

DESCRIPTION

AN AGENT FOR MEASURING ENDOTOXIN

TECHNICAL FIELD

[0001]

The present invention relates to an endotoxin-measuring agent, a method for producing the measuring agent, and a method for measuring endotoxin in a sample.

BACKGROUND ART

[0002]

Endotoxin is a lipopolysaccharide existing on the outer membrane of the cell wall of Gram-negative bacteria, and known to be a strong pyrogen. Further, it is known that even a small amount of endotoxin causes various disease states due to bacterial infection, such as release of inflammatory cytokines due to macrophage activation and induction of endotoxin shock, in addition to fever. Therefore, detection of endotoxin in pharmaceuticals such as those for injection; water; medical equipments; and the like is important. Further, endotoxin is considered to be the main cause of shock in Gram-negative bacterial infection, and hence, the presence or absence of infection and/or a pharmaceutical effect can be judged by measuring endotoxin in the blood.

[0003]

Further, it is known that infection of American horseshoe crab (*Limulus polyphemus*) with Gram-negative

bacteria causes intravascular coagulation, and this phenomenon has been used for detection of endotoxin.

[0004]

That is, a method for measuring endotoxin using a blood cell extract of a horseshoe crab (horseshoe crab amebocyte lysate; hereinafter also referred to as "lysate") is known (e.g., Non-patent Document 1). This method is called "limulus test", and uses a cascade reaction of various proteins existing in the lysate, which reaction is caused by contacting of endotoxin with the lysate. A schematic diagram of the cascade reaction is shown in Fig. 1.

[0005]

Upon contacting of endotoxin with the lysate, factor C existing in the lysate is activated to produce active-type factor C. This active-type factor C activates factor B existing in the lysate, to produce active-type factor B. This active-type factor B then activates a proclotting enzyme existing in the lysate, to produce a clotting enzyme.

[0006]

This clotting enzyme hydrolyzes a specific portion in the coagulogen molecule existing in the lysate. By this, coagulin gel is produced, to cause coagulation of the lysate. Thus, by measuring the coagulation reaction of the lysate, endotoxin can be measured.

[0007]

Further, also by allowing a clotting enzyme to react

with a synthetic substrate to cause color reaction, endotoxin can be measured. For example, a clotting enzyme reacts with a synthetic substrate t-butoxycarbonyl-leucyl-glycyl-arginyl-pNA (Boc-Leu-Gly-Arg-pNA) to hydrolyze its amide bond, and thereby pNA is released. Thus, by preliminarily including the synthetic substrate in the reaction system, endotoxin can be quantified by measurement of the absorbance (405 nm) of the coloring substance (pNA).

[0008]

Further, it is known that the cascade reaction system can be reconstructed using factor C, factor B, and a proclotting enzyme, which were purified from lysate of a Japanese horseshoe crab (Non-patent Document 2).

[0009]

Further, a case wherein a recombinant factor C derived from a Southeast Asian horseshoe crab *Carcinoscorpius rotundicauda*; and a recombinant factor B and a recombinant proclotting enzyme derived from a Japanese horseshoe crab *Tachypleus tridentatus*; were used to reconstruct the cascade reaction system is known (Patent Document 1).

[0010]

Further, a system for detecting endotoxin by using a recombinant factor C derived from a Southeast Asian horseshoe crab *Carcinoscorpius rotundicauda* and a substrate that reacts with active-type factor C to release a fluorescent substance is known (Patent Document

2). This system is commercially available as an endotoxin detection system (commercial name: PyroGene (registered trademark); Lonza).

[0011]

However, in order to use the lysate, or the naturally occurring factor C, factor B, and proclotting enzyme prepared therefrom, it is necessary to capture horseshoe crabs and collect blood therefrom. Hence, in view of conservation of biological resources or the like, it is difficult to supply these components unlimitedly. Therefore, a technique to easily and rapidly produce a reagent for detection of endotoxin at a low cost has been demanded.

[0012]

Further, in cases where a recombinant factor C, recombinant factor B, and recombinant proclotting enzyme are used, any of the above-described cases requires 1 hour or more for the measurement, and a detection sensitivity in the order of 0.001 EU/mL has not been achieved. Therefore, a technique to rapidly and highly sensitively measure endotoxin has been demanded.

PRIOR ART DOCUMENTS

Patent Documents

[0013]

[Patent Document 1] WO 2008/004674

[Patent Document 2] US 6,849,426 B

Non-patent Documents

[0014]

[Non-patent Document 1] Iwanaga S., Curr Opin Immunol. 1993 Feb; 5(1): 74-82.

[Non-patent Document 2] Nakamura T. et al., J Biochem. 1986 Mar; 99(3): 847-57.

SUMMARY OF THE INVENTION

[0015]

The present invention aims to provide a method for rapidly and highly sensitively measuring endotoxin. The present invention also aims to provide an endotoxin-measuring agent to be used in the method, and a method for producing the agent.

[0016]

The present inventors discovered that endotoxin can be rapidly and highly sensitively measured by using a recombinant factor C (His-tag free), recombinant factor B and recombinant proclotting enzyme, which were derived from a Japanese horseshoe crab *Tachypleus tridentatus* and expressed using insect cells as a host, thereby completing the present invention.

[0017]

That is, the present invention is as follows.

[1]

An endotoxin-measuring agent comprising the proteins (1) to (3) below, each of which is a recombinant protein obtainable by being expressed using insect cells as a host:

(1) a factor C derived from *Tachypleus tridentatus*, which factor C does not have His-tag sequence at the C-terminus;

(2) a factor B of a horseshoe crab; and

(3) a proclotting enzyme of a horseshoe crab.

[2]

The measuring agent according to [1], wherein said factor B and said proclotting enzyme are derived from *Tachypleus tridentatus*.

[3]

The measuring agent according to [1] or [2], wherein said factor C is the protein (A) or (B) below; said factor B is the protein (C) or (D) below; and said proclotting enzyme is the protein (E) or (F) below:

(A) a protein comprising the amino acid sequence shown in SEQ ID NO:2;

(B) a protein comprising the amino acid sequence shown in SEQ ID NO:2 but which includes substitution, deletion, insertion, or addition of one or several amino acid residues, which protein has factor C activity;

(C) a protein comprising the amino acid sequence shown in SEQ ID NO:4;

(D) a protein comprising the amino acid sequence shown in SEQ ID NO:4 but which includes substitution, deletion, insertion, or addition of one or several amino acid residues, which protein has factor B activity;

(E) a protein comprising the amino acid sequence shown in SEQ ID NO:6;

(F) a protein comprising the amino acid sequence shown in SEQ ID NO:6 but which includes substitution, deletion, insertion, or addition of one or several amino acid residues, which protein has proclotting enzyme activity.

[4]

A method for producing the measuring agent according to any one of [1] to [3], the method comprising the steps (A) to (C) below:

(A) a step of incorporating each of the DNAs (1) to (3) below into a viral DNA:

(1) a DNA encoding a factor C derived from *Tachypleus tridentatus*, which factor C does not have His-tag sequence at the C-terminus;

(2) a DNA encoding a factor B of a horseshoe crab; and

(3) a DNA encoding a proclotting enzyme of a horseshoe crab;

(B) a step of infecting insect cells with the virus into which said each DNA was incorporated; and

(C) a step of allowing the insect cells infected with said each virus to express the protein encoded by said each DNA.

[5]

A method for producing the measuring agent according to any one of [1] to [3], the method comprising the steps (A) to (C) below:

(A) a step of incorporating each of the DNAs (1) to

(3) below into a vector:

(1) a DNA encoding a factor C derived from *Tachypleus tridentatus*, which factor C does not have His-tag sequence at the C-terminus;

(2) a DNA encoding a factor B of a horseshoe crab; and

(3) a DNA encoding a proclotting enzyme of a horseshoe crab;

(B) a step of introducing the vector, into which said each DNA was incorporated, into insect cells to incorporate said each DNA into the chromosome of the insect cells; and

(C) a step of allowing the insect cells, into which said each DNA was incorporated, to express the protein encoded by said each DNA.

[6]

The method according to [4] or [5], wherein said DNA encoding factor C is the DNA (A) or (B) below; said DNA encoding factor B is the DNA (C) or (D) below; and said DNA encoding proclotting enzyme is the DNA (E) or (F) below:

(A) a DNA comprising the nucleotide sequence shown in SEQ ID NO:1;

(B) a DNA which hybridizes with the complementary sequence of the full length or a part of the nucleotide sequence shown in SEQ ID NO:1 under stringent conditions, and encodes a protein having factor C activity.

(C) a DNA comprising the nucleotide sequence shown in

SEQ ID NO:3 or 8;

(D) a DNA which hybridizes with the complementary sequence of the full length or a part of the nucleotide sequence shown in SEQ ID NO:3 or 8 under stringent conditions, and encodes a protein having factor B activity.

(E) a DNA comprising the nucleotide sequence shown in SEQ ID NO:5 or 9;

(F) a DNA which hybridizes with the complementary sequence of the full length or a part of the nucleotide sequence shown in SEQ ID NO:5 or 9 under stringent conditions, and encodes a protein having proclotting enzyme activity.

[7]

A method for measuring endotoxin in a test sample, the method comprising a step of mixing the measuring agent according to any one of [1] to [3] with the test sample and a step of measuring progress of cascade reaction.

[8]

The method according to [7], which comprises a step of adding a substrate for detection of progress of cascade reaction to a reaction system.

[9]

The method according to [8], which further comprises a step of calculating the endotoxin level in the test sample on the basis of reaction of said substrate.

[0018]

By the present invention, endotoxin can be rapidly and highly sensitively measured. For example, in one embodiment of the present invention, a detection sensitivity in the order of 0.0005 EU/mL can be achieved with only 30 minutes of measurement. Further, in the present invention, the expressed recombinant factor C, recombinant factor B, and recombinant proclotting enzyme can be used without purification, and hence, an endotoxin-measuring agent comprising these recombinant proteins can be simply and rapidly produced at a low cost.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019]

[Fig. 1] Fig. 1 is a diagram showing the cascade reaction system in a limulus test.

[Fig. 2] Fig. 2 is a diagram showing the structure of the vector pIZ/V5-His and the position of insertion of each of the genes. The arrow in the upper part indicates the position of insertion of the genes.

[Fig. 3] Fig. 3 is a photograph showing the expression levels of various factor Cs.

[Fig. 4] Fig. 4 is a diagram showing the activities of various factor Cs.

[Fig. 5] Fig. 5 is a photograph showing the stability of the factor C expressed by the viral method.

[Fig. 6] Fig. 6 is a photograph showing the stability of the factor C expressed by the stably expressing cell

method.

[Fig. 7] Fig. 7 is a diagram showing the effect of treatment by hollow fiber membrane filtration on the reactivities of the factors expressed by the viral method.

[Fig. 8] Fig. 8 is a diagram showing the reactivity of the endotoxin-measuring agent containing the factors expressed by the viral method.

[Fig. 9] Fig. 9 is a diagram showing the reactivity of the endotoxin-measuring agent containing the factors expressed by the stably expressing cell line method. (a) The reactivity at the endotoxin concentration of 0 to 0.1 EU/mL. (b) The reactivity at the endotoxin concentration of 0 to 0.01 EU/mL.

[Fig. 10] Fig. 10 is a photograph showing the purities and concentrations of the purified recombinant factor C and the purified naturally-occurring factor C.

[Fig. 11] Fig. 11 is a calibration curve showing a relationship between band intensities and amounts of BSA.

[Fig. 12] Fig. 12 is a diagram showing the activities of the purified recombinant factor C and the purified naturally-occurring factor C.

MODE FOR CARRYING OUT THE INVENTION

[0020]

In the present invention, a series of reactions wherein endotoxin activates factor C to produce active-type factor C; the active-type factor C activates factor

B to produce active-type factor B; and the active-type factor B activates a proclotting enzyme to produce a clotting enzyme; may be referred to as "cascade reaction".

[0021]

(1) Endotoxin-measuring Agent of Present Invention

The endotoxin-measuring agent of the present invention comprises factor C, factor B, and a proclotting enzyme. The factor C, factor B, and proclotting enzyme comprised in the endotoxin-measuring agent of the present invention may be hereinafter referred to as "factor C of the present invention", "factor B of the present invention" and "proclotting enzyme of the present invention", respectively. Further, the factor C, factor B, and proclotting enzyme may be collectively referred to as "factors".

[0022]

All of the factor C of the present invention, factor B of the present invention, and proclotting enzyme of the present invention are recombinant proteins obtainable by being expressed using insect cells as a host.

[0023]

The factor C of the present invention is a factor C derived from a Japanese horseshoe crab *Tachypleus tridentatus*. The factor C of the present invention is characterized in that it does not have a His-tag attached at the C-terminus. Further, the factor C of the present invention preferably does not have a V5-tag at the C-

terminus. Further, the factor C of the present invention more preferably does not have any peptide attached at the C-terminus. Further, the factor C of the present invention especially preferably does not have any peptide attached at either terminus. An amino acid sequence of the factor C of *Tachypleus tridentatus* is shown in SEQ ID NO:2. A nucleotide sequence of the gene encoding the factor C of *Tachypleus tridentatus* is shown in SEQ ID NO:1.

[0024]

The factor C of the present invention may be a variant of the protein having the amino acid sequence shown in SEQ ID NO:2 as long as the variant has the factor C activity.

[0025]

The "factor C activity" means an activity by which factor C becomes active-type factor C in the presence of endotoxin, to activate factor B. The fact that the factor C of the present invention "has the factor C activity" can be confirmed, for example, by using the factor C of the present invention in combination with a suitable factor B and a suitable proclotting enzyme, and detecting the progress of the cascade reaction in the presence of endotoxin. More particularly, the protein of SEQ ID NO:4 may be used as the suitable factor B, and the protein of SEQ ID NO:6 may be used as the suitable proclotting enzyme. The progress of the cascade reaction can be measured using the later-mentioned substrate for

detection.

[0026]

The factor C of the present invention may be a protein comprising the amino acid sequence shown in SEQ ID NO:2 but which includes substitution, deletion, insertion, or addition of one or several amino acid residues as long as the factor C has the factor C activity. The meaning of the term "one or several" varies depending on the positions of the amino acid residues in the three-dimensional structure of the protein and the types of the amino acid residues, and, more particularly, the term means preferably 1 to 20, more preferably 1 to 10, still more preferably 1 to 5, especially preferably 1 to 3. The above-described substitution, deletion, insertion, or addition of one or several amino acids is a conservative mutation that maintains the normal function of the protein. A representative example of the conservative mutation is a conservative substitution. The conservative substitution is, for example, a mutation wherein substitution takes place mutually among Phe, Trp, and Tyr, if the substitution site is an aromatic amino acid; among Leu, Ile, and Val, if the substitution site is a hydrophobic amino acid; between Gln and Asn, if the substitution site is a polar amino acid; among Lys, Arg, and His, if the substitution site is a basic amino acid; between Asp and Glu, if the substitution site is an acidic amino acid; and between Ser and Thr, if it the substitution site an

amino acid having a hydroxyl group. Examples of substitutions considered as conservative substitutions include, specifically, substitution of Ser or Thr for Ala, substitution of Gln, His, or Lys for Arg, substitution of Glu, Gln, Lys, His, or Asp for Asn, substitution of Asn, Glu, or Gln for Asp, substitution of Ser or Ala for Cys, substitution of Asn, Glu, Lys, His, Asp, or Arg for Gln, substitution of Gly, Asn, Gln, Lys, or Asp for Glu, substitution of Pro for Gly, substitution of Asn, Lys, Gln, Arg, or Tyr for His, substitution of Leu, Met, Val, or Phe for Ile, substitution of Ile, Met, Val, or Phe for Leu, substitution of Asn, Glu, Gln, His, or Arg for Lys, substitution of Ile, Leu, Val, or Phe for Met, substitution of Trp, Tyr, Met, Ile, or Leu for Phe, substitution of Thr or Ala for Ser, substitution of Ser or Ala for Thr, substitution of Phe or Tyr for Trp, substitution of His, Phe, or Trp for Tyr, and substitution of Met, Ile, or Leu for Val. Further, the above-described substitution, deletion, insertion, addition, inversion or the like may also include a naturally occurring mutation due to difference in the individual, strain, or species among the horseshoe crabs from which the gene was derived.

[0027]

Further, the factor C of the present invention may be a protein which has a homology or identity of not less than 80%, preferably not less than 90%, more preferably not less than 95%, still more preferably not less than

97%, especially preferably not less than 99% to the full length of the amino acid sequence of factor C as described above, for example, to the full length of the amino acid sequence shown in SEQ ID NO:2, and has the factor C activity.

[0028]

The gene encoding the factor C of the present invention is not particularly restricted as long as the gene encodes the factor C of the present invention as described above. The gene encoding the factor C of the present invention may be a probe prepared based on a known gene sequence, for example, a DNA which hybridizes with the complementary sequence of the full length or a part of the nucleotide sequence shown in SEQ ID NO:1 under stringent conditions and encodes a protein having the factor C activity. The term "stringent conditions" herein means conditions under which the so-called specific hybrid is formed but a non-specific hybrid is not formed. Examples of the conditions include conditions under which highly homologous DNAs hybridize to each other, for example, DNAs not less than 80% homologous, preferably not less than 90% homologous, more preferably not less than 95% homologous, still more preferably not less than 97% homologous, especially preferably not less than 99% homologous, hybridize to each other, while DNAs less homologous than the above do not hybridize to each other; and conditions under which washing is carried out once, more preferably 2 or 3

times, at a salt concentration and temperature corresponding to 60°C, 1×SSC, and 0.1% SDS; preferably 60°C, 0.1×SSC, and 0.1% SDS; more preferably 68°C, 0.1×SSC, and 0.1% SDS; which are normal washing conditions in Southern hybridization.

[0029]

Further, the combinations of the codons in the gene encoding the factor C of the present invention may be modified such that the gene is optimized for being expressed in insect cell. The optimization can be carried out using, for example, a generally available contract service. The gene encoding the factor C of the present invention may be a variant of a DNA whose combinations of the codons are optimized for expression in insect cells.

[0030]

The above description on variants of the gene and protein may be applied similarly to the factor B and proclotting enzyme of the present invention, and to the genes encoding those.

[0031]

The factor B of the present invention is a factor B derived from a horseshoe crab. Further, the proclotting enzyme of the present invention is a proclotting enzyme derived from a horseshoe crab. Examples of the horseshoe crab include a Japanese horseshoe crab *Tachypleus tridentatus*, American horseshoe crab *Limulus polyphemus*, Southeast Asian horseshoe crab *Carcinoscorpius*

rotundicauda and Southeast Asian horseshoe crab *Tachypleus gigas*. The above factors are preferably derived from, among those horseshoe crabs, the Japanese horseshoe crab *Tachypleus tridentatus*.

[0032]

Amino acid sequences of the factor B and proclotting enzyme of *Tachypleus tridentatus* are shown in SEQ ID NOs:4 and 6, respectively. Nucleotide sequences of the genes encoding the factor B and proclotting enzyme of *Tachypleus tridentatus* are shown in SEQ ID NOs:3 and 5, respectively.

[0033]

The factor B of the present invention may be a variant of the factor B of any of the above-described horseshoe crabs, for example, a variant of the protein having the amino acid sequence shown in SEQ ID NO:4, as long as the factor B of the present invention has the factor B activity. Further, the gene encoding the factor B of the present invention is not particularly restricted as long as the gene encodes the factor B of the present invention as described above. The above description on factor C is also applied *mutatis mutandis* to the variants of the gene and protein.

[0034]

The "factor B activity" means an activity by which factor B becomes active-type factor B in the presence of active-type factor C, to change a proclotting enzyme into its active form, a clotting enzyme. The fact that the

factor B of the present invention "has the factor B activity" can be confirmed, for example, by using the factor B of the present invention in combination with a suitable factor C and a suitable proclotting enzyme, and detecting the progress of the cascade reaction in the presence of endotoxin. More particularly, the protein of SEQ ID NO:2 may be used as the suitable factor C, and the protein of SEQ ID NO:6 may be used as the suitable proclotting enzyme. The progress of the cascade reaction can be measured using the later-mentioned substrate for detection.

[0035]

The proclotting enzyme of the present invention may be a variant of the proclotting enzyme of any of the above-described horseshoe crabs, for example, a variant of the protein having the amino acid sequence shown in SEQ ID NO:6, as long as the proclotting enzyme of the present invention has the proclotting enzyme activity. Further, the gene encoding the proclotting enzyme of the present invention is not particularly restricted as long as the gene encodes the proclotting enzyme of the present invention as described above. The above description on factor C is also applied *mutatis mutandis* to the variants of the gene and protein.

[0036]

The "proclotting enzyme activity" means an activity by which a proclotting enzyme is changed to a clotting enzyme in the presence of active-type factor B, to react

with the later-mentioned substrate for detection. The "activity to react with a substrate for detection" means, for example, an activity to react with coagulogen to cause coagulation, and an activity to react with Boc-Leu-Gly-Arg-pNA to release pNA. The fact that the proclotting enzyme of the present invention "has the proclotting enzyme activity" can be confirmed, for example, by using the clotting enzyme of the present invention in combination with a suitable factor C and a suitable factor B, and detecting the progress of the cascade reaction in the presence of endotoxin. More particularly, the protein of SEQ ID NO:2 may be used as the suitable factor C, and the protein of SEQ ID NO:4 may be used as the suitable factor B. The progress of the cascade reaction can be measured using the later-mentioned substrate for detection.

[0037]

To the factor B of the present invention and/or the proclotting enzyme of the present invention, an arbitrary peptide or the like may be added as long as the factors have the factor B activity and the proclotting enzyme activity, respectively. Examples of such a peptide include tag sequences such as His-tag and V5-tag. Similarly to the factor C of the present invention, the factor B of the present invention and/or the proclotting enzyme of the present invention to be employed may be any of those wherein His-tag is not added to the C-terminus, those wherein V5-tag is not added to the C-terminus,

those wherein no peptide is added to the C-terminus at all, and those wherein no peptide is added to either terminus at all.

[0038]

Further, the combinations of the codons in the gene encoding the factor B of the present invention and/or the gene encoding the proclotting enzyme of the present invention may be modified such that the gene(s) is/are optimized for being expressed in insect cells. Examples of the DNA that encodes the factor B of SEQ ID NO:4 and has combinations of the codons optimized for expression in insect cells include the DNA of SEQ ID NO:8. Examples of the DNA that encodes the proclotting enzyme of SEQ ID NO:6 and has combinations of the codons optimized for expression in insect cells include the DNA of SEQ ID NO:9. Each of the gene encoding the factor B of the present invention and/or the gene encoding the proclotting enzyme of the present invention may be a variant of a DNA whose combinations of the codons are optimized for expression in insect cells.

[0039]

The endotoxin-measuring agent of the present invention may consist of the factor C of the present invention, the factor B of the present invention, and the proclotting enzyme of the present invention.

[0040]

The endotoxin-measuring agent of the present invention may comprise a substrate for detection of the

progress of the cascade reaction. In the present invention, such a substrate may be referred to as "substrate for detection".

[0041]

Examples of the substrate for detection include coagulogen. Due to contact of coagulogen with a clotting enzyme, coagulation occurs to produce coagulin. The progress of the coagulation reaction may be assayed by measuring the turbidity of the reaction solution. Coagulogen can be recovered from a horseshoe crab blood cell extract (lysate). Also, because a nucleotide sequence of the gene encoding coagulogen has been clarified (Miyata, et al., PROTEIN, NUCLEIC ACID AND ENZYME, Extra Edition, No. 29, pp. 30-43 (1986)), coagulogen can be produced according to a conventional method by genetic engineering.

[0042]

As the substrate for detection, a synthetic substrate may also be used. The synthetic substrate is not particularly restricted as long as the substrate has a property suitable for detection, such as a property by which catalytic reaction of a clotting enzyme causes development of color or emission of fluorescence. Examples of the synthetic substrate include substrates represented by the general formula X-Y-Z (wherein X represents a protecting group, Y represents a peptide, and Z represents a dye bound to Y via an amide bond). In cases where endotoxin exists in the reaction system,

catalytic reaction of a clotting enzyme, which is yielded as a result of the cascade reaction, cleaves the amide bond between Y and Z, to release the dye Z, leading to development of color or emission of fluorescence. The protecting group X is not particularly restricted, and a known protecting group for peptides may be suitably used.

Examples of such a protecting group include the t-butoxycarbonyl group and the benzoyl group. The dye Z is not particularly restricted, and may be either a dye which can be detected under visible light or a fluorescent dye. Examples of the dye Z include pNA (para-nitroaniline), MCA (7-methoxycoumarin-4-acetic acid), DNP (2,4-dinitroaniline), and Dansyl dyes. Examples of the peptide Y include Leu-Gly-Arg (LGR), Ile-Glu-Gly-Arg (IEGR) (SEQ ID NO:12), and Val-Pro-Arg (VPR). The released dye Z may be measured by a method selected depending on the property of the dye.

[0043]

Further, the endotoxin-measuring agent of the present invention may also comprise a component other than the factors and the substrate for detection, as long as the agent can be used for measurement of endotoxin. Such a component is not particularly restricted, and may be selected in consideration of preservability, ease of handling, and stability of the factors and the substrate for detection. The endotoxin-measuring agent of the present invention may comprise, for example, a pH-buffering agent and/or salt. Examples of the pH-

buffering agent include HEPES buffer, MES buffer, Tris buffer, and GTA wide-range buffer. Organic solvents such as alcohols, esters, ketones, and amides may also be comprised in the endotoxin-measuring agent of the present invention.

[0044]

The endotoxin-measuring agent of the present invention may be formulated into an arbitrary form including, for example, a solid form, liquid form, and gel form. For the formulation, additives normally used as formulation carriers such as vehicles; binders; disintegrants; lubricants; stabilizers; correctives; diluents; surfactants; and solvents may be used. The endotoxin-measuring agent of the present invention may be used for measuring endotoxin as it is, or after being diluted, dispersed, or dissolved in water, physiological saline, buffer, or the like. Needless to say, the resulting formulation obtained by such dilution, dispersion, or dissolution is also within the scope of the endotoxin-measuring agent of the present invention.

[0045]

In the endotoxin-measuring agent of the present invention, the factors and the other components may exist as a mixture(s) or may separately exist. For example, the factors may be mixed at an arbitrary ratio to be formulated, or may be separately formulated.

[0046]

The concentrations of the factors and the other

components in the endotoxin-measuring agent of the present invention are not particularly restricted, and preferably adjusted such that the concentrations are within the later-mentioned preferred ranges when endotoxin is measured. The concentration of each of the factors in the endotoxin-measuring agent of the present invention (in terms of the solution prepared before contacting with the test sample) is, for example, preferably 20 to 100 $\mu\text{g/mL}$, more preferably 40 to 80 $\mu\text{g/mL}$, especially preferably about 60 $\mu\text{g/mL}$.

[0047]

The endotoxin-measuring agent of the present invention may be provided as an endotoxin-measuring kit. The endotoxin-measuring kit is not particularly restricted as long as the kit contains the endotoxin-measuring agent of the present invention.

[0048]

(2) Method for Producing Endotoxin-measuring Agent of Present Invention

The factors to be comprised in the endotoxin-measuring agent of the present invention can be produced by being expressed using insect cells as a host.

[0049]

The insect cells are not particularly restricted as long as the cells can express the factors, and cells normally used for expression of a heterologous protein may be suitably used. Examples of such insect cells include Sf9, Sf21, SF+, and High-Five. The insect cells

are preferably Sf9.

[0050]

The culture conditions under which the insect cells are cultured are not particularly restricted as long as the insect cells can be cultured under the conditions, and culture conditions normally used for culturing insect cells may be used after, if necessary, appropriately modified. For example, as a culture medium, one normally used for culturing insect cells may be used. Examples of such a medium include commercially available serum-free media for insect cells. More particularly, Sf900 II medium (Invitrogen) or the like may be suitably used. The cultivation may be carried out, for example, at 27°C to 28°C with shaking.

[0051]

The method for expressing the factors using insect cells as a host is not particularly restricted as long as the factors can be expressed thereby, and a method normally used for expression of a heterologous protein can be suitably used. For example, each factor can be expressed by infecting insect cells with a virus into which a gene encoding the factor was incorporated (viral method). Alternatively, each factor can be expressed by introducing a vector, into which a gene encoding the factor was incorporated, into insect cells, thereby incorporating the gene into the chromosome of the host (stably expressing cell line method).

[0052]

<Viral Method>

The virus to be used in the viral method is not particularly restricted as long as insect cells can be infected with the virus and the factors can be expressed thereby, and a virus normally used for expression of a protein in insect cells can be suitably used. Examples of such a virus include baculovirus. The baculovirus is preferably nucleopolyhedrovirus (NPV). Examples of the NPV include AcNPV (*Autographa californica* NPV) and BmNPV (*Bombix mori* NPV). The NPV is preferably AcNPV.

[0053]

Introduction of the nucleic acid into the virus can be carried out by a conventional method, for example, by homologous recombination using a transfer vector. Examples of the transfer vector include pPSC8 (Protein Sciences), pFastBac (Invitrogen), and pVL1393 (Pharmingen). The transfer vector is preferably pPSC8.

[0054]

By infecting, by a conventional method, insect cells with a virus into which the gene encoding each factor was incorporated, insect cells that harbor the virus and express the factor can be obtained.

[0055]

<Stably Expressing Cell Line Method>

By incorporating the gene encoding each factor into the chromosome of insect cells, a stably expressing cell line, which stably expresses the factor, can be obtained.

The method of construction of the stably expressing cell

line is not particularly restricted, and the construction can be carried out by a conventional method. For example, the stably expressing cell line can be constructed using the pIZ/V5-His vector (Invitrogen) according to the manual.

[0056]

In any case, the expressing cells are constructed such that the expressed factor C has the C-terminus to which Histag is not attached. Further, in cases where each factor is to be expressed without addition of any peptide, which is not restricted to His-tag at the C-terminus of the factor C, the expressing cells may be constructed such that no peptide is added.

[0057]

In any case, the factors may be expressed together by a single type of expressing cells, or expressing cells may be constructed for each factor to express the respective factors separately.

[0058]

Whether or not each factor is expressed can be confirmed by measuring the activity of the factor. Whether or not each factor is expressed can also be confirmed by measuring the amount of mRNA transcribed from the gene encoding the factor, or by detecting the factor by Western blotting using an antibody.

[0059]

Each expressed factor may be recovered as a solution containing the factor, to be used as a component of the

endotoxin-measuring agent of the present invention. The solution containing the factor may be, for example, a culture broth, culture supernatant, or cell extract, or a mixture thereof. Each factor may be used either after purification or without purification. In the present invention, an endotoxin-measuring agent having a sufficiently high performance can be provided even by using cell culture supernatant containing each expressed factor as it is without purification of the factor. In cases where each factor is to be purified, the purification may be carried out, for example, by a known method used for purification of a protein. Examples of such a method include ammonium sulfate precipitation, gel filtration chromatography, ion-exchange chromatography, hydrophobic interaction chromatography, and hydroxyapatite chromatography. In cases where a tag such as His-tag is attached to each factor, the factor may also be purified by affinity chromatography using affinity against the tag.

[0060]

In cases where each factor was produced by the viral method, the virus is preferably eliminated. The method of elimination of the virus is not particularly restricted, and the elimination may be carried out by a conventional method. For example, the virus may be eliminated through a hollow-fiber filtration membrane having a pore size of 500 kDa.

[0061]

(3) Method of Present Invention for Measuring Endotoxin.

By mixing the endotoxin-measuring agent of the present invention with a test sample, the cascade reaction proceeds in cases where the test sample contains endotoxin. By measuring the progress of the cascade reaction, the endotoxin in the test sample can be measured. That is, the present invention provides a method for measuring endotoxin in a test sample, which method comprises a step of mixing the endotoxin-measuring agent of the present invention with a test sample, and a step of measuring the progress of the cascade reaction (hereinafter referred to as "first embodiment").

[0062]

In the first embodiment, each factor comprised in the endotoxin-measuring agent of the present invention may have been contained in the reaction system from the beginning of the step of mixing the endotoxin-measuring agent of the present invention with a test sample, or may be sequentially added to the reaction system.

[0063]

For example, the step of mixing the endotoxin-measuring agent of the present invention with a test sample may comprise the following steps (A) to (C):

(A) a step of adding the factor C of the present invention to the reaction system;

(B) a step of adding the factor B of the present invention to the reaction system; and

(C) a step of adding the proclotting enzyme of the

present invention to the reaction system.

[0064]

Steps (A) to (C) may be carried out separately, partially at the same time, or totally at the same time. Steps (A) to (C) may be carried out in an arbitrary order. For example, Step (A) may be followed by Step (B), which may then be followed by Step (C).

[0065]

In the first embodiment, the progress of the cascade reaction can be measured by adding a substrate for detection to the reaction system and then measuring the reaction of the substrate (coloring, coagulation, or the like). The substrate for detection may have been contained in the reaction system from the beginning of the step of mixing the endotoxin-measuring agent of the present invention with a test sample, or may be added to the reaction system during the progress or after completion of the step. The first embodiment, of course, includes cases where the endotoxin-measuring agent of the present invention which preliminarily contains a substrate for detection is employed.

[0066]

As long as the cascade reaction proceeds in cases where endotoxin is contained in the test substance, the factor B and the proclotting enzyme of the present invention themselves may not necessarily contact with the test sample. That is, another embodiment of the method of the present invention for measuring endotoxin

(hereinafter referred to as "second embodiment") is a method for measuring endotoxin in a test substance, which method comprises Steps (A) to (D) below.

(A) a step of mixing the factor C of the present invention with a test sample;

(B) a step of mixing the factor B of the present invention with the factor C after the mixing thereof in Step A;

(C) a step of mixing the proclotting enzyme of the present invention with the factor B after the mixing thereof in Step B; and

(D) a step of measuring the progress of the cascade reaction.

[0067]

In the second embodiment, Steps (A) to (D) may proceed separately, partially at the same time, or totally at the same time. For example, after beginning Step A, the factor B and/or proclotting enzyme may be added to the reaction system during the progress or after completion of the step. Alternatively, after beginning Step B, the proclotting enzyme may be added to the reaction system during the progress or after completion of the step. Alternatively, all the 3 factors may be contained in the reaction system from the beginning of Step A. Alternatively, for example, the factor C after the contacting in Step A may be recovered to be used in Step B, and the factor B after the contacting in Step B may be recovered to be used in Step C.

[0068]

In the second embodiment, the progress of the cascade reaction may be measured by adding a substrate for detection to the reaction system and then measuring reaction of the substrate (coloring, coagulation, or the like). The substrate for detection may be contained in the reaction system from the beginning of Step A, or may be added to the reaction system during the progress or after completion of each step.

[0069]

The method of the present invention for measuring endotoxin may comprise another arbitrary step as long as the cascade reaction proceeds in cases where the test sample contains endotoxin. For example, the method of the present invention for measuring endotoxin may comprise a step of adding a substrate for detection to the reaction system, or a step of mixing a clotting enzyme produced by the cascade reaction with a substrate for detection. Further, for example, the method of the present invention for measuring endotoxin may comprise a step of calculating the endotoxin level in the test sample on the basis of reaction of the substrate for detection.

[0070]

In the method of the present invention for measuring endotoxin, the reaction is preferably carried out in an aqueous solvent such as water or a buffer.

[0071]

In the method of the present invention for measuring endotoxin, the concentration of each factor in the reaction solution is not particularly restricted as long as the cascade reaction proceeds in cases where endotoxin is contained in the test sample, and may be set appropriately depending on the property of the factor and/or the like. For example, the concentration of each factor is usually 10 to 50 $\mu\text{g/mL}$, preferably 20 to 40 $\mu\text{g/mL}$, more preferably about 30 $\mu\text{g/mL}$, in terms of the final concentration.

[0072]

In the method of the present invention for measuring endotoxin, the concentration of the substrate for detection in the reaction solution is not particularly restricted as long as the cascade reaction proceeds in cases where endotoxin is contained in the test sample, and may be set appropriately depending on the property of the substrate for detection and/or the like. For example, in cases where the substrate for detection is a synthetic substrate, the concentration of the substrate for detection is usually 0.001 mM to 100 mM, preferably 0.01 mM to 10 mM, in terms of the final concentration.

[0073]

In any embodiment, the reaction system may contain an arbitrary component(s) other than the endotoxin-measuring agent in the first embodiment or the factors in the second embodiment, substrate for detection, and test sample, as long as the cascade reaction proceeds in cases

where endotoxin is contained in the test sample. For example, the reaction system may contain a pH-buffering agent and/or salt. Examples of the pH-buffering agent include HEPES buffer, MES buffer, Tris buffer, and GTA wide-range buffer. Organic solvents such as alcohols, esters, ketones, and amides may also be contained in the reaction system.

[0074]

The pH of the reaction solution is not particularly restricted as long as the cascade reaction proceeds in cases where endotoxin is contained in the test sample, and may be set appropriately depending on the property of each factor. For example, the pH of the reaction solution is usually 5 to 10, preferably 7 to 8.5.

[0075]

The reaction temperature is not particularly restricted as long as the cascade reaction proceeds in cases where endotoxin is contained in the test sample, and may be set appropriately depending on the property of each factor. The reaction temperature is, for example, usually 10°C to 80°C, preferably 20°C to 50°C. For example, the reaction temperature may be room temperature.

[0076]

The reaction time is not particularly restricted, and may be set appropriately depending on conditions such as the property of each factor and the reaction temperature. The reaction time is, for example, usually 5 minutes to 1

hour, preferably 15 minutes to 45 minutes. For example, the reaction time may be 30 minutes.

[0077]

In any embodiment, during the process of reaction, the test sample, factors, and other components may be additionally added, individually or in an arbitrary combination, to the reaction system. These components may be added at once or in a plurality of times, or may be added continuously. Constant conditions may be employed from the beginning of the reaction to the end of the reaction, or conditions may be changed during the process of reaction.

[0078]

By measuring reaction of the substrate for detection (coloring, coagulation, or the like), the progress of the cascade reaction due to existence of endotoxin can be measured, and hence the endotoxin in the test substance can be measured. The reaction of the substrate for detection (coloring, coagulation, or the like) may be measured by a method depending on the substrate for detection employed.

[0079]

In cases where the measurement of endotoxin is carried out quantitatively, an endotoxin standard sample whose concentration is known may be used to obtain a correlation data between the endotoxin level and the degree of reaction of the substrate for detection (degree of coloring, coagulation, or the like), and, endotoxin

existing in the test sample may be quantified on the basis of the correlation data. The correlation data may be, for example, a calibration curve. The quantification may be carried out either by the kinetic method or by the end point method.

[0080]

The test sample to be subjected to the measurement of endotoxin is not particularly restricted, and examples thereof include medical water, pharmaceuticals, infusion solutions, blood preparations, medical equipments, medical apparatuses, cosmetics, foods and beverages, environmental samples (e.g., airs, rivers, and soils), biological components (e.g., bloods, body fluids, and tissues), naturally occurring proteins, recombinant proteins, nucleic acids, and carbohydrates. The test sample may be subjected to the measurement of endotoxin by mixing, dispersing, or dissolving the test sample as it is or an extract or washing solution of the test sample in a reaction system.

EXAMPLES

[0081]

The present invention will now be described by way of Examples more particularly. However, these are merely examples of the present invention, and the scope of the present invention is not limited to these.

[0082]

Example 1: Production of Endotoxin-measuring Agent of

Present Invention

(1-1) Method Using Virus (hereinafter referred to as "viral method")

In the present Example, a recombinant baculovirus into which the gene encoding each of factor C, factor B, and a proclotting enzyme was incorporated was used to express the factor in insect cells, and an endotoxin-measuring agent was thereby produced.

[0083]

(1-1-1) Preparation of Recombinant Baculovirus

As a DNA encoding His-tag-attached factor C (His-tag-attached factor C gene), the DNA of SEQ ID NO:7 was totally synthesized using a generally available contract service (TAKARA BIO INC.). The His-tag-attached factor C is the factor C of a Japanese horseshoe crab shown in SEQ ID NO:2 wherein a 6×His-tag is attached to the C-terminus. The DNA was inserted between the recognition sites of restriction enzymes *NruI* and *SmaI* of a transfer vector pPSC8 (Protein Sciences), to obtain a vector for recombination. Using the vector for recombination, the His-tag-attached factor C gene was incorporated into a baculovirus AcNPV, to prepare a recombinant baculovirus.

[0084]

Further, using a primer FC-N-Pst (SEQ ID NO:10) and a primer FC-notag-R-Bam (SEQ ID NO:11), and the above-described DNA encoding the His-tag-attached factor C as a template, PCR was carried out to prepare a DNA encoding factor C wherein the nucleotide sequence encoding the

His-tag sequence at the 3'-end was removed (His-tag-free factor C gene). The DNA encodes the Japanese horseshoe crab factor C shown in SEQ ID NO:2, wherein no His tag is attached at the C-terminus. Also for the His-tag-free factor C gene, a recombinant baculovirus was prepared by the same method as described above.

[0085]

As a DNA encoding factor B (factor B gene), the DNA of SEQ ID NO:8 was totally synthesized using a generally available contract service (TAKARA BIO INC.). The DNA encodes the Japanese horseshoe crab factor B shown in SEQ ID NO:4 (His-tag free), and the combinations of its codons are optimized for expression in insect cells. Also for the factor B gene, a recombinant baculovirus was prepared by the same method as described above. However, the position of insertion in the pPSC8 vector was between the recognition sites of restriction enzymes *Pst*I and *Kpn*I.

[0086]

As a DNA encoding the proclotting enzyme (proclotting enzyme gene), the DNA of SEQ ID NO:9 was totally synthesized using a generally available contract service (TAKARA BIO INC.). The DNA encodes the Japanese horseshoe crab proclotting enzyme shown in SEQ ID NO:6 (His-tag free), and the combinations of its codons are optimized for expression in insect cells. Also for the proclotting enzyme gene, a recombinant baculovirus was prepared by the same method as described above. However,

the position of insertion in the pPSC8 vector was between the recognition sites of restriction enzymes XbaI and BglII.

[0087]

(1-1-2) Infection of Insect Cells (Sf9 Cells) with Recombinant Baculovirus

Sf9 cells (Novagen) were inoculated in a medium at 1.5×10^6 cells/mL, and the recombinant baculovirus, into which the DNA encoding the His-tag-attached factor C was introduced, was added to the medium, to infect the cells with the virus. As the medium for the Sf9 cells, Sf900 II medium (Invitrogen) supplemented with antibiotics (antibiotics-antifungal agents ($\times 100$); Invitrogen) (final concentration, $\times 1$) (1 L) was used. The multiplicity of infection (MOI) of the virus was set to 1.0. Thereafter, the obtained cells were cultured at 28°C for 48 hours with shaking.

[0088]

Similarly, Sf9 cells were infected with the virus into which the DNA encoding the His-tag-free factor C was introduced.

[0089]

Further, Sf9 cells were infected also with each of the virus to which the DNA encoding the factor B was introduced and the virus to which the DNA encoding the proclotting enzyme was introduced. In these cases, MOI was set to 0.5, and the culturing time was 72 hours.

[0090]

(1-1-3) Recovery of Solution of Expressed Recombinant Protein

Each of the culture broths obtained after the above culturing was centrifuged at 4°C at 3000×g for 30 minutes to obtain the supernatant, which was then stored at -80°C.

[0091]

(1-1-4) Removal of Impurities and Viruses from Recombinant Protein Solution

Each of the supernatants which had been stored frozen as described above was thawed, and applied to a filter having a pore size of 0.1 µm (Cup Filter (Millipore)). Filtration was carried out with suction, and the solution which had passed through the filter was recovered. Each recovered supernatant was applied to a hollow fiber filtration membrane having a pore size of 500 kDa (polyether sulfone hollow fiber membrane; Spectrum Labs) and filtered using the Kros Flow TFF pump filtration system (Spectrum Labs). Each solution which had passed through the membrane was recovered.

[0092]

(1-1-5) Preparation of Reagent

At 4°C, 560 mL of each solution obtained in the above (1-1-4) (wherein factor C, factor B, or proclotting enzyme is contained), 134 mL of distilled water, 126 mL of 6.66 mM aqueous solution of a synthetic substrate (Boc-Leu-Gly-Arg-pNA) (final concentration, 0.3 mM) and 560 mL of 15% aqueous dextran solution (final

concentration, 3%) were mixed together. This mixture was aliquoted in 5 mL-volumes into vials and freeze-dried, to provide the endotoxin-measuring agent 1.

[0093]

(1-2) Method Using Plasmid (hereinafter also referred to as "stably expressing cell line method")

In the present Example, a gene encoding each of the factor C, factor B, and proclotting enzyme was incorporated into the chromosome of insect cells to construct a stably expressing cell line, and each factor was then expressed, thereby producing an endotoxin-measuring agent.

[0094]

(1-2-1) Preparation and Cultivation of Stably Expressing Cell Line

Each of the His-tag-free factor C gene, factor B gene (SEQ ID NO:8), and proclotting enzyme gene (SEQ ID NO:9) used in the above-described viral method was introduced into Sf9 cells (Invitrogen) using the pIZ vector kit (Invitrogen).

[0095]

More particularly, each of the DNAs was firstly incorporated between the *EcoRV* and *MluI* recognition sites in a vector pIZ/V5-His comprised in the kit, and the resulting each vector was mixed with Cellfectin comprised in the kit, followed by introduction of the vector into Sf9 cells. The position of incorporation of the DNAs in pIZ/V5-His, and the like are shown in Fig. 2. In the

region indicated by a thick arrow shown at the top in Fig. 2, each one of the DNAs was incorporated. As the medium for the Sf9 cells, Sf900 III medium (Invitrogen) supplemented with antibiotics (antibiotics-antifungal agents ($\times 100$); Invitrogen) (final concentration, $\times 1$) and Zeocin antibiotic (Invitrogen) (final concentration, 50 $\mu\text{g/mL}$) was used. The density of the thus obtained cell line, into which each DNA was introduced, was adjusted to 6×10^5 cells/mL (1L) in the medium, and the cells were cultured at 28°C for 96 hours with shaking.

[0096]

It should be noted that, although a His tag sequence is contained in pIZ/V5-His, all of the above described DNAs have a stop codon, so that all of the factor C, factor B, and proclotting enzyme are expressed without addition of His-tag.

[0097]

(1-2-2) Recovery of Solution of Recombinant Protein,
Removal of Impurities, and Preparation of Reagent

Each culture broth obtained after the above-described culturing was processed in the same manner as described in "(1-1-3) Recovery of Solution of Expressed Recombinant Protein", "(1-1-4) Removal of Impurities and Viruses from Recombinant Protein Solution" and "(1-1-5) Preparation of Reagent" for the viral method. However, the process of filtration using a hollow fiber filtration membrane in "(1-1-4) Removal of Impurities and Viruses from Recombinant Protein Solution" was not carried out. The

thus obtained measuring agent was provided as the endotoxin-measuring agent 2.

[0098]

Example 2: Properties and the Like of Expressed Proteins

(2-1) Comparison of Expression Level of Factor C

The expression level was compared among the His-tag-free factor Cs obtained by the viral method and the stably expressing cell line method, and the His-tag-attached factor C obtained by the viral method.

[0099]

The expression level was evaluated by sampling 0.5, 1.5, 5, or 15 μ L of the solution corresponding to the one after the filtration and before the preparation of the reagent in Example 1 and subjecting the sampled solutions to 5-20% polyacrylamide gel electrophoresis (under non-reducing conditions) in the presence of SDS and then to Western blotting using an anti-factor C antibody (2C12, obtained from Prof. Shun-ichiro Kawabata, Department of Biology, Graduate School of Sciences, Kyushu University).

[0100]

The results are shown in Fig. 3. The results indicate that the expression levels of the His-tag-free factor Cs were lower than the expression level of the His-tag-attached factor C. Further, the intensities of the bands on the Western blot in Fig. 3 were measured using a densitometer, and, the volume ratio of each solution with which equal concentrations of the factor Cs are attained was calculated on the basis of relative

values of the measured intensities. The volume ratio was 50 as for the His-tag free factor C obtained by the viral method, 17 as for the His-tag free factor C obtained by the stably expressing cell line method, and 7 as for the His-tag-attached factor C obtained by the viral method.

[0101]

(2-2) Comparison of Activity of Factor C

The proclotting enzyme-activating capacity of each of the factor C solutions was studied using an equal amount of factor C.

[0102]

More particularly, each of the His-tag-attached factor C solution obtained by the viral method (0.7 μ L or 5 μ L), His-tag-free factor C solution obtained by the viral method (5 μ L), and His-tag-free factor C solution obtained by the stably expressing cell line method (1.7 μ L) was placed in a well of a 96-well plate. Thereafter, the factor B-containing solution (5 μ L) and the proclotting enzyme-containing solution (5 μ L) obtained after the filtration through the 0.1 μ m filter in (1-1-4) in the viral method in Example 1, and Boc-Leu-Gly-Arg-pNA (final concentration, 0.3 mM), Tris-HCl (pH 8.0) (final concentration, 100 mM), and 50 μ L of endotoxin (product name "USP-Reference Standard Endotoxin" (USP-RSE); commercially available from Seikagaku Biobusiness Corporation) (sample concentration: 0, 0.05, or 0.5 EU/mL) were added to each well such that the total volume in the well became 100 μ L, and mixed together, followed

by incubation at 37°C for 3 hours, during which the absorbance at 405 nm was measured with time. As a negative control, distilled water was used. The rate of increase in the absorbance (the absorbance change rate) reflects the proclotting enzyme-activating capacity. The term "EU" means the "endotoxin unit", which is a unit representing the amount of endotoxin (this also applies hereinafter).

[0103]

The results are shown in Fig. 4. In Fig. 4, "DW" means distilled water; "Virus + His tag (x1)" means the His-tag-attached factor C solution obtained by the viral method (0.7 μ L); "Virus + His tag (x7)" means the same solution (5 μ L); "Virus No tag (x1)" means the His-tag free factor C solution obtained by the viral method; and "Stable Sf9 No tag (x1)" means the His-tag free factor C solution obtained by the stably expressing cell line method.

[0104]

As a result, activation of the proclotting enzyme was not observed or hardly observed in the His-tag-attached factor C solution containing the equal amount of factor C (0.7 μ L), and even in the solution containing about 7 times the amount of factor C (5 μ L). On the other hand, the His-tag-free factor C showed a remarkable proclotting enzyme-activating capacity irrespective of whether it was obtained by the viral method or by the stably expressing cell line method.

[0105]

From the above results, it was shown that a recombinant factor C molecule expressed without addition of His-tag sequence has a much stronger proclotting enzyme-activating capacity than a recombinant factor C molecule expressed with addition of His-tag sequence. Further, it was shown that each of the expressed proteins can be used without purification, in the state where the protein is contained in the culture supernatant.

[0106]

(2-3) Comparison of Stability of Expressed Factor C

(2-3-1) Stability of Factor C Expressed by Viral Method

In the step of culturing the virus-infected cells at 28°C with shaking in (1-1-2) in the viral method in Example 1, the supernatant was recovered after 48 hours, 72 hours, and 96 hours of cultivation, and each recovered supernatant was subjected to 5-20% polyacrylamide gel electrophoresis (under non-reducing conditions) in the presence of SDS, followed by evaluation of the remaining amount of the factor C by Western blotting using the anti-factor C antibody (2C12, which is the same as the one used above). Further, sampling and analysis were separately carried out in the same manner for the supernatant obtained by adding a protease inhibitor (leupeptin at a final concentration of 0.5 µg/mL + pepstatin A at a final concentration of 0.7 µg/mL) to the culture broth after 24 hours of the infection with the virus.

[0107]

The results are shown in Fig. 5. As a result, it was shown that the factor C expressed by the viral method was decomposed with time during the cultivation. Further, it was shown that decomposition of the factor C also occurred to some extent in the case where the protease inhibitor was added.

[0108]

(2-3-2) Stability of Factor C Expressed by Stably Expressing Cell Line Method

Similarly, in the step of culturing the stably expressing cell line at 28°C with shaking in (1-2-1) in the stably expressing cell line method in Example 1, the supernatant was recovered after 72 hours, 96 hours, 120 hours, 144 hours, and 168 hours of cultivation, and each recovered supernatant was subjected to 5-20% polyacrylamide gel electrophoresis (under non-reducing conditions) in the presence of SDS, followed by evaluation of the remaining amount of the factor C by Western blotting using the anti-factor C antibody (2C12, which is the same as the one used above). Further, the supernatant obtained after 48 hours of cultivation by the viral method was also applied.

[0109]

The results are shown in Fig. 6. As a result, the factor C expressed by the stably expressing cell line method has not been decomposed in the absence of a protease inhibitor even after 168 hours of cultivation.

By this, it was shown that use of the stable expressing cell line method can prevent decomposition of factor C.

[0110]

(2-4) Study on Whether Treatment by Hollow Fiber Membrane Filtration Is Necessary

Whether the treatment by a hollow fiber filtration membrane in (1-1-4) in the viral method is necessary was studied. Using each solution sampled before filtration through the hollow fiber membrane in (1-1-4) and each solution sampled after filtration therethrough (3 lots) in (1-1-4), the rate of increase in the absorbance (the absorbance change rate) was measured in the same manner as in the above (2-2), by adding endotoxin (USP-RSE) to a final concentration of 0 or 0.05 EU/mL.

[0111]

The results are shown in Fig. 7. In Fig. 7, "unfiltered solution" means a solution remained in the hollow fiber membrane cartridge without being filtered. In the cases where each solution sampled after filtration through the hollow fiber membrane was used, the degree of activation of the proclotting enzyme was low when the concentration of endotoxin was 0 EU/mL (in other words, the blank value upon endotoxin measurement was low), and that is, an excellent result was obtained. By contrast, it was revealed that, in the cases where each solution sampled before filtration through the hollow fiber membrane ("before filtration" or "unfiltered solution") was used, the degree of activation of the proclotting

enzyme was high even in the cases of 0 EU/mL of endotoxin, which leads to a high blank value upon endotoxin measurement.

[0112]

By contrast, by the stably expressing cell line method, the degree of activation of the proclotting enzyme in the case of 0 EU/mL of endotoxin (the blank value upon endotoxin measurement) was kept low even without such a process of filtration through the hollow fiber filtration membrane (Fig. 4).

[0113]

From these results, it was shown that, while filtration through a hollow fiber filtration membrane is indispensable in cases where the viral method is used, such filtration is not necessary in cases where the stably expressing cell line method is used.

[0114]

Example 3: Measurement of Endotoxin Using Endotoxin-Measuring Agent of Present Invention

(3-1) Measurement Using Endotoxin-measuring Agent 1

To the endotoxin-measuring agent 1 (freeze-dried product), 3.3 mL of 100 mM Tris buffer (pH 8.0) was added, to dissolve the agent. In this solution, the protein concentration of each of the culture supernatants containing the factor C, factor B, and proclotting enzyme was about 60 µg/mL.

Into each well of a 96-well microtiter plate, 50 µL of an endotoxin solution at a concentration of 0, 0.001,

0.01, or 0.1 EU/mL was aliquoted, and 50 μ L of the endotoxin-measuring agent solution prepared by dissolving the agent was added to the each well, followed by mixing the resulting mixture. The mixture was then incubated at 37°C for 30 minutes, and the endotoxin concentration was measured according to the reaction rate method in which the absorbance at 405 nm was measured with time during the incubation. In this reaction solution, the protein concentration of each of the culture supernatants containing the factor C, factor B, and proclotting enzyme was about 30 μ g/mL.

[0115]

The results are shown in Fig. 8. As a result, it was shown that, in cases where the factors expressed by the viral method are used, the absorbance change rate linearly increases within the range of 0.001 to 0.10 EU/mL as the concentration of endotoxin increases.

[0116]

(3-2) Measurement Using Endotoxin-measuring Agent 2

To the endotoxin-measuring agent 2 (freeze-dried product), 3.3 mL of 100 mM Hepes buffer (pH 7.6) was added, to dissolve the agent. In this solution, the protein concentration of each of the culture supernatants containing the factor C, factor B, and proclotting enzyme was about 60 μ g/mL.

Into each well of a 96-well microtiter plate, 50 μ L of an endotoxin solution at a concentration of 0, 0.0005, 0.001, 0.005, 0.01, or 0.1 EU/mL was aliquoted, and 50 μ L

of the endotoxin-measuring agent solution prepared by dissolving the agent was added to the each well, followed by mixing the resulting mixture. The mixture was then incubated at 37°C for 30 minutes, and the endotoxin concentration was measured according to the reaction rate method in which the absorbance at 405 nm was measured with time during the incubation. In this reaction solution, the protein concentration of each of the culture supernatants containing the factor C, factor B, and proclotting enzyme was about 30 µg/mL.

[0117]

The results are shown in Fig. 9. As a result, it was shown that, in cases where the factors expressed by the stably expressing cell method are used, the absorbance change rate linearly increases within the range of 0.0005 to 0.1 EU/mL as the concentration of endotoxin increases.

[0118]

Based on the above results, with either of the endotoxin-measuring agents, quantification of endotoxin at a concentration of 0.001 EU/mL was possible within 30 minutes. Further, with the endotoxin-measuring agent 2, endotoxin at a concentration of 0.0005 EU/mL was able to be measured within 30 minutes. Thus, it was shown that the endotoxin-measuring agents of the present invention enable more rapid and sensitive quantification of endotoxin compared to the conventional methods (by which the measurement takes not less than 1 hour, and the detection sensitivity of 0.001 EU/mL has not been

achieved). Further, it was shown that, in either case, the expressed factors can be used for a measuring agent as they are without purification.

[0119]

Example 4: Difference in Activity between Recombinant Factor C Protein and Naturally Occurring Factor C Protein
(4-1) Purification of Recombinant Factor C Protein and Naturally Occurring Factor C Protein

By covalently bonding 2 mg of the anti-factor C antibody (2C12, which is the same as the one used above) to sepharose column 1ml (GE Healthcare), 2 factor-C antibody columns were prepared. The preparation was carried out according to the method described in the attached instructions. To 76 mL of culture supernatant containing recombinant factor C protein derived by the stably expressing cell method that was prepared by the same process as described in the above "(1-2-2) Recovery of Recombinant Protein", an equal amount of 20 mM Tris-HCl buffer (pH 8.0) containing 2 M sodium chloride and 2 mM EDTA was added to dilute the culture supernatant, followed by subjecting the resulting dilution to one of the factor-C antibody columns. Similarly, to 76 mL of an extract of horseshoe crab blood cells, an equal amount of 20 mM Tris-HCl buffer (pH 8.0) containing 2 M sodium chloride and 2 mM EDTA was added to dilute the extract, followed by subjecting the resulting dilution to the other of the factor-C antibody columns. The both columns were washed sequentially with 20 mL each of 20 mM Tris-

HCl buffer (pH 8.0) containing 200 mM or 450 mM sodium chloride, and elution was then carried out with 50 mM glycine buffer (pH 2.5). Into a 1.5 mL tube in which 0.025 mL of 1M Trizma base (Sigma) was preliminarily placed, 1 mL of each eluted fraction was collected, to return the pH of the eluted solution to neutral.

[0120]

(4-2) Comparison of Concentration between Purified Recombinant and Naturally Occurring Factor C Proteins

The eluted fractions of the purified recombinant and naturally occurring factor C proteins were subjected to separation by 5-20% polyacrylamide gel electrophoresis (under non-reducing conditions) in the presence of SDS. In this process, purified bovine serum albumin (=BSA) whose concentration is known was also subjected to separation on the same gel as samples for concentration reference (Fig. 10). The intensities of BSA bands on the gel stained with Coomassie brilliant blue were quantified with a densitometer, and plotted against the concentrations of the BSA protein, to prepare a calibration curve (Fig. 11). Based on the band intensities of the purified factor C proteins and the calibration curve, the concentrations of the purified factor C proteins were approximated. As a result, it was revealed that, as for the purified samples, the naturally occurring factor C protein had about 4 times the concentration of the recombinant factor C protein.

[0121]

(4-3) Comparison of Activity between Purified Recombinant and Naturally Occurring Factor C Proteins

A comparison of the activity was made using the purified recombinant and naturally occurring factor C proteins. In this comparison, the protein concentration was equalized between the purified factor Cs based on the results of (4-2). Into each well of a 96-well microtiter plate, 50 μ L of an endotoxin solution at a concentration of 0, 0.05, 0.1, or 0.5 EU/mL was aliquoted. Reagents and culture supernatants were added to the each well such that 50 mM Tris buffer (pH 8.0), 0.2 μ g/mL purified factor C protein, 30 μ g/mL protein of each of culture supernatants containing the recombinant factor B and recombinant proclotting enzyme, and 0.3 mM of the synthetic substrate Boc-Leu-Gly-Arg-pNA were contained in the reaction solution, whose total volume was adjusted to 100 μ L by addition of water for injection. The reaction solution was incubated at 37°C for 30 minutes, and the analysis was carried out according to the reaction rate method in which the absorbance at 405 nm was measured with time during the incubation.

[0122]

As a result, it was revealed that the purified recombinant factor C protein had about twice the activity of the purified naturally occurring factor C protein (Fig. 12). The above results suggest that the recombinant factor C has a higher specific activity than the naturally occurring factor C.

INDUSTRIAL APPLICABILITY

[0123]

By the present invention, endotoxin can be rapidly and highly sensitively measured. Further, by the present invention, an endotoxin-measuring agent can be simply and rapidly produced at a low cost. Therefore, the present invention can be extremely effectively used for detection of endotoxin.

[0124]

DESCRIPTION OF SEQUENCE LISTING

SEQ ID NO:1 DNA sequence of factor C gene of Japanese horseshoe crab

SEQ ID NO:2 Amino acid sequence of factor C of Japanese horseshoe crab

SEQ ID NO:3 DNA sequence of factor B gene of Japanese horseshoe crab

SEQ ID NO:4 Amino acid sequence of factor B of Japanese horseshoe crab

SEQ ID NO:5 DNA sequence of proclotting enzyme gene of Japanese horseshoe crab

SEQ ID NO:6 Amino acid sequence of proclotting enzyme of Japanese horseshoe crab

SEQ ID NO:7 DNA sequence of His-tag-attached factor C gene

SEQ ID NO:8 DNA sequence of factor B gene whose codons are optimized for expression in insect cells

SEQ ID NO:9 DNA sequence of proclotting enzyme gene

whose codons are optimized for expression in insect cells

SEQ ID NO:10 Primer for preparation of His-tag-free
factor C gene

SEQ ID NO:11 Primer for preparation of His-tag-free
factor C gene

SEQ ID NO:12 Peptide sequence

CLAIMS

1. An endotoxin-measuring agent comprising the proteins (1) to (3) below, each of which is a recombinant protein obtainable by being expressed using insect cells as a host:

(1) a factor C derived from *Tachypleus tridentatus*, which factor C does not have His-tag sequence at the C-terminus;

(2) a factor B of a horseshoe crab; and

(3) a proclotting enzyme of a horseshoe crab.

2. The measuring agent according to claim 1, wherein said factor B and said proclotting enzyme are derived from *Tachypleus tridentatus*.

3. The measuring agent according to claim 1 or 2, wherein said factor C is the protein (A) or (B) below; said factor B is the protein (C) or (D) below; and said proclotting enzyme is the protein (E) or (F) below:

(A) a protein comprising the amino acid sequence shown in SEQ ID NO:2;

(B) a protein comprising the amino acid sequence shown in SEQ ID NO:2 but which includes substitution, deletion, insertion, or addition of one or several amino acid residues, which protein has factor C activity;

(C) a protein comprising the amino acid sequence shown in SEQ ID NO:4;

(D) a protein comprising the amino acid sequence shown in SEQ ID NO:4 but which includes substitution, deletion, insertion, or addition of one or several amino acid residues, which protein has factor B activity;

(E) a protein comprising the amino acid sequence shown in SEQ ID NO:6;

(F) a protein comprising the amino acid sequence shown in SEQ ID NO:6 but which includes substitution, deletion, insertion, or addition of one or several amino acid residues, which protein has proclotting enzyme activity.

4. A method for producing the measuring agent according to any one of claims 1 to 3, the method comprising the steps (A) to (C) below:

(A) a step of incorporating each of the DNAs (1) to (3) below into a viral DNA:

(1) a DNA encoding a factor C derived from *Tachyplesus tridentatus*, which factor C does not have His-tag sequence at the C-terminus;

(2) a DNA encoding a factor B of a horseshoe crab; and

(3) a DNA encoding a proclotting enzyme of a horseshoe crab;

(B) a step of infecting insect cells with the virus into which said each DNA was incorporated; and

(C) a step of allowing the insect cells infected with said each virus to express the protein encoded by said

each DNA.

5. A method for producing the measuring agent according to any one of claims 1 to 3, the method comprising the steps (A) to (C) below:

(A) a step of incorporating each of the DNAs (1) to (3) below into a vector:

(1) a DNA encoding a factor C derived from *Tachypleus tridentatus*, which factor C does not have His-tag sequence at the C-terminus;

(2) a DNA encoding a factor B of a horseshoe crab; and

(3) a DNA encoding a proclotting enzyme of a horseshoe crab;

(B) a step of introducing the vector, into which said each DNA was incorporated, into insect cells to incorporate said each DNA into the chromosome of the insect cells; and

(C) a step of allowing the insect cells, into which said each DNA was incorporated, to express the protein encoded by said each DNA.

6. The method according to claim 4 or 5, wherein said DNA encoding factor C is the DNA (A) or (B) below; said DNA encoding factor B is the DNA (C) or (D) below; and said DNA encoding proclotting enzyme is the DNA (E) or (F) below:

(A) a DNA comprising the nucleotide sequence shown in

SEQ ID NO:1;

(B) a DNA which hybridizes with the complementary sequence of the full length or a part of the nucleotide sequence shown in SEQ ID NO:1 under stringent conditions, and encodes a protein having factor C activity.

(C) a DNA comprising the nucleotide sequence shown in SEQ ID NO:3 or 8;

(D) a DNA which hybridizes with the complementary sequence of the full length or a part of the nucleotide sequence shown in SEQ ID NO:3 or 8 under stringent conditions, and encodes a protein having factor B activity.

(E) a DNA comprising the nucleotide sequence shown in SEQ ID NO:5 or 9;

(F) a DNA which hybridizes with the complementary sequence of the full length or a part of the nucleotide sequence shown in SEQ ID NO:5 or 9 under stringent conditions, and encodes a protein having proclotting enzyme activity.

7. A method for measuring endotoxin in a test sample, the method comprising a step of mixing the measuring agent according to any one of claims 1 to 3 with the test sample and a step of measuring progress of cascade reaction.

8. The method according to claim 7, which comprises a step of adding a substrate for detection of progress of

cascade reaction to a reaction system.

9. The method according to claim 8, which further comprises a step of calculating the endotoxin level in the test sample on the basis of reaction of said substrate.

SEQUENCE LISTING

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<150> US61/447, 556

<151> 2011-02-28

<160> 12

<170> PatentIn version 3.1

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1 5 10 15

gcc caa caa atg cgt cca gtt cag tcc aga gga gta gat ctg ggc ttg 96

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Phe Asn Val Pro Met Lys Gln Cys Thr Tyr Phe Tyr Arg Trp Arg Pro

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Tyr Cys Lys Pro Cys Asp Asp Leu Glu Ala Lys Asp Ile Cys Pro Lys

65 70 75 80

tac aaa cga tgt caa gag tgt aag gct ggt ctt gat agt tgt gtt act 288

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Cys	Pro	Pro	Asn	Lys	Tyr	Gly	Thr	Trp	Cys	Ser	Gly	Glu	Cys	Gln	Cys		
			100					105					110				
aag	aat	gga	ggg	atc	tgt	gac	cag	agg	aca	gga	gct	tgt	acc	tgt	cgt		384
Lys	Asn	Gly	Gly	Ile	Cys	Asp	Gln	Arg	Thr	Gly	Ala	Cys	Thr	Cys	Arg		
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Asp	Arg	Tyr	Glu	Gly	Ala	His	Cys	Glu	Ile	Leu	Lys	Gly	Cys	Pro	Leu		
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Val	Ala	Arg	Ile	Ser	Cys	Leu	Pro	Asn	Gly	Gln	Trp	Ser	Ser	Phe	Pro		
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Pro	Lys	Cys	Ile	Arg	Glu	Cys	Ala	Lys	Val	Ser	Ser	Pro	Glu	His	Gly		
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Phe	Ser	Cys	Asp	Ser	Pro	Tyr	Tyr	Leu	Ile	Gly	Gln	Glu	Thr	Leu	Thr		
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Cys	Gln	Gly	Asn	Gly	Gln	Trp	Ser	Gly	Gln	Ile	Pro	Gln	Cys	Lys	Lys		
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Thr	Leu	Lys	Cys	Asn	Pro	Asp	Gly	Ser	Trp	Ser	Gly	Ser	Gln	Pro	Ser	
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Cys	Val	Lys	Val	Ala	Asp	Arg	Glu	Val	Asp	Cys	Asp	Ser	Lys	Ala	Val	
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Asp	Phe	Leu	Asp	Asp	Val	Gly	Glu	Pro	Val	Arg	Ile	His	Cys	Pro	Ala	
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Gln Asn Pro Pro Val Pro Ser Tyr Gly Ser Val Glu Ile Lys Pro Pro			
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 Pro Gln Thr Ile Asp Tyr Ser Cys Ser Pro Gly Phe Lys Leu Lys Gly

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Lys	Val	Asn	Ala	Pro	Ser	Gly	Asn	Met	Ile	Glu	Gly	Ala	Thr	Leu	Arg				
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Phe	Ser	Cys	Asp	Ser	Pro	Tyr	Tyr	Leu	Ile	Gly	Gln	Glu	Thr	Leu	Thr				
225					230					235					240				
Cys	Gln	Gly	Asn	Gly	Gln	Trp	Ser	Gly	Gln	Ile	Pro	Gln	Cys	Lys	Lys				
			245					250				255							
Leu	Val	Phe	Cys	Pro	Asp	Leu	Asp	Pro	Val	Asn	His	Ala	Glu	His	Gln				
		260					265					270							
Val	Lys	Ile	Gly	Val	Glu	Gln	Lys	Tyr	Gly	Gln	Phe	Pro	Gln	Gly	Thr				
	275					280					285								
Glu	Val	Thr	Tyr	Thr	Cys	Ser	Gly	Asn	Tyr	Phe	Leu	Met	Gly	Phe	Asn				
	290					295				300									
Thr	Leu	Lys	Cys	Asn	Pro	Asp	Gly	Ser	Trp	Ser	Gly	Ser	Gln	Pro	Ser				
305					310					315					320				
Cys	Val	Lys	Val	Ala	Asp	Arg	Glu	Val	Asp	Cys	Asp	Ser	Lys	Ala	Val				
			325						330				335						
Asp	Phe	Leu	Asp	Asp	Val	Gly	Glu	Pro	Val	Arg	Ile	His	Cys	Pro	Ala				
		340					345					350							
Gly	Cys	Ser	Leu	Thr	Ala	Gly	Thr	Val	Trp	Gly	Thr	Ala	Ile	Tyr	His				
	355					360					365								
Glu	Leu	Ser	Ser	Val	Cys	Arg	Ala	Ala	Ile	His	Ala	Gly	Lys	Leu	Pro				
	370				375					380									
Asn	Ser	Gly	Gly	Ala	Val	His	Val	Val	Asn	Asn	Gly	Pro	Tyr	Ser	Asp				
385					390					395					400				
Phe	Leu	Gly	Ser	Asp	Leu	Asn	Gly	Ile	Lys	Ser	Glu	Glu	Leu	Lys	Ser				
			405						410					415					
Leu	Ala	Arg	Ser	Phe	Arg	Phe	Asp	Tyr	Val	Ser	Ser	Ser	Thr	Ala	Gly				
		420					425						430						
Arg	Ser	Gly	Cys	Pro	Asp	Gly	Trp	Phe	Glu	Val	Glu	Glu	Asn	Cys	Val				
	435					440					445								
Tyr	Val	Thr	Ser	Lys	Gln	Arg	Ala	Trp	Glu	Arg	Ala	Gln	Gly	Val	Cys				
	450				455					460									
Thr	Asn	Met	Ala	Ala	Arg	Leu	Ala	Val	Leu	Asp	Lys	Asp	Leu	Ile	Pro				
465					470					475					480				
Ser	Ser	Leu	Thr	Glu	Thr	Leu	Arg	Gly	Lys	Gly	Leu	Thr	Thr	Thr	Trp				
			485					490					495						

Ile Gly Leu His Arg Leu Asp Ala Glu Lys Pro Phe Val Trp Glu Leu
 500 505 510
 Met Asp Arg Ser Asn Val Val Leu Asn Asp Asn Leu Thr Phe Trp Ala
 515 520 525
 Ser Gly Glu Pro Gly Asn Glu Thr Asn Cys Val Tyr Leu Asp Ile Arg
 530 535 540
 Asp Gln Leu Gln Pro Val Trp Lys Thr Lys Ser Cys Phe Gln Pro Ser
 545 550 555 560
 Ser Phe Ala Cys Met Met Asp Leu Ser Asp Arg Asn Lys Ala Lys Cys
 565 570 575
 Asp Asp Pro Gly Pro Leu Glu Asn Gly His Ala Thr Leu His Gly Gln
 580 585 590
 Ser Ile Asp Gly Phe Tyr Ala Gly Ser Ser Ile Arg Tyr Ser Cys Glu
 595 600 605
 Val Leu His Tyr Leu Ser Gly Thr Glu Thr Val Thr Cys Thr Thr Asn
 610 615 620
 Gly Thr Trp Ser Ala Pro Lys Pro Arg Cys Ile Lys Val Ile Thr Cys
 625 630 635 640
 Gln Asn Pro Pro Val Pro Ser Tyr Gly Ser Val Glu Ile Lys Pro Pro
 645 650 655
 Ser Arg Thr Asn Ser Ile Ser Arg Val Gly Ser Pro Phe Leu Arg Leu
 660 665 670
 Pro Arg Leu Pro Leu Pro Leu Ala Arg Ala Ala Lys Pro Pro Pro Lys
 675 680 685
 Pro Arg Ser Ser Gln Pro Ser Thr Val Asp Leu Ala Ser Lys Val Lys
 690 695 700
 Leu Pro Glu Gly His Tyr Arg Val Gly Ser Arg Ala Ile Tyr Thr Cys
 705 710 715 720
 Glu Ser Arg Tyr Tyr Glu Leu Leu Gly Ser Gln Gly Arg Arg Cys Asp
 725 730 735
 Ser Asn Gly Asn Trp Ser Gly Arg Pro Ala Ser Cys Ile Pro Val Cys
 740 745 750
 Gly Arg Ser Asp Ser Pro Arg Ser Pro Phe Ile Trp Asn Gly Asn Ser
 755 760 765
 Thr Glu Ile Gly Gln Trp Pro Trp Gln Ala Gly Ile Ser Arg Trp Leu
 770 775 780
 Ala Asp His Asn Met Trp Phe Leu Gln Cys Gly Gly Ser Leu Leu Asn
 785 790 795 800
 Glu Lys Trp Ile Val Thr Ala Ala His Cys Val Thr Tyr Ser Ala Thr
 805 810 815
 Ala Glu Ile Ile Asp Pro Ser Gln Phe Lys Ile Tyr Leu Gly Lys Tyr

820	825	830
Tyr Arg Asp Asp Ser Arg Asp Asp Asp Tyr Val Gln Val Arg Glu Ala		
835	840	845
Leu Glu Ile His Val Asn Pro Asn Tyr Asp Pro Gly Asn Leu Asn Phe		
850	855	860
Asp Ile Ala Leu Ile Gln Leu Lys Thr Pro Val Thr Leu Thr Thr Arg		
865	870	875
Val Gln Pro Ile Cys Leu Pro Thr Asp Ile Thr Thr Arg Glu His Leu		
885	890	895
Lys Glu Gly Thr Leu Ala Val Val Thr Gly Trp Gly Leu Asn Glu Asn		
900	905	910
Asn Thr Tyr Ser Glu Met Ile Gln Gln Ala Val Leu Pro Val Val Ala		
915	920	925
Ala Ser Thr Cys Glu Glu Gly Tyr Lys Glu Ala Asp Leu Pro Leu Thr		
930	935	940
Val Thr Glu Asn Met Phe Cys Ala Gly Tyr Lys Lys Gly Arg Tyr Asp		
945	950	955
Ala Cys Ser Gly Asp Ser Gly Gly Pro Leu Val Phe Ala Asp Asp Ser		
965	970	975
Arg Thr Glu Arg Arg Trp Val Leu Glu Gly Ile Val Ser Trp Gly Ser		
980	985	990
Pro Ser Gly Cys Gly Lys Ala Asn Gln Tyr Gly Gly Phe Thr Lys Val		
995	1000	1005
Asn Val Phe Leu Ser Trp Ile Arg Gln Phe Ile		
1010	1015	

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 <212> DNA
 <213> Tachypleus tridentatus

<220>
 <221> CDS
 <222> (1)..(1203)

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1 5 10 15	
ttg ggt aac aaa gtt agt aga gtg ggg gtc ctc ttc ccc aag aca cgg	96

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Leu Gly Asn Lys Val Ser Arg Val Gly Val Leu Phe Pro Lys Thr Arg	
20 25 30	
aac gac aat gag tgt aca gca aga ggg gga ttg aaa gga tcc tgc aaa	144
Asn Asp Asn Glu Cys Thr Ala Arg Gly Gly Leu Lys Gly Ser Cys Lys	
35 40 45	
tcc ctc ata gac tgt cct agt gtc ttg gct acg ttg aag gac agt ttt	192
Ser Leu Ile Asp Cys Pro Ser Val Leu Ala Thr Leu Lys Asp Ser Phe	
50 55 60	
cct gtc gtt tgc tct tgg aat ggt cga ttt cag cct att gtc tgc tgt	240
Pro Val Val Cys Ser Trp Asn Gly Arg Phe Gln Pro Ile Val Cys Cys	
65 70 75 80	
cct gat gca ata gca cca cca cct gta acc aca aca gct gta act gta	288
Pro Asp Ala Ile Ala Pro Pro Pro Val Thr Thr Thr Ala Val Thr Val	
85 90 95	
ata tct aca aaa gaa cca aag ctt cca aga tta cat ata tca ggt tgt	336
Ile Ser Thr Lys Glu Pro Lys Leu Pro Arg Leu His Ile Ser Gly Cys	
100 105 110	
gga aaa aga aaa gtc aaa ata gat att aca act gtt gga cgc tct gga	384
Gly Lys Arg Lys Val Lys Ile Asp Ile Thr Thr Val Gly Arg Ser Gly	
115 120 125	
tca cca ata ctt cct ccg ata tct act cct caa aat tca aca ggt ggg	432
Ser Pro Ile Leu Pro Pro Ile Ser Thr Pro Gln Asn Ser Thr Gly Gly	
130 135 140	
aga gga att att gct gga ggc gta gaa gcc aaa att ggc gcg tgg cct	480
Arg Gly Ile Ile Ala Gly Gly Val Glu Ala Lys Ile Gly Ala Trp Pro	
145 150 155 160	
tgg atg gca gct gtt ttt gtg aaa aac ttt ggc att ggc aga ttc cac	528
Trp Met Ala Ala Val Phe Val Lys Asn Phe Gly Ile Gly Arg Phe His	
165 170 175	
tgt gct ggt agc ata atc agt aac aag tac att ttg tca gct gcc cac	576
Cys Ala Gly Ser Ile Ile Ser Asn Lys Tyr Ile Leu Ser Ala Ala His	
180 185 190	
gcc ttc ctt atc gga ggt cga aag ttg acc cca act cgc tta gct gtc	624
Ala Phe Leu Ile Gly Gly Arg Lys Leu Thr Pro Thr Arg Leu Ala Val	
195 200 205	
cgt gtg gga ggc cac tac ata aag agg ggt caa gag tat cca gtg aaa	672
Arg Val Gly Gly His Tyr Ile Lys Arg Gly Gln Glu Tyr Pro Val Lys	
210 215 220	
gac gtg att atc cat cct cat tat gta gaa aag gag aac tac aat gat	720
Asp Val Ile Ile His Pro His Tyr Val Glu Lys Glu Asn Tyr Asn Asp	
225 230 235 240	

11/20

ata gcc ata atc gag tta aaa gag gaa ctg aac ttt acg gac ttg gtc	768
Ile Ala Ile Ile Glu Leu Lys Glu Glu Leu Asn Phe Thr Asp Leu Val	
245 250 255	
aat cct ata tgt ctc cct gat cca gag aca gta acg gat cca tta aaa	816
Asn Pro Ile Cys Leu Pro Asp Pro Glu Thr Val Thr Asp Pro Leu Lys	
260 265 270	
gac aga att gtg act gca gcg gga tgg ggc gat ctg gat ttc tcc ggt	864
Asp Arg Ile Val Thr Ala Ala Gly Trp Gly Asp Leu Asp Phe Ser Gly	
275 280 285	
cca cgg agc caa gtt cta cgt gag gta agc atc cca gtt gtt cca gtt	912
Pro Arg Ser Gln Val Leu Arg Glu Val Ser Ile Pro Val Val Pro Val	
290 295 300	
gat aaa tgt gat caa gcc tat gag aaa ctc aac acc cct tca cta aaa	960
Asp Lys Cys Asp Gln Ala Tyr Glu Lys Leu Asn Thr Pro Ser Leu Lys	
305 310 315 320	
aat ggg ata acg aat aac ttc ctt tgc gct gga ttg gaa gaa gga ggg	1008
Asn Gly Ile Thr Asn Asn Phe Leu Cys Ala Gly Leu Glu Glu Gly Gly	
325 330 335	
aaa gac gct tgc caa ggc gat tct ggt gga ccg ttg atg cta gtg aac	1056
Lys Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Met Leu Val Asn	
340 345 350	
aac act agg tgg ata gta gta gga gtt gtg tcg ttc ggg cac aag tgt	1104
Asn Thr Arg Trp Ile Val Val Gly Val Val Ser Phe Gly His Lys Cys	
355 360 365	
gcc gag gaa gga tat cct ggt gtg tac tcg cgc gta gcg agt tac cta	1152
Ala Glu Glu Gly Tyr Pro Gly Val Tyr Ser Arg Val Ala Ser Tyr Leu	
370 375 380	
gac tgg atc gcg aaa gtt acg aac tcg tta gat cat gcc gtc act aac	1200
Asp Trp Ile Ala Lys Val Thr Asn Ser Leu Asp His Ala Val Thr Asn	
385 390 395 400	
taa	1203

<210> 4

<211> 400

<212> PRT

<213> Tachypleus tridentatus

<400> 4

Met Thr Trp Ile Cys Val Ile Thr Leu Phe Ala Leu Ala Ser Ala Thr

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			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							
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				
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 <213> Tachypleus tridentatus

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1 5 10 15	
tct gtg gtt aga tgc agt act ctc agc aga cag cgt aga cag ttt gtt	96
Ser Val Val Arg Cys Ser Thr Leu Ser Arg Gln Arg Arg Gln Phe Val	
20 25 30	
ttc cct gac gag gaa gaa ctt tgc tca aac cga ttt act gaa gaa gga	144
Phe Pro Asp Glu Glu Glu Leu Cys Ser Asn Arg Phe Thr Glu Glu Gly	
35 40 45	
aca tgc aaa aat gtc ttg gat tgt aga ata ctt tta caa aaa aat gat	192
Thr Cys Lys Asn Val Leu Asp Cys Arg Ile Leu Leu Gln Lys Asn Asp	
50 55 60	
tat aat tta ctc aaa gaa tca ata tgc ggc ttt gaa ggc ata aca ccc	240
Tyr Asn Leu Leu Lys Glu Ser Ile Cys Gly Phe Glu Gly Ile Thr Pro	
65 70 75 80	
aaa gtt tgt tgt ccg aaa tca agc cat gta att tca agt aca cag gca	288
Lys Val Cys Cys Pro Lys Ser Ser His Val Ile Ser Ser Thr Gln Ala	
85 90 95	
cct cca gaa acc act acg act gaa cgc cca cca aaa cag ata cca ccc	336
Pro Pro Glu Thr Thr Thr Thr Glu Arg Pro Pro Lys Gln Ile Pro Pro	
100 105 110	
aat ctt cat gaa gtg tgt gga att cac aat act aca act acc agg att	384

Asn	Leu	His	Glu	Val	Cys	Gly	Ile	His	Asn	Thr	Thr	Thr	Thr	Arg	Ile		
	115						120					125					
att	gga	ggt	cgg	gaa	gca	cct	att	gga	gcc	tgg	ccg	tgg	atg	act	gct	432	
Ile	Gly	Gly	Arg	Glu	Ala	Pro	Ile	Gly	Ala	Trp	Pro	Trp	Met	Thr	Ala		
	130						135					140					
gtc	tac	ata	aaa	caa	gga	gga	atc	aga	agt	gtt	cag	tgt	ggt	ggc	gca	480	
Val	Tyr	Ile	Lys	Gln	Gly	Gly	Ile	Arg	Ser	Val	Gln	Cys	Gly	Gly	Ala		
	145					150				155				160			
ctt	gtc	act	aac	agg	cac	gtg	att	aca	gct	tcg	cac	tgt	gtt	gta	aac	528	
Leu	Val	Thr	Asn	Arg	His	Val	Ile	Thr	Ala	Ser	His	Cys	Val	Val	Asn		
			165					170				175					
agt	gca	gga	aca	gat	gtg	atg	cca	gct	gat	gta	ttc	tcg	gtt	cgt	ctg	576	
Ser	Ala	Gly	Thr	Asp	Val	Met	Pro	Ala	Asp	Val	Phe	Ser	Val	Arg	Leu		
		180					185				190						
ggt	gaa	cac	aat	tta	tac	agt	acc	gat	gac	gat	tcg	aat	cca	ata	gat	624	
Gly	Glu	His	Asn	Leu	Tyr	Ser	Thr	Asp	Asp	Asp	Ser	Asn	Pro	Ile	Asp		
	195					200					205						
ttt	gca	gtt	acg	tcg	gtg	aaa	cat	cac	gaa	cac	ttt	gta	ctc	gcg	acg	672	
Phe	Ala	Val	Thr	Ser	Val	Lys	His	His	Glu	His	Phe	Val	Leu	Ala	Thr		
	210					215					220						
tat	ttg	aat	gac	atc	gca	att	cta	acg	tta	aat	gac	aca	gtt	acg	ttt	720	
Tyr	Leu	Asn	Asp	Ile	Ala	Ile	Leu	Thr	Leu	Asn	Asp	Thr	Val	Thr	Phe		
	225				230				235			240					
aca	gac	aga	att	cga	ccc	att	tgt	cta	cct	tat	cgt	aag	ttg	aga	tac	768	
Thr	Asp	Arg	Ile	Arg	Pro	Ile	Cys	Leu	Pro	Tyr	Arg	Lys	Leu	Arg	Tyr		
			245				250				255						
gat	gat	cta	gca	atg	aga	aaa	ccg	ttt	atc	act	gga	tgg	gga	aca	aca	816	
Asp	Asp	Leu	Ala	Met	Arg	Lys	Pro	Phe	Ile	Thr	Gly	Trp	Gly	Thr	Thr		
	260					265					270						
gca	ttt	aac	ggc	cca	tct	agt	gca	gtg	ttg	aga	gaa	gta	cag	tta	cca	864	
Ala	Phe	Asn	Gly	Pro	Ser	Ser	Ala	Val	Leu	Arg	Glu	Val	Gln	Leu	Pro		
	275					280					285						
ata	tgg	gaa	cac	gag	gcc	tgt	aga	cag	gcc	tac	gag	aag	gat	tta	aat	912	
Ile	Trp	Glu	His	Glu	Ala	Cys	Arg	Gln	Ala	Tyr	Glu	Lys	Asp	Leu	Asn		
	290					295				300							
att	aca	aac	gtg	tat	atg	tgt	gct	ggc	ttt	gca	gat	ggc	ggg	aag	gat	960	
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					

15/20

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 tag 1128
 Trp Ile Ala Glu His Met Val
 370 375

<210> 6
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 <212> PRT
 <213> Tachypleus tridentatus

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 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 His 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
 325 330 335
 Gly Glu Phe Tyr Leu Ile Gly Ile Val Ser Phe Gly Lys Lys Cys Ala
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 Leu Pro Gly Phe Pro Gly Val Tyr Thr Lys Val Thr Glu Phe Leu Asp
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<211> 3078

<212> DNA

<213> Tachypleus tridentatus

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 cgatggaggc cttattgtaa accatgtgat gacctggagg ctaaggacat ttgtccaaag 240
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agctgtctcc	caaatggaca	gtggagtagc	tttccacca	aatgtattcg	agaatgtgcc	600
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<212> DNA

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1