt tat aaa att act aat     1019
Gln Phe Asp Phe Glu His Leu Glu Asn Asn Val Tyr Lys Ile Thr Asn
310                 315                 320

aaa aaa agt gga aaa tct ttg gat gtt tat aat ttt ggg act gag aat     1067
Lys Lys Ser Gly Lys Ser Leu Asp Val Tyr Asn Phe Gly Thr Glu Asn
325                 330                 335

ggt gtt aga atc caa cag tgg tca tat gga ggg gct cgc aat cag cag     1115
Gly Val Arg Ile Gln Gln Trp Ser Tyr Gly Gly Ala Arg Asn Gln Gln
340                 345                 350

ttt act gta caa agt gtt ggt gat ggt tat tat aag att att cca cgc     1163
Phe Thr Val Gln Ser Val Gly Asp Gly Tyr Tyr Lys Ile Ile Pro Arg
355                 360                 365

ggc agt gga aag tta gtg gaa gta gca gat ttt agt aaa gat gca gga     1211
Gly Ser Gly Lys Leu Val Glu Val Ala Asp Phe Ser Lys Asp Ala Gly
370                 375                 380                 385

ggg aag ata caa caa tgg tct gat aac aac caa tta tct gga cag tgg     1259
Gly Lys Ile Gln Gln Trp Ser Asp Asn Asn Gln Leu Ser Gly Gln Trp
390                 395                 400

aaa ctt att aaa agt aaa agt tat tct aaa tta att cag gca gaa agt     1307
Lys Leu Ile Lys Ser Lys Ser Tyr Ser Lys Leu Ile Gln Ala Glu Ser
405                 410                 415

tat ttt gat tcc tca aaa gta caa ttg gaa gat acc tca gat gta gga     1355
Tyr Phe Asp Ser Ser Lys Val Gln Leu Glu Asp Thr Ser Asp Val Gly
420                 425                 430

ggt ggg aag aat gtt aaa tgt gat aat gaa gga gcc tgg atg gct tat     1403
Gly Gly Lys Asn Val Lys Cys Asp Asn Glu Gly Ala Trp Met Ala Tyr
435                 440                 445

aag gat att gat ttc ccc agt tca ggt aat tat cga ata gaa tac aga     1451
Lys Asp Ile Asp Phe Pro Ser Ser Gly Asn Tyr Arg Ile Glu Tyr Arg
450                 455                 460                 465

gta gca agt gaa cgt gca gga gga aag ctg tct ctg gat ttg aat gca     1499
Val Ala Ser Glu Arg Ala Gly Gly Lys Leu Ser Leu Asp Leu Asn Ala
470                 475                 480

ggc tct ata gtt ctt ggc atg ctg gat gtt cct tca aca gga gga tgg     1547
Gly Ser Ile Val Leu Gly Met Leu Asp Val Pro Ser Thr Gly Gly Trp
485                 490                 495

cag aag tgg acc acc att tcc cat aca gtg aat gtg gat tca ggt aca     1595
Gln Lys Trp Thr Thr Ile Ser His Thr Val Asn Val Asp Ser Gly Thr
500                 505                 510

tat aac ttg ggg atc tat gtt caa cga gcc agc tgg aat atc aac tgg     1643
Tyr Asn Leu Gly Ile Tyr Val Gln Arg Ala Ser Trp Asn Ile Asn Trp
515                 520                 525
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ata aag att aca aaa ata cct gaa cag tca aat ttg aat caa ggg cgt     1691
Ile Lys Ile Thr Lys Ile Pro Glu Gln Ser Asn Leu Asn Gln Gly Arg
530                 535                 540                 545

cgt aat tct aaa tta att cag gca gaa agt tat ttt agt tac tca gaa     1739
Arg Asn Ser Lys Leu Ile Gln Ala Glu Ser Tyr Phe Ser Tyr Ser Glu
550                 555                 560

gta caa ctg gaa gat acc tta gat gta gga ggt gga aag aat gtt aaa     1787
Val Gln Leu Glu Asp Thr Leu Asp Val Gly Gly Gly Lys Asn Val Lys
565                 570                 575

tgt gat aaa gaa ggg gcc tgg atg gct tac aag gat att gat ttc ccc     1835
Cys Asp Lys Glu Gly Ala Trp Met Ala Tyr Lys Asp Ile Asp Phe Pro
580                 585                 590

agt tca gga agt tat cga gta gaa tac aga gtg gca agt gaa cgt gca     1883
Ser Ser Gly Ser Tyr Arg Val Glu Tyr Arg Val Ala Ser Glu Arg Ala
595                 600                 605

gga gga aag ctg tcc cta gat ttg aat gca ggc tct ata gtg ctt ggc     1931
Gly Gly Lys Leu Ser Leu Asp Leu Asn Ala Gly Ser Ile Val Leu Gly
610                 615                 620                 625

atg ctg gat att cct tca aca gga gga ttg cag aag tgg acc acc att     1979
Met Leu Asp Ile Pro Ser Thr Gly Gly Leu Gln Lys Trp Thr Thr Ile
630                 635                 640

tct cat ata gtg aat gtg gat tta ggt aca tat aac ttg gga att tat     2027
Ser His Ile Val Asn Val Asp Leu Gly Thr Tyr Asn Leu Gly Ile Tyr
645                 650                 655

gtt caa aaa gcc agt tgg aat atc aat tgg att aga att aca aaa gtg     2075
Val Gln Lys Ala Ser Trp Asn Ile Asn Trp Ile Arg Ile Thr Lys Val
660                 665                 670

tag gatacaagag caaaccaatt gtattatttt gaagaaacaa cagctgttga          2128

ccataatctt tgttcattga gaatttatcc aactgttata gaatctatca cctttccaga   2188

tgtacgcatt gctgatggtt ttgaactaat aaatgaggag attataagtg ctaatgtgtt   2248

tgttatatct ttaatttta aaaacaaatt atcaactaac ttttcaattc aggcatggtg   2308

tttctctttt taatctgtat ttctaataaa ttaatgtctt taagagttgt tttgtttaca   2368

ataaataaag tttgattgtg tgggat                                       2394
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<210> SEQ ID NO 18
<211> LENGTH: 673
<212> TYPE: PRT
<213> ORGANISM: Tachypleus tridentatus

<400> SEQUENCE: 18

```
Met Leu Val Leu Leu Cys Cys Val Val Leu His Val Gly Val Ala Arg
1               5                   10                  15

Ile Cys Cys Ser His Glu Pro Lys Trp Gln Leu Val Trp Ser Asp Glu
20                  25                  30

Phe Thr Asn Gly Ile Ser Ser Asp Trp Glu Phe Glu Met Gly Asn Gly
35                  40                  45

Leu Asn Gly Trp Gly Asn Asn Glu Leu Gln Tyr Tyr Arg Arg Glu Asn
50                  55                  60

Ala Gln Val Glu Gly Gly Lys Leu Val Ile Thr Ala Lys Arg Glu Asp
65                  70                  75                  80

Tyr Asp Gly Phe Lys Tyr Thr Ser Ala Arg Leu Lys Thr Gln Phe Asp
85                  90                  95

Lys Ser Trp Lys Tyr Gly Lys Ile Glu Ala Lys Met Ala Ile Pro Ser
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100                 105                 110

Phe Arg Gly Val Trp Val Met Phe Trp Met Ser Gly Asp Asn Thr Asn
115                 120                 125

Tyr Val Arg Trp Pro Ser Ser Gly Glu Ile Asp Phe Ile Glu His Arg
130                 135                 140

Asn Thr Asn Asn Glu Lys Val Arg Gly Thr Ile His Trp Ser Thr Pro
145                 150                 155                 160

Asp Gly Ala His Ala His His Asn Arg Glu Ser Asn Thr Asn Gly Ile
165                 170                 175

Asp Tyr His Ile Tyr Ser Val Glu Trp Asn Ser Ser Ile Val Lys Trp
180                 185                 190

Phe Val Asn Gly Asn Gln Tyr Phe Glu Val Lys Ile Gln Gly Gly Val
195                 200                 205

Asn Gly Lys Ser Ala Phe Arg Asn Lys Val Phe Val Ile Leu Asn Met
210                 215                 220

Ala Ile Gly Gly Asn Trp Pro Gly Phe Asp Val Ala Asp Glu Ala Phe
225                 230                 235                 240

Pro Ala Lys Met Tyr Ile Asp Tyr Val Arg Val Tyr Gln Asp Ala Ser
245                 250                 255

Thr Ser Ser Pro Val Gly Asp Thr Ser Leu Asp Gly Tyr Tyr Phe Val
260                 265                 270

Gln Asn Arg His Ser Glu Leu Tyr Leu Asp Val Thr Asp Ala Ser Asn
275                 280                 285

Glu Asp Gly Ala Phe Leu Gln Gln Trp Ser Tyr Ser Gly Asn Glu Asn
290                 295                 300

Gln Gln Phe Asp Phe Glu His Leu Glu Asn Asn Val Tyr Lys Ile Thr
305                 310                 315                 320

Asn Lys Lys Ser Gly Lys Ser Leu Asp Val Tyr Asn Phe Gly Thr Glu
325                 330                 335

Asn Gly Val Arg Ile Gln Gln Trp Ser Tyr Gly Gly Ala Arg Asn Gln
340                 345                 350

Gln Phe Thr Val Gln Ser Val Gly Asp Gly Tyr Tyr Lys Ile Ile Pro
355                 360                 365

Arg Gly Ser Gly Lys Leu Val Glu Val Ala Asp Phe Ser Lys Asp Ala
370                 375                 380

Gly Gly Lys Ile Gln Gln Trp Ser Asp Asn Asn Gln Leu Ser Gly Gln
385                 390                 395                 400

Trp Lys Leu Ile Lys Ser Lys Ser Tyr Ser Lys Leu Ile Gln Ala Glu
405                 410                 415

Ser Tyr Phe Asp Ser Ser Lys Val Gln Leu Glu Asp Thr Ser Asp Val
420                 425                 430

Gly Gly Gly Lys Asn Val Lys Cys Asp Asn Glu Gly Ala Trp Met Ala
435                 440                 445

Tyr Lys Asp Ile Asp Phe Pro Ser Ser Gly Asn Tyr Arg Ile Glu Tyr
450                 455                 460

Arg Val Ala Ser Glu Arg Ala Gly Gly Lys Leu Ser Leu Asp Leu Asn
465                 470                 475                 480

Ala Gly Ser Ile Val Leu Gly Met Leu Asp Val Pro Ser Thr Gly Gly
485                 490                 495

Trp Gln Lys Trp Thr Thr Ile Ser His Thr Val Asn Val Asp Ser Gly
500                 505                 510
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Thr Tyr Asn Leu Gly Ile Tyr Val Gln Arg Ala Ser Trp Asn Ile Asn
515                 520                 525

Trp Ile Lys Ile Thr Lys Ile Pro Glu Gln Ser Asn Leu Asn Gln Gly
530                 535                 540

Arg Arg Asn Ser Lys Leu Ile Gln Ala Glu Ser Tyr Phe Ser Tyr Ser
545                 550                 555                 560

Glu Val Gln Leu Glu Asp Thr Leu Asp Val Gly Gly Gly Lys Asn Val
565                 570                 575

Lys Cys Asp Lys Glu Gly Ala Trp Met Ala Tyr Lys Asp Ile Asp Phe
580                 585                 590

Pro Ser Ser Gly Ser Tyr Arg Val Glu Tyr Arg Val Ala Ser Glu Arg
595                 600                 605

Ala Gly Gly Lys Leu Ser Leu Asp Leu Asn Ala Gly Ser Ile Val Leu
610                 615                 620

Gly Met Leu Asp Ile Pro Ser Thr Gly Gly Leu Gln Lys Trp Thr Thr
625                 630                 635                 640

Ile Ser His Ile Val Asn Val Asp Leu Gly Thr Tyr Asn Leu Gly Ile
645                 650                 655

Tyr Val Gln Lys Ala Ser Trp Asn Ile Asn Trp Ile Arg Ile Thr Lys
660                 665                 670

Val
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<210> SEQ ID NO 19
<211> LENGTH: 1979
<212> TYPE: DNA
<213> ORGANISM: Tachypleus tridentatus
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (101)..(1030)

<400> SEQUENCE: 19

```
gaagacaaga gagttgaaac aaccatagcc tgtttgctta tgactttcaa taagagatac     60

tcggcttaaa gggaactgac ttattcgtag aggctatacc atg gat atc agt ttc      115
Met Asp Ile Ser Phe
1               5

ctg gtt ttt atc aca ctg tct atg gct ctc ttc tcg agc aac gtg aca      163
Leu Val Phe Ile Thr Leu Ser Met Ala Leu Phe Ser Ser Asn Val Thr
10                  15                  20

gga acg tca gta aca tca agg gta cga cgt gga ata aat gaa aaa cat      211
Gly Thr Ser Val Thr Ser Arg Val Arg Arg Gly Ile Asn Glu Lys His
25                  30                  35

tgt ggg ttc cga cca gta att aca aga att att ggt gga gga ata gcg      259
Cys Gly Phe Arg Pro Val Ile Thr Arg Ile Ile Gly Gly Gly Ile Ala
40                  45                  50

acg cct cat tca tgg ccg tgg atg gtt gga att ttc aaa gta aat cct      307
Thr Pro His Ser Trp Pro Trp Met Val Gly Ile Phe Lys Val Asn Pro
55                  60                  65

cac cgt ttc ctt tgt ggt gga tct att att aat aaa gtc tct gtt gtt      355
His Arg Phe Leu Cys Gly Gly Ser Ile Ile Asn Lys Val Ser Val Val
70                  75                  80                  85

act gcc gcc cat tgt ctt gtg acg cag ttt gga aac aga cag aat tat      403
Thr Ala Ala His Cys Leu Val Thr Gln Phe Gly Asn Arg Gln Asn Tyr
90                  95                  100

tct atc ttc gta aga gtt gga gcc cat gac ata gac aat tcg ggt aca      451
Ser Ile Phe Val Arg Val Gly Ala His Asp Ile Asp Asn Ser Gly Thr
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105                 110                 115

aat tat caa gtg gat aaa gtt att gtt cac cag ggc tac aaa cac cat      499
Asn Tyr Gln Val Asp Lys Val Ile Val His Gln Gly Tyr Lys His His
120                 125                 130

tca cac tac tac gat atc ggt ttg att tta ctc tcg aaa cca gtc gaa      547
Ser His Tyr Tyr Asp Ile Gly Leu Ile Leu Leu Ser Lys Pro Val Glu
135                 140                 145

tac aac gac aaa ata cag cct gtc tgt att cct gag ttc aac aaa cct      595
Tyr Asn Asp Lys Ile Gln Pro Val Cys Ile Pro Glu Phe Asn Lys Pro
150                 155                 160                 165

cac gtg aac ttg aac aat att aag gtc gtc att act ggt tgg ggt gtt      643
His Val Asn Leu Asn Asn Ile Lys Val Val Ile Thr Gly Trp Gly Val
170                 175                 180

act ggg aaa gct act gag aaa cgt aac gtt ctt cgt gaa ttg gag ttg      691
Thr Gly Lys Ala Thr Glu Lys Arg Asn Val Leu Arg Glu Leu Glu Leu
185                 190                 195

ccc gtg gtt aca aac gaa cag tgc aac aaa tct tat cag aca ctc cca      739
Pro Val Val Thr Asn Glu Gln Cys Asn Lys Ser Tyr Gln Thr Leu Pro
200                 205                 210

ttc tca aaa ttg aac cga gga atc act aac gac atg att tgt gcg ggg      787
Phe Ser Lys Leu Asn Arg Gly Ile Thr Asn Asp Met Ile Cys Ala Gly
215                 220                 225

ttt ccg gaa gga ggg aaa gat gct tgt cag ggc gac tct ggt ggt ccc      835
Phe Pro Glu Gly Gly Lys Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro
230                 235                 240                 245

ctg atg tat cag aat cca aca aca gga aga gtg aaa ata gtt gga gtt      883
Leu Met Tyr Gln Asn Pro Thr Thr Gly Arg Val Lys Ile Val Gly Val
250                 255                 260

gta tca ttt ggg ttc gaa tgt gct cgt ccc aac ttc ccc ggt gtt tac      931
Val Ser Phe Gly Phe Glu Cys Ala Arg Pro Asn Phe Pro Gly Val Tyr
265                 270                 275

acg cgc ctc tcg agc tac gtt aac tgg ctc cag gaa atc acc ttc gga      979
Thr Arg Leu Ser Ser Tyr Val Asn Trp Leu Gln Glu Ile Thr Phe Gly
280                 285                 290

cag tca ctc gct tct tta ttt gaa gtt gta cca ata ttt ata ccc gag     1027
Gln Ser Leu Ala Ser Leu Phe Glu Val Val Pro Ile Phe Ile Pro Glu
295                 300                 305

tga gactgaagat aaatattgaa gagaaatcta gaataatgta caatataaga          1080

agcctgaaat tactgaaata gaaaggcgcg tgatgagaaa tacgtttcaa attttatttt   1140

ttattaactt tattgtgttt aactattctt tacgtgggac atgaaatata aatctttatt   1200

tcttctttat atactttaga ttttcatttc atctatcttt atcagttttg taatgttact   1260

aataatattt cttatggcac ggatcgagcc tcgtgaatca cagtaaataa taataattat   1320

aaaatcacac attattaaaa gcaatagcat tcagagtgag taacatataa acttcactat   1380

gagtggactt ttttattcac attttaagtt cattactaac tgttgggagg tctttatatt   1440

gttgtatatt tatatattaa ttaggttggt ttagtacatt gttgttaatg gtggaatagg   1500

gcgtaggttt taaatgtgtt tgcaaaaaaa caaacaaaac aagtaatggt ggatgatggt   1560

tccaaagtaa ccgaaagaac actttgaaca ttttttataca aaaatttatg ttttaaaata   1620

cgagtatata caatcgatct ctaagtacaa gaaaaactga agtgttcatt caggtttaac   1680

agtgcaactt aaatcaacag ttagttgttc actaaacatt acaatttgat cctttataaa   1740

cgctaatact gtttaaacag tcagtaataa tacagtatca tagcatatca tatatgaagg   1800
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tattttaaca ttctatatac aaagccagaa ttgaaaacgg taatattttg tacgattagt   1860

gaattattgt ttttaagaac aaactggtat caaatttaaa atatgaatct gtgatttaat   1920

atttttttaca acgttctaac ttaccacttt tgttgtgaat aaaggtgttt acaaatgga    1979


<210> SEQ ID NO 20
<211> LENGTH: 309
<212> TYPE: PRT
<213> ORGANISM: Tachypleus tridentatus

<400> SEQUENCE: 20

Met Asp Ile Ser Phe Leu Val Phe Ile Thr Leu Ser Met Ala Leu Phe
1               5                   10                  15

Ser Ser Asn Val Thr Gly Thr Ser Val Thr Ser Arg Val Arg Arg Gly
20                  25                  30

Ile Asn Glu Lys His Cys Gly Phe Arg Pro Val Ile Thr Arg Ile Ile
35                  40                  45

Gly Gly Gly Ile Ala Thr Pro His Ser Trp Pro Trp Met Val Gly Ile
50                  55                  60

Phe Lys Val Asn Pro His Arg Phe Leu Cys Gly Gly Ser Ile Ile Asn
65                  70                  75                  80

Lys Val Ser Val Val Thr Ala Ala His Cys Leu Val Thr Gln Phe Gly
85                  90                  95

Asn Arg Gln Asn Tyr Ser Ile Phe Val Arg Val Gly Ala His Asp Ile
100                 105                 110

Asp Asn Ser Gly Thr Asn Tyr Gln Val Asp Lys Val Ile Val His Gln
115                 120                 125

Gly Tyr Lys His His Ser His Tyr Tyr Asp Ile Gly Leu Ile Leu Leu
130                 135                 140

Ser Lys Pro Val Glu Tyr Asn Asp Lys Ile Gln Pro Val Cys Ile Pro
145                 150                 155                 160

Glu Phe Asn Lys Pro His Val Asn Leu Asn Asn Ile Lys Val Val Ile
165                 170                 175

Thr Gly Trp Gly Val Thr Gly Lys Ala Thr Glu Lys Arg Asn Val Leu
180                 185                 190

Arg Glu Leu Glu Leu Pro Val Val Thr Asn Glu Gln Cys Asn Lys Ser
195                 200                 205

Tyr Gln Thr Leu Pro Phe Ser Lys Leu Asn Arg Gly Ile Thr Asn Asp
210                 215                 220

Met Ile Cys Ala Gly Phe Pro Glu Gly Gly Lys Asp Ala Cys Gln Gly
225                 230                 235                 240

Asp Ser Gly Gly Pro Leu Met Tyr Gln Asn Pro Thr Thr Gly Arg Val
245                 250                 255

Lys Ile Val Gly Val Val Ser Phe Gly Phe Glu Cys Ala Arg Pro Asn
260                 265                 270

Phe Pro Gly Val Tyr Thr Arg Leu Ser Ser Tyr Val Asn Trp Leu Gln
275                 280                 285

Glu Ile Thr Phe Gly Gln Ser Leu Ala Ser Leu Phe Glu Val Val Pro
290                 295                 300

Ile Phe Ile Pro Glu
305
```

1. A nucleic acid fragment encoding a pro-clotting enzyme derived from a horseshoe crab.

2. The nucleic acid fragment according to claim **1**, wherein the horseshoe crab is selected from among *Tachypleus tridentatus, Limulus polyphemus, Tachypleus gigas*, and *Tachypleus rotundicauda.*

3. The nucleic acid fragment according to claim **1**, wherein the nucleic acid fragment encoding a pro-clotting enzyme derived from a horseshoe crab is selected from the following nucleic acid fragments (A) to (C):

(A) a DNA fragment encoding a protein having an amino acid sequence defined by SEQ ID NO: 4,

(B) a DNA fragment encoding a protein having an amino acid sequence defined by SEQ ID NO: 4 in which one or several amino acid residues are deleted, substituted, inserted, or translocated, and having activity of a pro-clotting enzyme derived from a horseshoe crab, and

(C) an RNA fragment produced through transcription of the DNA fragment (A) or (B).

4. The nucleic acid fragment according to claim **1**, wherein the nucleic acid fragment encoding a pro-clotting enzyme derived from a horseshoe crab is selected from the following nucleic acid fragments (a) to (c):

(a) a DNA fragment having a nucleotide sequence defined by nucleotides 1 to 1143 in SEQ ID NO: 3,

(b) a DNA fragment having a nucleotide mutation in a nucleotide sequence containing a nucleotide sequence defined by nucleotides 1 to 1143 in SEQ ID NO: 3, wherein the mutation causes deletion, substitution, insertion, or translocation of one or several amino acid residues in an amino acid sequence of a protein encoded by the nucleotide sequence defined by nucleotides 1 to 1143 in SEQ ID NO: 3, and a protein expressed by the DNA fragment having a nucleotide mutation has activity of a pro-clotting enzyme derived from a horseshoe crab, and

(c) an RNA fragment produced through transcription of the DNA fragment (a) or (b).

5. A virus harboring a nucleic acid fragment according to claim **1**.

6. The virus according to claim **5**, wherein the virus is baculovirus.

7. The virus according to claim **6**, wherein the baculovirus is nuclear polyhedrosis virus.

8. A cell harboring a virus according to claim **5**.

9. The cell according to claim **8**, which is a cell derived from an insect.

10. A method of producing a pro-clotting enzyme derived from a horseshoe crab, the method comprising the steps of growing a cell according to claim **8**, and preparing the pro-clotting enzyme from the growth product.

11. A pro-clotting enzyme produced through a method according to claim **10**.

12. A method of detecting an endotoxin, wherein the method comprises:

causing a serine protease precursor to be co-present with a pro-clotting enzyme according to claim **11** in an endotoxin-detection sample, said serine protease precursor expressing an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with the endotoxin; and

detecting the endotoxin present in the sample through employing, as an index, an enzymatic activity in conversion of a pro-clotting enzyme to a clotting enzyme.

13. The method of detecting an endotoxin according to claim **12**, wherein the serine protease precursor which expresses an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with the endotoxin comprises a serine protease precursor which expresses an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with activated factor C, and factor C derived from a horseshoe crab and/or recombinant factor C.

14. The method of detecting an endotoxin according to claim **13**, wherein the serine protease precursor which expresses an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with activated factor C is factor B derived from a horseshoe crab and/or recombinant factor B.

15. A method of detecting an endotoxin, wherein the method comprises:

causing a serine protease precursor to be co-present with a pro-clotting enzyme according to claim **11** in an endotoxin-detection sample, said serine protease precursor expressing an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with the endotoxin; and

detecting the endotoxin present in the sample through employing, as an index, an enzymatic activity in conversion of a pro-clotting enzyme to a clotting enzyme,

wherein a pro-clotting enzyme according to claim **11**, recombinant factor C, and recombinant factor B are exclusively caused to be co-present as cascade reaction proteins.

16. An endotoxin-detection kit for carrying out a detection method, wherein the method comprises:

causing a serine protease precursor to be co-present with a pro-clotting enzyme according to claim **11** in an endotoxin-detection sample, said serine protease precursor expressing an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with the endotoxin; and

detecting the endotoxin present in the sample through employing, as an index, an enzymatic activity in conversion of a pro-clotting enzyme to a clotting enzyme,

wherein the kit comprises, as essential components, a pro-clotting enzyme according to claim **11**, and a serine protease precursor which expresses an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with the endotoxin.

17. The endotoxin-detection kit according to claim **16**, wherein the serine protease precursor which expresses an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with the endotoxin comprises a serine protease precursor which expresses an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with activated factor C, and factor C derived from a horseshoe crab and/or recombinant factor C.

18. The endotoxin-detection kit according to claim **17**, wherein the serine protease precursor which expresses an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with activated factor C is factor B derived from a horseshoe crab and/or recombinant factor B.

19. An endotoxin-detection kit for carrying out a detection method, wherein the method comprises:

causing a serine protease precursor to be co-present with a pro-clotting enzyme according to claim **11** in an endotoxin-detection sample, said serine protease precursor

expressing an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with the endotoxin; and

detecting the endotoxin present in the sample through employing, as an index, an enzymatic activity in conversion of a pro-clotting enzyme to a clotting enzyme,

wherein the kit comprises, as essential components, a pro-clotting enzyme according to claim **11**, and a serine protease precursor which expresses an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with the endotoxin,

wherein the kit contains, as cascade reaction proteins, exclusively a pro-clotting enzyme according to claim **11**, recombinant factor C, and recombinant factor B.

20. A method of detecting (1→3)-β-D-glucan, wherein the method comprises:

causing a serine protease precursor to be co-present with a pro-clotting enzyme according to claim **11** in a (1→3)-β-D-glucan-detection sample, said serine protease precursor expressing an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with (1→3)-β-D-glucan; and

detecting (1→3)-β-D-glucan present in the sample through employing, as an index, enzymatic activity in conversion of a pro-clotting enzyme to a clotting enzyme.

21. The method of detecting (1→3)-β-D-glucan according to claim **20**, wherein the serine protease precursor which expresses an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with (1→3)-β-D-glucan comprises factor G derived from a horseshoe crab and/or recombinant factor G.

22. A method of detecting (1→3)-β-D-glucan, wherein the method comprises:

causing a serine protease precursor to be co-present with a pro-clotting enzyme according to claim **11** in a (1→3)-β-D-glucan-detection sample, said serine protease precursor expressing an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with (1→3)-β-D-glucan; and

detecting (1→3)-β-D-glucan present in the sample through employing, as an index, enzymatic activity in conversion of a pro-clotting enzyme to a clotting enzyme,

wherein a pro-clotting enzyme according to claim **11** and recombinant factor G are exclusively caused to be co-present as cascade reaction proteins.

23. A (1→3)-β-D-glucan-detection kit for carrying out a detection method, wherein the method comprises:

causing a serine protease precursor to be co-present with a pro-clotting enzyme according to claim **11** in a (1→3)-β-D-glucan-detection sample, said serine protease precursor expressing an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with (1→3)-β-D-glucan; and

detecting (1→3)-β-D-glucan present in the sample through employing, as an index, enzymatic activity in conversion of a pro-clotting enzyme to a clotting enzyme,

comprising, as essential components, a pro-clotting enzyme according to claim **11**, and a serine protease precursor which expresses an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with (1→3)-β-D-glucan.

24. The (1→3)-β-D-glucan-detection kit according to claim **23**, wherein the serine protease precursor which expresses an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with (1→3)-β-D-glucan comprises factor G derived from a horseshoe crab and/or recombinant factor G.

25. A (1→3)-β-D-glucan-detection kit for carrying out a detection method, wherein the method comprises:

causing a serine protease precursor to be co-present with a pro-clotting enzyme according to claim **11** in a (1→3)-β-D-glucan-detection sample, said serine protease precursor expressing an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with (1→3)-β-D-glucan; and

detecting (1→3)-β-D-glucan present in the sample through employing, as an index, enzymatic activity in conversion of a pro-clotting enzyme to a clotting enzyme,

the kit comprising, as essential components, a pro-clotting enzyme according to claim **11**, and a serine protease precursor which expresses an activity of converting a pro-clotting enzyme to a clotting enzyme upon contact with (1→3)-β-D-glucan,

wherein the kit contains, as cascade reaction proteins, exclusively a pro-clotting enzyme according to claim **11** and recombinant factor G.

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