

DESCRIPTION

HORSESHOE CRAB FACTOR B VARIANT

Technical Field

[0001]

The present invention relates to a horseshoe crab factor B variant.

Background Art

[0002]

A means for detecting a microorganism-derived substance in the hygiene management of medicines and food products or in the diagnosis of animals including human beings, and measuring the extent of microorganism contamination, is important. Regarding the means for measuring the extent of microorganism contamination, Limulus test is popularly used. The Limulus test is a technology for measuring the extent of microorganism contamination by using an endotoxin (LPS) or (1→3)-β-D-glucan as an object substance for measurement, and is a measurement method of utilizing the property that a protease precursor carried by horseshoe crabs is activated by these object substances for measurement.

[0003]

Regarding the Limulus test, a method of using a

horseshoe crab hemocyte extract (horseshoe crab amebocyte lysate; hereinafter, simply referred to as "lysate") is widely used. This method is a method of utilizing a cascade reaction that proceeds when serine protease precursors (factor C, factor B, and proclotting enzyme) come into contact with an endotoxin, or when serine protease precursors (factor G and proclotting enzyme) come into contact with (1→3)-β-D-glucan, the serine protease precursors are sequentially activated.

[0004]

Non Patent Literature 1 discloses Tachypleus tridentatus factor B isolated from the lysate. Furthermore, Non Patent Literature 2 discloses an amino acid sequence of a polypeptide of Tachypleus tridentatus factor B. However, a factor B variant having enzyme characteristics that are superior to the factor B itself, is not disclosed in any of these literatures.

Citation List

Non Patent Literature

[0005]

Non Patent Literature 1: Nakamura, T., Horiuchi, T., Morita, T., Iwanaga, S (1986) J. Biochem. 99, 847-57

Non Patent Literature 2: Muta, T., Oda, T., Iwanaga, S. (1993) J. Biol. Chem. 268, 21384-8

Summary of Invention

Technical Problem

[0006]

An object of the present invention is to provide a technology relating to a horseshoe crab factor B variant. Particularly, it is an object of the present invention to provide a polypeptide of a horseshoe crab factor B variant; a nucleic acid encoding the polypeptide; a vector retaining the nucleic acid; a cell retaining the nucleic acid and/or the vector; a method for producing the polypeptide; a method for measuring an endotoxin by using the polypeptide; a reagent for endotoxin measurement including the polypeptide as a constituent component; and a kit for endotoxin measurement including the polypeptide or the reagent as a component part.

Solution to Problem

[0007]

The inventors of the present invention conducted a thorough investigation in order to solve the problems described above, and as a result, the inventors found that when an amino acid residue at a particular site in the amino acid sequence of a polypeptide of horseshoe crab factor B is modified, a horseshoe crab factor B variant

having protease activity superior to that of the factor B itself may be obtained. Furthermore, the present inventors also found that a horseshoe crab factor B variant having thermal stability that is superior to that of the factor B itself may be obtained by the finding as described above. Thus, the present inventors completed the present invention based on these findings.

[0008]

The problems described above can be solved by the present invention including the following embodiments.

[1]

A polypeptide having an amino acid sequence in which the amino acid residue at the 193-position in an amino acid sequence of a polypeptide of horseshoe crab factor B is substituted with a cysteine (Cys) residue.

[2]

A polypeptide represented by any one of the following (A) to (D):

(A) a polypeptide having an amino acid sequence represented by the following (A1) or (A2):

(A1) an amino acid sequence represented by amino acid numbers 1 to 400 of SEQ ID NO: 7; and

(A2) an amino acid sequence represented by amino acid numbers 24 to 400 of SEQ ID NO: 7,

(B) a polypeptide having an amino acid sequence

represented by the following (B1) or (B2):

(B1) an amino acid sequence represented by amino acid numbers 1 to 400 of SEQ ID NO: 10; and

(B2) an amino acid sequence represented by amino acid numbers 24 to 400 of SEQ ID NO: 10,

(C) a polypeptide having an amino acid sequence including substitution, deletion, insertion, and/or addition of one amino acid residue or a plurality of amino acid residues in the amino acid sequence of the polypeptide represented by the item (A) or (B) described above (provided that the cysteine (Cys) residue at the 193-position is neither substituted nor deleted), the polypeptide having the function of horseshoe crab factor B, and

(D) a polypeptide having an amino acid sequence of a fusion polypeptide in which a peptide tag is added to the polypeptide represented by any one of the items (A) to (C) described above, the polypeptide having the function of horseshoe crab factor B.

[3]

A nucleic acid encoding the polypeptide according to [1] or [2].

[4]

A DNA represented by any one of the following (a) to (d):

(a) a DNA having a base sequence represented by any one of the following (a1) to (a4):

(a1) a base sequence represented by base numbers 1 to 1200 of SEQ ID NO: 5;

(a2) a base sequence represented by base numbers 70 to 1200 of SEQ ID NO: 5;

(a3) a base sequence represented by base numbers 1 to 1200 of SEQ ID NO: 6; and

(a4) a base sequence represented by base numbers 70 to 1200 of SEQ ID NO: 6,

(b) a DNA having a base sequence represented by any one of the following (b1) to (b4):

(b1) a base sequence represented by base numbers 1 to 1200 of SEQ ID NO: 8;

(b2) a base sequence represented by base numbers 70 to 1200 of SEQ ID NO: 8;

(b3) a base sequence represented by base numbers 1 to 1200 of SEQ ID NO: 9; and

(b4) a base sequence represented by base numbers 70 to 1200 of SEQ ID NO: 9,

(c) a DNA hybridizing with a DNA including a base sequence complementary to the DNA represented by the item (a) or (b) described above under stringent conditions (provided that the bases represented by base numbers 577 to 579 are conserved), the DNA encoding a polypeptide having

the function of horseshoe crab factor B, and

(d) a DNA having a base sequence of a fusion DNA in which a peptide tag-encoding DNA is added to the DNA represented by any one of the items (a) to (c) described above, the DNA encoding a polypeptide having the function of horseshoe crab factor B.

[5]

A vector retaining the nucleic acid according to the item [3] and/or the DNA according to the item [4].

[6]

A cell retaining the nucleic acid according to the item [3], the DNA according to the item [4], and/or the vector according to the item [5].

[7]

A method for producing a polypeptide, the method including a step of producing a polypeptide having the function of horseshoe crab factor B using the cell according to the item [6].

[8]

A polypeptide obtained by the method according to the item [7].

[9]

A method for measuring an endotoxin, the method including steps of the following (1) and (2):

(1) a step of mixing the polypeptide according to

the item [1], [2], or [8] with horseshoe crab factor C and a test sample; and

(2) a step of measuring protease activity of the polypeptide.

[10]

A reagent for endotoxin measurement, including the polypeptide according to the item [1], [2], or [8] as a constituent component.

[11]

A kit for endotoxin measurement, including the polypeptide according to the item [1], [2] or [8], or the reagent according to the item [10], as a component part.

Advantageous Effects of Invention

[0009]

According to the present invention, a horseshoe crab factor B variant having protease activity superior to that of the factor B itself can be provided. Furthermore, according to the present invention, a horseshoe crab factor B variant having thermal stability superior to that of the factor B itself.

Brief Description of Drawings

[0010]

[FIG. 1] FIG. 1 is a diagram showing the specific

activity (units/ μ mol) of *Tachypleus tridentatus* factor B (TFB) and a *Tachypleus tridentatus* factor B variant (Murasame-TFB).

[FIG. 2] FIG. 2 is a diagram showing the thermal stability of *Tachypleus tridentatus* factor B (TFB) (graph represented by ○) and *Tachypleus tridentatus* factor B variant (Murasame-TFB) (graph represented by ●).

Description of Embodiments

[0011]

The term "Limulus factor" as used for the present invention refers individually or collectively to factor C, factor B, and proclotting enzyme. Furthermore, the term "Limulus reagent" as used for the present invention refers to a reagent that includes any arbitrary Limulus factor as a constituent component and is used in a Limulus test.

[0012]

According to the present invention, a series of reactions by which factor C that has come into contact with an endotoxin is autocatalytically changed to an activated form (activated factor C), and factor B is cleaved by the protease activity of the activated factor C and is thereby changed to activated factor B, may be referred to as "cascade reaction". Furthermore, according to the present invention, a series of reactions by which also includes, in

addition to these series of reactions, a reaction by which proclotting enzyme is cleaved by the protease activity of the activated factor B and is thereby changed to a clotting enzyme, may also be referred to as "cascade reaction".

[0013]

<1> Polypeptide of present invention

The polypeptide of the present invention is a polypeptide in which an amino acid residue at a particular site in the amino acid sequence of a polypeptide of horseshoe crab factor B is modified. The polypeptide of the present invention is specifically a polypeptide having an amino acid sequence in which the amino acid residue at the 193-position in the amino acid sequence of a polypeptide of horseshoe crab factor B is substituted with a cysteine (Cys) residue.

[0014]

It is known that a sequence of twenty-three residues at the N-terminal in the amino acid sequence of a polypeptide of horseshoe crab factor B function as a signal sequence (Non Patent Literature 2). Therefore, in regard to the polypeptide of the present invention, those ordinarily skilled in the art can understand that in addition to a polypeptide in an embodiment of having the signal sequence, a polypeptide in an embodiment of not having the signal sequence (polypeptide in an embodiment of

not having the twenty-three residues at the N-terminal) and a polypeptide in an embodiment of having another signal sequence (signal sequence other than the signal sequence inherently possessed by horseshoe crabs) also constitute one embodiment of the polypeptide of the present invention.

[0015]

In the present invention, the type of horseshoe crab is not particularly limited. Regarding horseshoe crab, four kinds, namely, *Tachypleus tridentatus*, *Limulus polyphemus*, *Carcinoscorpius rotundicauda*, and *Tachypleus gigas*, are known, and these horseshoe crabs may be mentioned as examples of the horseshoe crab according to the present invention. The polypeptide of the present invention is, for example, a polypeptide having an amino acid sequence in which the amino acid residue at the 193-position in the amino acid sequence of a polypeptide of factor B for such a horseshoe crab is substituted with a cysteine (Cys) residue.

[0016]

According to the present invention, it is preferable that the horseshoe crab is *Tachypleus tridentatus*, *Limulus polyphemus*, or *Carcinoscorpius rotundicauda*, and it is more preferable that the horseshoe crab is *Tachypleus tridentatus* or *Limulus polyphemus*.

[0017]

The polypeptide of the present invention is specifically exemplified by a polypeptide represented by any one of the following (A) to (D).

(A) a polypeptide having an amino acid sequence represented by the following (A1) or (A2):

(A1) an amino acid sequence represented by amino acid numbers 1 to 400 of SEQ ID NO: 7; and

(A2) an amino acid sequence represented by amino acid numbers 24 to 400 of SEQ ID NO: 7,

(B) a polypeptide having an amino acid sequence represented by the following (B1) or (B2):

(B1) an amino acid sequence represented by amino acid numbers 1 to 400 of SEQ ID NO: 10; and

(B2) an amino acid sequence represented by amino acid numbers 24 to 400 of SEQ ID NO: 10,

(C) a polypeptide having an amino acid sequence including substitution, deletion, insertion, and/or addition of one amino acid residue or a plurality of amino acid residues in the amino acid sequence of the polypeptide represented by the item (A) or (B) (provided that the cysteine (Cys) residue at the 193-position is neither substituted nor deleted), the polypeptide having the function of horseshoe crab factor B, and

(D) a polypeptide having an amino acid sequence of a fusion polypeptide in which a peptide tag is added to the

polypeptide represented by any one of the items (A) to (C), the polypeptide having the function of horseshoe crab factor B.

[0018]

The "polypeptide having an amino acid sequence" as used for the present invention includes the "polypeptide consisting of the amino acid sequence" as an embodiment.

[0019]

The amino acid sequence set forth in SEQ ID NO: 7 for the item (A) is an amino acid sequence including substitution of the amino acid residue at the 193-position in the amino acid sequence of a polypeptide of *Tachypleus tridentatus* factor B (SEQ ID NO: 2) with a cysteine (Cys) residue.

[0020]

The amino acid sequence set forth in SEQ ID NO: 10 for the item (B) is an amino acid sequence including substitution of the amino acid residue at the 193-position in the amino acid sequence of a polypeptide of *Limulus polyphemus* factor B (SEQ ID NO: 4) with a cysteine (Cys) residue.

[0021]

The amino acid sequence of the polypeptide shown in the item (C) is an amino acid sequence including substitution, deletion, insertion, and/or addition of one

amino acid residue or a plurality of amino acid residues in the amino acid sequence of the polypeptide shown in the item (A) or (B), with the cysteine (Cys) residue at the 193-position being neither substituted nor deleted (the Cys residue at the 193-position is conserved). Meanwhile, the cysteine (Cys) residue at the 193-position represents the position of the cysteine (Cys) residue to be conserved in the amino acid sequence set forth in SEQ ID NO: 7 or SEQ ID NO: 10. Therefore, the cysteine (Cys) residue that is conserved in an amino acid sequence including substitution, deletion, insertion, and/or addition of one amino acid residue or a plurality of amino acid residues in the amino acid sequence of the polypeptide represented by the item (A) or (B) (that is, the amino acid sequence represented by the item (C)) is a cysteine (Cys) residue at a position corresponding to the cysteine (Cys) residue at the 193-position in the alignment of the amino acid sequence with the original amino acid sequence (amino acid sequence of the polypeptide represented by the item (A) or (B)).

[0022]

The term "a plurality" as used for the item (C) means the number (total number) of amino acid residues to the extent that even if the polypeptide is subjected to substitution, deletion, insertion, and/or addition, the polypeptide does not lose the function of horseshoe crab

factor B. The term "a plurality" may be, for example, a number of preferably 10% or less, more preferably 5% or less, even more preferably 2% or less, and particularly preferably 1% or less, with respect to the total number of amino acid residues that constitute the polypeptide.

[0023]

Therefore, in the case of the amino acid sequence of the polypeptide represented by the item (A1) or (B1), since the total number of amino acid residues is 400, "a plurality" may be preferably 2 to 40, more preferably 2 to 20, even more preferably 2 to 8, and particularly preferably 2 to 4. Furthermore, in the case of the amino acid sequence of the polypeptide represented by the item (A2) or (B2), since the total number of amino acid residues is 377, "a plurality" may be preferably 2 to 37, more preferably 2 to 18, even more preferably 2 to 7, and particularly preferably 2 to 3. The term "a plurality" as used for the item (C) may be, as the number of specific individuals, an integer such as two, three, four, five, six, seven, or eight.

[0024]

The terms "substitution, deletion, insertion, and/or addition" as used for the item (C) are, for example, conservative mutations. A representative example of conservative mutation is conservative substitution.

Conservative mutation is a mutation resulting from the following: in a case in which the site of substitution is an aromatic amino acid, substitution occurs between Phe, Trp, and Tyr; in a case in which the site of substitution is a hydrophobic amino acid, substitution occurs between Leu, Ile, and Val; in a case in which the site of substitution is a polar amino acid, substitution occurs between Gln and Asn; in a case in which the site of substitution is a basic amino acid, substitution occurs between Lys, Arg, and His; in a case in which the site of substitution is an acid amino acid, substitution occurs between Asp and Glu; and in a case in which the site of substitution is an amino acid having a hydroxyl group, substitution occurs between Ser and Thr. Specific examples of a substitution that is regarded as conservative substitution include substitution of Ala with Ser or Thr; substitution of Arg with Gln, His, or Lys; substitution of Asn with Glu, Gln, Lys, His, or Asp; substitution of Asp with Asn, Glu, or Gln; substitution of Cys with Ser or Ala; substitution of Gln with Asn, Glu, Lys, His, Asp, or Arg; substitution of Glu with Gly, Asn, Gln, Lys, or Asp; substitution of Gly with Pro; substitution of His with Asn, Lys, Gln, Arg, or Tyr; substitution of Ile with Leu, Met, Val, or Phe; substitution of Leu with Ile, Met, Val, or Phe; substitution of Lys with Asn, Glu, Gln, His, or Arg;

substitution of Met with Ile, Leu, Val, or Phe;
substitution of Phe with Trp, Tyr, Met, Ile, or Leu;
substitution of Ser with Thr or Ala; substitution of Thr
with Ser or Ala; substitution of Trp with Phe or Tyr;
substitution of Tyr with His, Phe, or Trp; and substitution
of Val with Met, Ile, or Leu.

[0025]

The polypeptide represented by the item (C) may be,
for example, a polypeptide having a similarity of
preferably 90% or higher, more preferably 95% or higher,
even more preferably 98% or higher, and particularly
preferably 99% or higher, to the amino acid sequence of the
polypeptide represented by the item (A) or (B), the
polypeptide having the function of horseshoe crab factor B
(provided that the cysteine (Cys) residue at the 193-
position may be conserved). Meanwhile, since the term
"similarity" as used herein is a concept including
"identity", similarity may be replaced with identity and
can be applied to suitable embodiments of the polypeptide.

[0026]

The polypeptide represented by the item (C) may have
any arbitrary amino acid residue substituted or deleted in
the amino acid sequence of the polypeptide represented by
the item (A) or (B) as long as the cysteine (Cys) residue
at the 193-position is neither substituted nor deleted (Cys

residue at the 193-position is conserved). However, it is preferable that other Cys residues that are conserved (exist at the same position) between the amino acid sequence of a polypeptide of *Tachypleus tridentatus* factor B (SEQ ID NO: 2) and the amino acid sequence of a polypeptide of *Limulus polyphemus* factor B (SEQ ID NO: 4) are also conserved (neither substituted nor deleted). Specifically, it is preferable for the polypeptide represented by the item (C) that the Cys residues at the 112-position, 177-position, 193-position, 260-position, 307-position, 329-position, 340-position, and 368-position in the amino acid sequence of the polypeptide represented by the item (A) or (B) are conserved (neither substituted nor deleted).

[0027]

The "peptide tag" as used for the item (D) means a peptide that is added to the polypeptide in order to facilitate detection or purification. The length and amino acid sequence of the "peptide tag" are not particularly limited as long as a fusion polypeptide in which a peptide tag is added to the polypeptide represented by any one of the items (A) to (C) has the function of horseshoe crab factor B. Examples of such a peptide tag include 6XHis peptide (His tag), FLAG peptide (FLAG tag), c-myc peptide (myc tag), protein A, maltose-binding protein (MBP), and

glutathione-S-transferase (GST).

[0028]

The peptide tag may be added directly to the polypeptide represented by any one of the items (A) to (C), or may be added to the polypeptide through any arbitrary linker. The "linker" as used herein may be any peptide linker including an arbitrary amino acid sequence. The length and amino acid sequence of the "peptide linker" as used herein are not particularly limited, similarly to the case of the peptide tag, as long as the fusion polypeptide in which a peptide tag is added to the polypeptide represented by any one of the items (A) to (C) has the function of horseshoe crab factor B.

[0029]

The peptide tag may be added to, for example, the N-terminal and/or the C-terminal of the polypeptide. The peptide tag may be added to the N-terminal side of the polypeptide only, may be added to the C-terminal side only, or may be added to both of the terminals. One kind of peptide tag may be added to the polypeptide, or two or more kinds of peptide tags may also be added. Furthermore, regarding the various kinds of peptide tags to be added to the polypeptide, one peptide tag from each kind may be independently added, or two or more peptide tags from each kind may be independently added.

[0030]

The "function of horseshoe crab factor B" according to the present invention means the function as a protease precursor possessed by horseshoe crab factor B. The "function of horseshoe crab factor B" specifically means the function of coming into contact with activated factor C to be changed to an activated form (activated factor B) and exhibiting the protease activity.

[0031]

The function of horseshoe crab factor B is, for example, a function of coming into contact with activated factor C to be changed to an activated form (activated factor B), cleaving a proclotting enzyme, and thereby changing the proclotting enzyme to a clotting enzyme. Furthermore, the function of horseshoe crab factor B is, for example, a function of coming into contact with activated factor C to be changed to an activated form (activated factor B), cleaving a substrate for detection, which serves as a substrate for the activated factor B, and thereby releasing a marker substance. In regard to the factor C, proclotting enzyme, and substrate for detection as used herein, for example, embodiments described in connection with the measurement method of the present invention that will be described below can be suitably used.

[0032]

Whether a polypeptide has the function of horseshoe crab factor B can be determined by, for example, evaluating whether the polypeptide exhibits protease activity when brought into contact with activated factor C. Specifically, whether a polypeptide has the function of horseshoe crab factor B can be determined by, for example, the method described in <Example 2> or <Example 5> that will be described below.

[0033]

Furthermore, whether a polypeptide has the function of horseshoe crab factor B can also be determined by, for example, evaluating, in a case in which a Limulus reagent configured to include the polypeptide in combination with factor C is used, whether a cascade reaction proceeds when the Limulus reagent is allowed to co-exist with an endotoxin.

[0034]

Furthermore, whether a polypeptide has the function of horseshoe crab factor B can be determined by, for example, evaluating, in a case in which a Limulus reagent configured to include the polypeptide in combination with factor C and a proclotting enzyme is used, whether a cascade reaction proceeds when the Limulus reagent is allowed to co-exist with an endotoxin.

[0035]

Therefore, the polypeptide represented by the item (C) can be obtained by, for example, employing the function of horseshoe crab factor B as a marker, and selecting a site where substitution, deletion, insertion, and/or addition of one amino acid residue or a plurality of amino acid residues can be carried out without losing the function of horseshoe crab factor B, from the amino acid sequence represented by the item (A) or (B). Furthermore, the polypeptide represented by the item (D) can be obtained by, for example, employing the function of horseshoe crab factor B as a marker and selecting a polypeptide to which a peptide tag can be added without losing the function of horseshoe crab factor B, from the polypeptides represented by any of the items (A) to (C).

[0036]

An embodiment of the polypeptide of the present invention is a polypeptide that exhibits protease activity that is superior to that of horseshoe crab factor B itself. Horseshoe crab factor B itself is specifically a polypeptide including the same amino acid sequence as that of naturally occurring horseshoe crab factor B. Horseshoe crab factor B itself is more specifically, for example, a polypeptide including an amino acid sequence in which the amino acid residue at the 193-position in the amino acid sequence represented by the item (A) or (B) is substituted

with an alanine (Ala) residue (polypeptide including the amino acid sequence set forth in SEQ ID NO: 2 or SEQ ID NO: 4). The polypeptide of the present invention may be a polypeptide characterized in that, for example, the protease activity (specific activity) exhibited when the polypeptide comes into contact with activated factor C to be changed to an activated form (activated factor B) is two times or more, preferably 5 times or more, and more preferably 10 times or more, compared to horseshoe crab factor B itself. Furthermore, the polypeptide of the present invention may be a polypeptide characterized in that, for example, the protease activity (specific activity) exhibited when the polypeptide comes into contact with activated factor C to be changed to an activated form (activated factor B) is 80 units/ μ mol or higher, preferably 200 units/ μ mol or higher, more preferably 300 units/ μ mol or higher, and particularly preferably 400 units/ μ mol or higher. Here, 1 unit corresponds to the protease activity capable of cleaving 1 μ mol of a substrate (substrate for detection) for 1 minute at 37°C. The measurement conditions for the enzyme activity (units/ μ mol) may be, for example, the conditions described in <Example 2> or <Example 5>.

[0037]

An embodiment of the polypeptide of the present

invention is a polypeptide having thermal stability superior to that of horseshoe crab factor B itself. The polypeptide of the present invention may be, for example, a polypeptide characterized in that the protease activity (specific activity) exhibited when the polypeptide that has been heated for 2 minutes at 50°C is brought into contact with activated factor C to be changed to an activated form (activated factor B) is 50% or higher, preferably 70% or higher, and more preferably 80% or higher, compared to the activity of the polypeptide that has not been heated. Furthermore, the polypeptide of the present invention may be, for example, a polypeptide characterized in that even after being heated for 2 minutes at 60°C, 70°C, 80°C, or 90°C, the polypeptide does not lose the function of horseshoe crab factor B.

[0038]

The polypeptide of the present invention may be, for example, a polypeptide characterized in that the protease activity (specific activity) exhibited when the polypeptide that has been heated for 2 minutes at 60°C is brought into contact with activated factor C to be changed to an activated form (activated factor B) is 30% or higher, preferably 50% or higher, and more preferably 70% or higher, compared to the activity of the polypeptide that has not been heated. Furthermore, the polypeptide of the present

invention may be, for example, a polypeptide characterized in that the protease activity (specific activity) exhibited when the polypeptide that has been heated for 2 minutes at 90°C is brought into contact with activated factor C to be changed to an activated form (activated factor B) is 20% or higher, preferably 30% or higher, and more preferably 40% or higher, compared to the activity of the polypeptide that has not been heated.

[0039]

The protease activity of the activated factor B can be measured by, for example, the method described in <Example 2> or <Example 5> that will be described below, by which the protease activity of factor B (activated factor B) that has been activated by being brought into contact with activated factor C is measured.

[0040]

Here, the amino acid sequence of the polypeptide is homologous to the Limulus factor of horseshoe crab, and high similarity is found between species. The similarity of amino acid sequences can be calculated using well-known computer software programs, and for example, the similarity can be calculated using the algorithm BLAST (Karlin, S., Altschul, SF. (1993) Proc. Natl. Acad. Sci. U.S.A., 90, 5873-7) or the algorithm FASTA (Pearson, WR. (1990) Methods. Enzymol. 183, 63-98). Specifically, the similarity of

amino acid sequences can be calculated using, for example, GENETYX (manufactured by Genetyx Corporation).

[0041]

For example, the polypeptide of *Tachypleus tridentatus* factor B (SEQ ID NO: 2, NCBI Accession No.: BAA03528.1) and the polypeptide of *Limulus polyphemus* factor B (SEQ ID NO: 4, NCBI Accession No.: XP_013784210.1) have a similarity of 98.5%.

[0042]

Furthermore, for example, the polypeptide of *Tachypleus tridentatus* factor C (SEQ ID NO: 12, NCBI Accession No.: BAA14315.1) and the polypeptide of *Limulus polyphemus* factor C (SEQ ID NO: 14) have a similarity of 99.5%. The polypeptide of *Tachypleus tridentatus* factor C and the polypeptide of *Carcinoscorpius rotundicauda* factor C (SEQ ID NO: 16, NCBI Accession No. AAB34361.1) have a similarity of 99.8%. The polypeptide of *Limulus polyphemus* factor C and the polypeptide of *Carcinoscorpius rotundicauda* factor C have a similarity of 99.4%.

[0043]

Furthermore, for example, the polypeptide of *Tachypleus tridentatus* proclotting enzyme (SEQ ID NO: 18, NCBI Accession No.: AAA30094.1) and the polypeptide of *Limulus polyphemus* proclotting enzyme (SEQ ID NO: 20, NCBI Accession No.: XP_013783518.1) have a similarity of 96.0%.

[0044]

Therefore, the technology of the present invention is verified by modification of the respective polypeptides of *Tachypleus tridentatus* factor B and *Limulus polyphemus* factor B in the Examples described below; however, a person ordinarily skilled in the art can understand that the technology of the present invention is also applicable to the polypeptides of other horseshoe crab factors B. For example, the amino acid sequences of the respective polypeptides of *Carcinoscorpius rotundicauda* factor B and *Tachypleus gigas* factor B, and the base sequences encoding the polypeptides are still not known; however, those ordinarily skilled in the art can understand that the technology of the present invention is also applicable to the polypeptides of these factors B.

[0045]

The polypeptide of the present invention may be an embodiment composed of the polypeptide of the present invention or may be an embodiment including other components. The "other components" as used herein are not particularly limited as long as they are not components that cause the polypeptide of the present invention to lose the function. Examples of the "other components" include a buffer agent, an alkali metal salt, an alkaline earth metal salt, and a surfactant. Examples of the "other components"

also include components other than the polypeptide of the present invention (a nucleic acid, a protein, a carbohydrate, a lipid, and the like), the components being derived from cells that produce the polypeptide of the present invention.

[0046]

The polypeptide of the present invention may have any arbitrary form. The polypeptide of the present invention may be, for example, in a solid form such as a freeze-dried product, or may be in a liquid form in a state of being dissolved in an arbitrary solvent such as an aqueous solvent.

[0047]

The weight concentration occupied by the polypeptide of the present invention in the polypeptide of the present invention may be, for example, 0.001% or more, 0.01% or more, 0.1% or more, 1% or more, 5% or more, 10% or more, 25% or more, or 50% or more. Furthermore, the weight concentration occupied by the polypeptide of the present invention in the polypeptide of the present invention may be, for example, 100% or less, 75% or less, 50% or less, 25% or less, 10% or less, 5% or less, or 1% or less.

[0048]

The polypeptide of the present invention can be produced by any known technique based on the description of

the present specification. The polypeptide of the present invention can be produced by, for example, a genetic engineering technique. Specifically, the polypeptide of the present invention can be produced by, for example, the production method of the present invention that will be described below. The polypeptide of the present invention may be the culture fluid itself obtainable by culturing cells according to the production method of the present invention, or may be a fraction obtained by purifying this culture fluid to a desired extent.

[0049]

<2> Nucleic acid of present invention

The nucleic acid of the present invention is a nucleic acid encoding the polypeptide of the present invention. The nucleic acid of the present invention is not particularly limited as long as it is a nucleic acid encoding the polypeptide of the present invention. The nucleic acid of the present invention includes all nucleic acids having base sequences that vary due to degeneracy (degeneration) of genetic codes (codons) but encode the same polypeptide, as long as the nucleic acids are nucleic acids encoding the polypeptide of the present invention. The term "nucleic acid" as used herein includes DNA and RNA.

[0050]

The nucleic acid of the present invention may be a

double-stranded nucleic acid or a single-stranded nucleic acid. In a case in which the nucleic acid of the present invention is a double-stranded nucleic acid, the nucleic acid may also be a hybrid strand formed from a DNA and an RNA. Furthermore, since the nucleic acid of the present invention is a nucleic acid encoding the polypeptide of the present invention, the nucleic acid of the present invention may be a nucleic acid having a sequence of an intron within the region encoding the polypeptide of the present invention, or may be a nucleic acid that does not have a sequence of an intron in that region. Furthermore, the nucleic acid of the present invention may be an mRNA (mRNA precursor or a mature mRNA) or may be a DNA (cDNA) synthesized by a reverse transcription reaction from an mRNA. The nucleic acid of the present invention may be an isolated nucleic acid.

[0051]

The nucleic acid of the present invention is, for example, a DNA having a base sequence in which the base of base number 577 in a base sequence of a cDNA encoding a polypeptide of horseshoe crab factor B is substituted with thymine (T), the base of base number 578 is substituted with guanine (G), and the base of base number 579 is substituted with thymine (T) or cytosine (C), the nucleic acid being a DNA encoding a polypeptide having the function

of horseshoe crab factor B. Specifically, the nucleic acid of the present invention may be, for example, a DNA represented by any one of the following (a) to (d).

(a) a DNA having a base sequence represented by any one of the following (a1) to (a4):

(a1) a base sequence represented by base numbers 1 to 1200 of SEQ ID NO: 5;

(a2) a base sequence represented by base numbers 70 to 1200 of SEQ ID NO: 5;

(a3) a base sequence represented by base numbers 1 to 1200 of SEQ ID NO: 6; and

(a4) a base sequence represented by base numbers 70 to 1200 of SEQ ID NO: 6,

(b) a DNA having a base sequence represented by any one of the following (b1) to (b4):

(b1) a base sequence represented by base numbers 1 to 1200 of SEQ ID NO: 8;

(b2) a base sequence represented by base numbers 70 to 1200 of SEQ ID NO: 8;

(b3) a base sequence represented by base numbers 1 to 1200 of SEQ ID NO: 9; and

(b4) a base sequence represented by base numbers 70 to 1200 of SEQ ID NO: 9,

(c) a DNA hybridizing with a DNA including a base sequence complementary to the DNA represented by the item

(a) or (b) under stringent conditions (provided that the bases represented by base numbers 577 to 579 are conserved), the DNA encoding a polypeptide having the function of horseshoe crab factor B, and

(d) a DNA having a base sequence of a fusion DNA in which a peptide tag-encoding DNA is added to the DNA represented by any one of the items (a) to (c), the DNA encoding a polypeptide having the function of horseshoe crab factor B.

[0052]

The "nucleic acid having a base sequence" as used for the present invention includes this "nucleic acid consisting of a base sequence" as an embodiment. Therefore, the "DNA having a base sequence" as used for the present invention includes this "DNA consisting of a base sequence" as an embodiment.

[0053]

The base sequence represented by SEQ ID NO: 5 for the item (a) is a base sequence in which, in a DNA (SEQ ID NO: 1) encoding a polypeptide of *Tachypleus tridentatus* factor B, the base of base number 577 is substituted with thymine (T), the base of base number 578 is substituted with guanine (G), and the base of base number 579 is substituted with thymine (T).

[0054]

The base sequence represented by SEQ ID NO: 6 for the item (a) is a base sequence in which, in the base sequence of a DNA (SEQ ID NO: 1) encoding a polypeptide of *Tachypleus tridentatus* factor B, the base of base number 577 is substituted with thymine (T), and the base of base number 578 is substituted with guanine (G).

[0055]

The base sequence represented by SEQ ID NO: 8 for the item (b) is a base sequence in which, in the base sequence of a DNA (SEQ ID NO: 3) encoding a polypeptide of *Limulus polyphemus* factor B, the base of base number 577 is substituted with thymine (T), and the base of base number 578 is substituted with guanine (G).

[0056]

The base sequence represented by SEQ ID NO: 9 for the item (b) is a base sequence in which, in the base sequence of a DNA (SEQ ID NO: 3) encoding a polypeptide of *Limulus polyphemus* factor B, the base of base number 577 is substituted with thymine (T), the base of base number 578 is substituted with guanine (G), and the base of base number 579 is substituted with cytosine (C).

[0057]

The "stringent conditions" employed for the item (c) mean conditions in which a specific hybrid is formed while a non-specific hybrid is not formed. Therefore, the

"stringent conditions" as used herein mean, for example, conditions in which a specific hybrid is formed for a DNA including a base sequence that is complementary to the DNA represented by the item (a) or (b). An example of the stringent conditions is conditions in which DNAs having high similarity, for example, DNAs having a similarity of 80% or higher, preferably 90% or higher, more preferably 95% or higher, even more preferably 98% or higher, and particularly preferably 99% or higher, hybridize with each other, and DNAs having a similarity lower than that do not hybridize with each other. An example of such conditions is, for example, conditions in which DNAs are washed once, preferably two or three times, at a salt concentration and a temperature corresponding to 60°C, 1×SSC, 0.1% SDS; preferably 60°C, 0.1×SSC, 0.1% SDS; and more preferably 68°C, 0.1×SSC, 0.1% SDS, which are conditions for washing in conventional Southern hybridization. Furthermore, another example of such conditions is conditions in which DNAs are hybridized at 42°C in a solution including 50% formamide, 4×SSC, 50 mM HEPES-NaOH (pH 7.0), 10×Denhardt's solution, and 100 µg/mL salmon sperm DNA, the hybrid is washed at room temperature with a solution including 2×SSC and 0.1% SDS, and the hybrid is further washed at 50°C with a solution including 0.1×SSC and 0.1% SDS (Sambrook J, et al., Molecular Cloning: A Laboratory Manual, Second Edition,

Cold Spring Harbor Laboratory Press (1989)).

[0058]

The DNA represented by the item (c) can be produced by, for example, performing substitution, deletion, insertion, and/or addition of a nucleic acid residue (hereinafter, collectively referred to as "introduction of mutation") for the DNA represented by the item (a) or (b), in a region other than base numbers 577 to 579 of the same base sequence. The "introduction of mutation" as used herein can be carried out by, for example, any known method.

[0059]

Regarding the method of performing the "introduction of mutation", a method of using a restriction enzyme and T4DNA ligase may be mentioned as an example. That is, a DNA having a mutation introduced therein can be obtained by subjecting the two terminals of a DNA fragment having a mutation introduced therein, to limited digestion using a restriction enzyme; mixing the resultant with a vector into which a DNA represented by the item (a) or (b) has been subcloned, the DNA having been subjected to limited digestion using the same restriction enzyme; and then subjecting the two to ligation using T4DNA ligase. The "DNA fragment having a mutation introduced therein" can be obtained by, for example, a PCR reaction using oligonucleotides into which such a mutation has been

introduced, as primers.

[0060]

Furthermore, another example of the method for implementing the "introduction of mutation" is a site-specific mutation introduction method. Examples of the site-specific mutation introduction method include methods of using a PCR reaction (Higuchi, R. (1989) in PCR technology (Erlich, H.A., ed.) Stockton Press, New York, pp. 61-70; Carter, P. (1987) *Methods Enzymol.*, 154, 382-403), and methods of using phages (Kramer, W., Fritz, H.J. (1987) *Methods Enzymol.*, 154, 350-67; Kunkel, T.A., Roberts, J.D., Zakour, R.A. (1987) *Methods Enzymol.*, 154, 367-82). Specifically, the site-specific mutation introduction method can be carried out by utilizing, for example, KOD-Plus-Mutagenesis Kit (manufactured by Toyobo Co., Ltd.).

[0061]

The DNA represented by the item (c) may have a mutation introduced into any arbitrary site in the base sequence of a DNA represented by the item (a) or (b) as long as no mutation is introduced into the sites represented by base numbers 577 to 579 (bases represented by base numbers 577 to 579 are conserved); however, it is preferable that the DNA represented by the item (c) is a DNA encoding a polypeptide in which other Cys residues that are conserved (exist at the same positions) between the

amino acid sequence of a polypeptide of *Tachypleus tridentatus* factor B (SEQ ID NO: 2) and the amino acid sequence of a polypeptide of *Limulus polyphemus* factor B (SEQ ID NO: 4) are also conserved (neither substituted nor deleted). In regard to the DNA represented by the item (c), specifically, it is preferable that base sequences (codons) represented by base numbers 334 to 336, base numbers 529 to 531, base numbers 577 to 579, base numbers 778 to 780, base numbers 919 to 921, base numbers 985 to 987, base numbers 1018 to 1020, and base numbers 1102 to 1104 are conserved (TGT or TGC).

[0062]

The embodiment of the "peptide tag" for the item (d) is the same as the embodiment concerning the "<1> Polypeptide of present invention" described above.

Therefore, as long as a polypeptide encoded by a DNA has the function of horseshoe crab factor B, a fusion DNA in which a peptide tag-encoding DNA is added to a DNA represented by the item (a), (b), or (c), is also included as an example of the nucleic acid of the present invention.

[0063]

Such a "fusion DNA in which a peptide tag-encoding DNA is added" can be produced by, for example, a method of using a restriction enzyme and T4DNA ligase. That is, the fusion DNA can be obtained by subjecting the two terminals

of a DNA fragment that encodes a peptide tag, to limited digestion using a restriction enzyme; mixing the resultant with a vector into which a DNA represented by any one of the items (a) to (c) has been subcloned, the DNA having been subjected to limited digestion using the same restriction enzyme; and then subjecting the two to ligation using T4DNA ligase.

[0064]

Furthermore, such a "fusion DNA in which a peptide-tag encoding DNA is added" can also be obtained by, for example, a PCR reaction that uses oligonucleotides having the base sequence of the peptide tag-encoding DNA as primers, and uses the DNA represented by any one of the items (a) to (c) as a template.

[0065]

The polypeptide encoded by the nucleic acid of the present invention can be produced by, for example, the production method of the present invention that will be described below. Regarding the production method of the present invention, specifically, a method of using a mammalian cell as described in <Example 1> or <Example 4> that will be described below may be mentioned as an example.

[0066]

The polypeptide encoded by the nucleic acid of the present invention is a polypeptide having the function of

horseshoe crab factor B. The definition for the "function of horseshoe crab factor B" as used herein is as described in connection with the above section "<1> Polypeptide of present invention". Therefore, by determining whether the polypeptide encoded by the nucleic acid has the function of horseshoe crab factor B according to the method described in the section "<1> Polypeptide of present invention", the DNA represented by the item (c) or (d) can be selected.

[0067]

The nucleic acid of the present invention can be produced by any known technique, based on the description of the present specification. The nucleic acid of the present invention can be produced by a genetic engineering technique. Specifically, the nucleic acid of the present invention can be produced by, for example, the PCR reaction described in <Example 1> or <Example 4> that will be described below. Furthermore, the nucleic acid of the present invention can also be produced by, for example, chemical total synthesis of the base sequence.

[0068]

<3> Vector of present invention

The vector of the present invention is a vector retaining the nucleic acid of the present invention. The type or number of the nucleic acid of the present invention retained by the vector of the present invention is not

particularly limited. One kind of the nucleic acid of the present invention may be retained by the vector of the present invention, or two or more kinds of the nucleic acids of the present invention may be retained.

Furthermore, regarding various kinds of the nucleic acids of the present invention to be retained by the vector of the present invention, one nucleic acid (one copy) from each kind may be independently retained, or two or more nucleic acids (two copies) from each kind may be independently retained.

[0069]

The "vector" as used herein means a nucleic acid molecule used for the amplification of the nucleic acid of the present invention and/or the expression of the polypeptide encoded by the nucleic acid of the present invention. According to the present invention, the vector is not particularly limited as long as the vector enables amplification of the nucleic acid of the present invention or the vector, or expression of the polypeptide that is encoded by the nucleic acid of the present invention in a cell into which this vector is introduced. Examples of such a vector include a phage, a plasmid, and a virus.

[0070]

The vector can be appropriately selected according to various conditions such as the kind of cell into which

this vector is introduced, and the desired amount of expression of the polypeptide encoded by the nucleic acid of the present invention. For example, in a case in which a prokaryotic cell such as a bacterial cell is utilized as the cell, a phage or a plasmid can be suitably utilized as the vector. Furthermore, in a case in which a eukaryotic cell such as an insect cell or a mammalian cell is utilized as the cell, a plasmid or a virus can be suitably utilized as the vector.

[0071]

Examples of the plasmid that can be utilized in mammalian cells include pCA7 (Takeda, M., Ohno, S., Seki, F., Nakatsu, Y., Tahara, M., Yanagi, Y. (2005) J. Virol. 79, 14346-54) and pCI-neo (manufactured by Promega Corporation). Examples of the plasmid that can be utilized in insect cells include pIZ-V5 (manufactured by Life Technologies Corp.). Examples of the plasmid that can be utilized in bacterial cells include pBlue Script II SK(+) (manufactured by Agilent Technologies, Inc.) and pET (manufactured by Takara Bio, Inc.).

[0072]

Examples of the virus that can be utilized in mammalian cells include animal viruses. Examples of the animal viruses include Sendai virus. Examples of the virus that can be utilized in insect cells include baculovirus.

Examples of the baculovirus include nuclear polyhedrosis virus (NPV).

[0073]

Examples of the phage that can be utilized in bacterial cells include bacteriophage. Examples of the bacteriophage include Lambda phage (λ phage) and T4 phage.

[0074]

The vector of the present invention can be obtained by, for example, introducing the nucleic acid of the present invention into a vector. Introduction of the nucleic acid of the present invention into a vector can be carried out by a conventional method.

[0075]

An example of a method for introducing the nucleic acid of the present invention into a vector may be a method of utilizing a multiple cloning site carried by the vector. The vector of the present invention can be obtained by, for example, selecting any two restriction enzyme sites from the restriction enzyme sites existing in a multiple cloning site carried by the vector, and subjecting the vector and the nucleic acid of the present invention to limited digestion using these restriction enzymes and then to ligation. Regarding the nucleic acid of the present invention for this use application, for example, a DNA obtainable by a PCR reaction that uses oligonucleotides

having a restriction enzyme site added to the 5'-terminal side as primers, and uses the nucleic acid of the present invention as a template, can be used. Furthermore, for example, the vector of the present invention can also be obtained by performing the introduction of a mutation such that the amino acid residue at the 193-position in the amino acid sequence of a polypeptide of horseshoe crab factor B is changed to a cysteine (Cys) residue, by an inverse PCR reaction using, as a template, a vector in which a DNA encoding the polypeptide of horseshoe crab factor B has been inserted. Specifically, an example of the method for producing the vector of the present invention is the method described in <Example 1> or <Example 4> that will be described below.

[0076]

<4> Cell of present invention

The cell of the present invention is a cell that retains the nucleic acid of the present invention and/or the vector of the present invention (hereinafter, collectively referred to as "vector or the like of the present invention"). Since the vector of the present invention is a vector having the nucleic acid of the present invention, the cell that retains the vector of the present invention also corresponds to a cell that retains the nucleic acid of the present invention.

[0077]

The cell of the present invention can be obtained by, for example, introducing the vector or the like of the present invention into a cell. Therefore, the cell of the present invention can be obtained by, for example, transforming (transducing) a cell using the vector or the like of the present invention. The cell according to the present invention is not particularly limited as long as the cell is capable of having the vector or the like of the present invention introduced therein, or capable of being transformed using this. It is preferable that the cell according to the present invention is an isolated cell.

[0078]

The type or number of the vector or the like of the present invention retained by the cell of the present invention is not particularly limited. One kind of the vector or the like of the present invention may be retained by the cell of the present invention, or two or more kinds of the vectors or the like of the present invention may be retained by the cell. Furthermore, regarding various kinds of the vectors of the present invention to be retained by the cell of the present invention, one vector (one copy) from each kind may be independently retained, or two or more vectors (two copies) from each kind may be independently retained.

[0079]

The cell of the present invention may retain the vector or the like of the present invention intrachromosomally, may retain the vector or the like of the present invention extrachromosomally, or may retain the vector or the like of the present invention both intrachromosomally and extrachromosomally. Specifically, the cell of the present invention may be a cell having the vector or the like of the present invention introduced therein, or a cell transformed using this.

[0080]

In regard to the cell of the present invention, the "cell" is, for example, a host cell. The "host cell" as used herein means a cell used as a host for amplifying the vector or the like of the present invention, and/or as a host for expressing the polypeptide of the present invention encoded by the vector or the like of the present invention.

[0081]

The cell according to the present invention can be selected as appropriate according to the purpose of using the vector or the like of the present invention.

[0082]

For example, in a case in which amplification of the vector or the like of the present invention is purported,

the cell is preferably a prokaryotic cell, and specifically, the cell is preferably a bacterial cell. Above all, it is preferable that the cell is *Escherichia coli* cell.

Examples of *Escherichia coli* include strain JM109 and strain DH5 α .

[0083]

Furthermore, for example, in a case in which expression of the polypeptide encoded by the vector or the like of the present invention is purported, for example, a cell that is usually used in order to express a polypeptide that is not inherently possessed by the cell can be used as the cell. Such a cell is, for example, preferably a eukaryotic cell, and it is preferable that the cell is specifically a mammalian cell, an insect cell, a plant cell, or a yeast cell. The eukaryotic cell is preferably a mammalian cell or an insect cell, and more preferably a mammalian cell.

[0084]

It is preferable that the mammalian cell is a cell of a primate or a cell of a rodent. Examples of the primate include human being, monkey, and chimpanzee. An example of the cell of a primate is a human cell. A specific example of the human cell is a human embryonic kidney cell-derived cell strain (HEK cell). An example of the HEK cell is a HEK293 cell. An example of the HEK293

cell is a HEK293S cell. An example of the HEK293S cell is HEK293S GnTI⁻ cell. Furthermore, examples of the rodent include hamster, mouse, rat, and guinea pig. An example of the cell of a rodent is a hamster cell. An example of the hamster is a Chinese hamster cell. An example of the Chinese hamster cell is a CHO cell. Examples of the CHO cell include CHO DG44 cell, CHO-K1 cell, and CHO-S cell.

[0085]

Introduction of a nucleic acid into a cell can be carried out by a conventional method. The method for introducing a nucleic acid into a cell in the present invention is not particularly limited as long as it is a method capable of introducing the vector or the like of the present invention into a cell. Specific examples of the method for introducing a nucleic acid into a cell include a calcium phosphate method, a lipofection method, a DEAE dextran method, an electroporation method, and a microinjection method. Transformation of the cell can be carried out by, for example, the method described in <Example 1> or <Example 4> that will be described below.

[0086]

<5> Production method of present invention

The production method of the present invention is a method for producing the polypeptide of the present invention using the cell of the present invention. The

production method of the present invention is specifically a method for producing a polypeptide, the method including a step of producing the polypeptide of the present invention by using the cell of the present invention (hereinafter, referred to as "production step"). The production step is, for example, a step of expressing the polypeptide of the present invention by culturing the cell of the present invention.

[0087]

The conditions for culturing the cell of the present invention in the production method of the present invention (hereinafter, simply referred to as "culture conditions") are not particularly limited as long as the conditions are conditions capable of exhibiting the polypeptide of the present invention in a cell. The culture conditions can be selected as appropriate according to various conditions such as the type of the cell and the desired amount of expression of the polypeptide of the present invention encoded by the vector or the like of the present invention. Culture of cells can be carried out by, for example, using a medium that is usually used for the culture of the relevant cell. Regarding the culture conditions, specifically, the conditions described in <Example 1> or <Example 4> that will be described below may be mentioned as an example.

[0088]

The production method of the present invention may further include another step as long as the method includes the production step. The "other step" as used herein is, for example, a step of collecting the polypeptide of the present invention produced in the production step (hereinafter, referred to as "collection step"). The "collection step" as used herein is a step of obtaining a fraction including the polypeptide of the present invention from the culture fluid of the cell.

[0089]

For example, in a case in which the polypeptide of the present invention is expressed in a form of being secreted extracellularly, the collection step may be a step of collecting the culture fluid itself, the supernatant obtained after centrifugation, or a fraction obtainable after purifying these by subjecting to a column or the like, as the polypeptide of the present invention.

[0090]

Furthermore, for example, in a case in which the polypeptide of the present invention is expressed in a form of being accumulated in the cell, the collection step may be a step of collecting the cell itself, a crushed product (cell debris) obtainable by crushing the cell or an extract, or a fraction obtainable after purifying these by

subjecting to a column or the like, as the polypeptide of the present invention.

[0091]

The term "crushing" in the above description can be carried out by a method selected as appropriate according to the type of the cell. Examples of the method of crushing include a method of performing homogenization, a method performing an ultrasonic treatment, a method of performing freezing and thawing, and a method of adding a surfactant. These techniques used for crushing can be used in combination as appropriate.

[0092]

The term "purification" in the above description can be carried out by a technique that is usually used for the purification of polypeptides. Examples of such a method include ammonium sulfate precipitation, gel permeation chromatography, ion exchange chromatography, hydrophobic interaction chromatography, hydroxyapatite chromatography, and affinity chromatography. These techniques can be used in combination as appropriate.

[0093]

Whether the polypeptide of the present invention is included in the fraction thus collected can be determined by, for example, measuring the presence or absence of a polypeptide having the function of horseshoe crab factor B

according to the method described in the section "<1> Polypeptide of present invention". Furthermore, whether the polypeptide of the present invention is included in the fraction thus collected can be determined by, for example, a method of using an antibody that binds to the polypeptide of the present invention.

[0094]

<6> Measurement method of present invention

The measurement method of the present invention is a method of performing the measurement of an endotoxin using the polypeptide of the present invention. According to the present invention, the term measurement is used as a collective name for detection, sensing, and quantitative determination. Therefore, the measurement method of the present invention may also be, for example, a method for detecting an endotoxin, a method for sensing an endotoxin, or a method for quantitatively determining an endotoxin.

[0095]

The measurement method of the present invention is, for example, a method for measuring an endotoxin, the method including the following steps (1) and (2):

(1) a step of mixing the polypeptide of the present invention with horseshoe crab factor C and a test sample; and

(2) a step of measuring protease activity of the

polypeptide.

[0096]

The step of the item (1) is a step of mixing the polypeptide of the present invention with horseshoe factor C and a test sample. In a case in which the test sample is a sample containing an endotoxin, a cascade reaction proceeds, in which factor C that has come into contact with the endotoxin is changed to activated factor C, and subsequently factor B is changed to activated factor B.

[0097]

In the step of the item (1) may further include an operation of mixing another substance, as long as the step includes the operation of mixing the polypeptide of the present invention with factor C and a test sample. Examples of the "other substance" as used herein include a proclotting enzyme, a buffer agent, an alkali metal salt, an alkaline earth metal salt, a surfactant, and a substrate for detection. In a case in which the step of the item (1) is a step of mixing the polypeptide of the present invention with factor C, a proclotting enzyme, and a test sample, and the test sample is a sample containing an endotoxin, a cascade reaction proceeds, in which factor C that has come into contact with the endotoxin is changed to activated factor C, factor B is changed to activated factor B, and the proclotting enzyme is changed to a clotting

enzyme.

[0098]

The "factor C" used for the present invention is not particularly limited as long as it is factor C having the function of horseshoe crab factor C. The definition for the "horseshoe crab" used herein is as described in the section "<1> Polypeptide of present invention". The "function of horseshoe crab factor C" means the function as a protease precursor possessed by horseshoe crab factor C. The "function of horseshoe crab factor C" means specifically the function of being changed to an activated form (activated factor C) in the co-presence of an endotoxin and exhibiting the protease activity. The function of horseshoe crab factor C is, for example, the function of being changed to an activated form (activated factor C) in the co-presence of an endotoxin, cleaving factor B, and thereby changing the factor B into an activated form (activated factor B).

[0099]

The "proclotting enzyme" according to the present invention is not particularly limited as long as it is a proclotting enzyme having the function of a horseshoe crab proclotting enzyme. The definition for the "horseshoe crab" use herein is as described in the section "<1> Polypeptide of present invention". The "function of a

horseshoe crab proclotting enzyme" means the function as a protease precursor possessed by a horseshoe crab proclotting enzyme. The "function of a horseshoe crab proclotting enzyme" means specifically the function of being changed to an activated form (clotting enzyme) in the co-presence of activated factor B and exhibiting protease activity. The function of a horseshoe crab proclotting enzyme is, for example, the function of being changed to an activated form (clotting enzyme) in the co-presence of activated factor B, cleaving coagulogen, and thereby forming coagulin gel. Furthermore, the function of a horseshoe crab proclotting enzyme is, for example, the function of being changed to an activated form (clotting enzyme) in the co-presence of activated factor B, cleaving a substrate for detection, which serves as a substrate for a clotting enzyme, and thereby releasing a marker substance.

[0100]

Factor C and the proclotting enzyme according to the present invention may be each independently a naturally occurring Limulus factor obtainable from horseshoe crab, may be a recombinant Limulus factor produced according to a genetic engineering technique, or may be a mixture including a naturally occurring Limulus factor and a recombinant Limulus factor at any arbitrary proportions.

[0101]

The naturally occurring Limulus factor may be a Limulus factor obtained by using hemocyte of horseshoe crab as a raw material, purifying as appropriate a lysate obtained by a conventional method, and preparatively separating the Limulus factor. Preparative separation of a Limulus factor can be carried out by, for example, referring to a method described in the literature (Nakamura, T., Horiuchi, T., Morita T., and Iwanaga, S. (1986) J. Biochem. 99, 847-57).

[0102]

A recombinant Limulus factor can be obtained by introducing a nucleic acid encoding a polypeptide of a Limulus factor into a cell and producing the Limulus factor in the cell. The base sequence of a nucleic acid encoding a Limulus factor can be obtained from a known database such as NCBI (www.ncbi.nlm.nih.gov).

[0103]

The production of a Limulus factor using a cell can be carried out using a known technique. Regarding the production of a Limulus factor using a cell, a method of using a mammalian cell as described in <Example 1> or <Example 4> that will be described below may be mentioned as an example. Furthermore, the production of a Limulus factor using a cell can also be carried out by, for example, referring to a method described in the literature (WO

2012/118226 or WO 2014/092079). Furthermore, the production of a Limulus factor using a cell can also be carried out, for example, by applying the method described in the section "<5> Production method of present invention" mutatis mutandis.

[0104]

Specific embodiments of the factor C and the proclotting enzyme according to the present invention can be presented by applying the description in the section "<1> Polypeptide of present invention" mutatis mutandis. For example, it is preferable that the Limulus factor according to the present invention is a recombinant. Furthermore, for example, the cell used for the production of the Limulus factor according to the present invention is preferably a mammalian cell or an insect cell, and more preferably a mammalian cell.

[0105]

Regarding the factor C according to the present invention, specifically, a polypeptide represented by any one of the following (1) to (5) may be mentioned as an example.

(1) A polypeptide having an amino acid sequence represented by SEQ ID NO: 12;

(2) a polypeptide having an amino acid sequence represented by SEQ ID NO: 14;

(3) a polypeptide having an amino acid sequence represented by SEQ ID NO: 16;

(4) a polypeptide having an amino acid sequence including substitution, deletion, insertion, and/or addition of one amino acid residue or a plurality of amino acid residues in the amino acid sequence represented by any one of the items (1) to (3), the polypeptide having the function of horseshoe crab factor C; and

(5) a polypeptide having the amino acid sequence of a fusion polypeptide in which a peptide tag is added to the polypeptide represented by any one of the items (1) to (4), the polypeptide having the function of horseshoe crab factor C.

[0106]

Regarding the proclotting enzyme according to the present invention, specifically, a polypeptide represented by any one of the following (6) to (9) may be mentioned as an example.

(6) A polypeptide having an amino acid sequence set forth in SEQ ID NO: 18;

(7) a polypeptide having an amino acid sequence set forth in SEQ ID NO: 20;

(8) a polypeptide having an amino acid sequence including substitution, deletion, insertion, and/or addition of one amino acid residue or a plurality of amino

acid residues in the amino acid sequence represented by the item (6) or (7), the polypeptide having the function of a horseshoe crab proclotting enzyme; and

(9) a polypeptide having the amino acid sequence of a fusion polypeptide in which a peptide tag is added to the polypeptide represented by any one of the items (6) to (8), the polypeptide having the function of a horseshoe crab proclotting enzyme.

[0107]

The amino acid sequence set forth in SEQ ID NO: 12 for the item (1) is the amino acid sequence of a polypeptide of *Tachyplesus tridentatus* factor C.

[0108]

The amino acid sequence set forth in SEQ ID NO: 14 for the item (2) is the amino acid sequence of a polypeptide of *Limulus polyphemus* factor C.

[0109]

The amino acid sequence set forth in SEQ ID NO: 16 for the item (3) is the amino acid sequence of a polypeptide of *Carcinoscopius rotundicauda* factor C.

[0110]

The amino acid sequence set forth in SEQ ID NO: 18 for the item (6) is the amino acid sequence of a polypeptide of *Tachyplesus tridentatus* proclotting enzyme.

[0111]

The amino acid sequence set forth in SEQ ID NO: 20 for the item (7) is the amino acid sequence of a polypeptide of *Limulus polyphemus* proclotting enzyme.

[0112]

Embodiments of the term "a plurality" and "substitution, deletion, insertion, and/or addition" as used for the items (4) and (8) are the same as the embodiments for the section "<1> Polypeptide of present invention".

[0113]

The polypeptide represented by the item (4) may be, for example, a polypeptide having a similarity of preferably 90% or higher, more preferably 95% or higher, even more preferably 98% or higher, and particularly preferably 99% or higher, with respect to the entire amino acid sequence represented by any one of the items (1) to (3), the polypeptide having the function of horseshoe crab factor C. Furthermore, the polypeptide represented by the item (8) may be, for example, a polypeptide having a similarity of preferably 90% or higher, more preferably 95% or higher, even more preferably 98% or higher, and particularly preferably 99% or higher, with respect to the entire amino acid sequence represented by the item (6) or (7), the polypeptide having the function of a horseshoe crab proclotting enzyme. In addition, the "similarity" as

used for these is a concept including the term "identity", and therefore, the term similarity can be replaced with the term identity and applied to suitable embodiments of the polypeptide.

[0114]

The embodiments of the "peptide tag" for the item (4) and (8) are the same as the embodiments for the section "<1> Polypeptide of present invention".

[0115]

The "substrate for detection" according to the present invention is a substrate used for measuring the presence or absence of an activated Limulus factor, the amount of the activated Limulus factor, or the progress of the cascade reaction. The substrate for detection may be a substrate for measuring activated factor B, or may be a substrate for measuring a clotting enzyme. The substrate for detection is not particularly limited as long as it is a substrate that serves as a substrate for an activated Limulus factor. The substrate for detection may be, for example, a protein, a peptide, or a derivative of any one of these.

[0116]

The protein may be a naturally occurring protein, or may be a recombinant protein. An example of the protein is coagulogen, which is a substrate for a clotting enzyme.

For example, naturally occurring coagulogen can be produced by preparatively separating the substance from a lysate. Furthermore, for example, a recombinant coagulogen can be produced by referring to a method described in the literature (Miyata, et al., Tanpakushitsu Kakusan Koso Bessatsu (Proteins, Nucleic acids, and Enzymes: Extra Issue) No. 29; p.30-43; 1986).

[0117]

The peptide may be, for example, a synthetic substrate that is chemically synthesized. The synthetic substrate is not particularly limited as long as it is a substrate suitable for measuring the presence or absence of an activated Limulus factor, the amount of the activated Limulus factor, or the progress of the cascade reaction. The synthetic substrate is preferably a derivative of a peptide.

[0118]

An example of the synthetic substrate is a substrate represented by general formula: Y-X-Z (wherein Y may or may not exist; in a case in which Y exists, Y represents a protective group; X represents a peptide; and Z represents a marker substance). It is desirable that such a synthetic substrate has a property by which the covalent bond between X and Z is cleaved by an activated Limulus factor, and a marker substance Z is released. In regard to the general

formula described above, it is preferable that the protective group (Y) is a protective group for an amino group at the N-terminal of a peptide. In regard to the general formula, it is preferable that the bond between Y and X is an amide bond formed between a carboxy group of the protective group and an α -amino group at the N-terminal of a peptide. Furthermore, in regard to the general formula, it is preferable that the bond between X and Z is an amide bond formed between a carboxy group at the C-terminal of a peptide and an amino group of the marker substance Z.

[0119]

The protective group (Y) is not particularly limited, and any known protective group that is applicable to the protection of a peptide can be used. Examples of the protective group (Y) include a tert-butoxycarbonyl group (Boc), a benzyloxycarbonyl group (Cbz), a benzyl group (Bzl), a benzoyl group (Bz), and an acetyl group (Ac).

[0120]

The peptide (X) is not particularly limited as long as it is a peptide having an amino acid sequence that serves as a substrate for an activated Limulus factor. It is preferable that the peptide is a substrate suitable for the measurement of a serine protease, and it is preferable that the peptide is a peptide having an Arg (R) residue at

the C-terminal.

[0121]

In a case in which the Limulus factor is factor B, it is preferable that the peptide is a peptide having an amino acid sequence represented by general formula: X-Thr-Arg (wherein X represents any arbitrary amino acid). Specifically, in a case in which the Limulus factor is factor B, it is preferable that the peptide is a peptide having an amino acid sequence represented by Leu-Thr-Arg (LTR) or Met-Thr-Arg (MTR).

[0122]

In a case in which the Limulus factor is a proclotting enzyme, it is preferable that the peptide is a peptide having an amino acid sequence represented by general formula: X-Gly-Arg (wherein X represents any arbitrary amino acid). Specifically, in a case in which the Limulus factor is a proclotting enzyme, it is preferable that the peptide is a peptide having an amino acids sequence represented by Leu-Gly-Arg (LGR) or Glu-Gly-Arg (EGR).

[0123]

The marker substance (Z) is not particularly limited, and any known marker substance that can be applied to the measurement of protease activity can be used. Regarding the marker substance, for example, a marker substance that

becomes, when released from a peptide, detectable through color development or fluorescence, can be used. Examples of such a marker substance include para-nitroaniline (pNA), 7-methoxycoumarin-4-acetic acid (MCA), and 2,4-dinitroaniline (DNP). Furthermore, regarding the marker substance, for example, a marker substance that becomes, when released from a peptide, detectable according to an electrochemical measurement method (voltammetry, amperometry, or the like), can be used. Examples of such a marker substance include p-aminophenol (pAP), p-methoxyaniline (pMA), N-methyl-p-phenylenediamine (MPDD), and N,N'-dimethyl-p-phenylenediamine (DMPD).

[0124]

In the step of (1) in the measurement method of the present invention as described above, the polypeptide of the present invention, a Limulus factor, a test sample, a substrate for detection, and other substances (a buffer agent and the like) may be added in an arbitrary order and mixed. For example, in the step of (1), the test sample may be added to a mixture of the polypeptide of the present invention, a Limulus factor, a substrate for detection, and other substances, and mixed with the mixture. Furthermore, for example, in the step of (1), the test sample may have a mixture of the polypeptide of the present invention, a Limulus factor, a substrate for detection, and other

substances added thereto and mixed. In the step of (1), mixing may be carried out, for example, in the inside (in a container) of a container having an opening at one end (a test tube, a vial, or the like). The test sample is not particularly limited, and examples include water for injection, a pharmaceutical product, an infusion liquid, a blood preparation, a medical instrument (medical tool), a quasi-drug, a cosmetic, as well as a food product, a beverage, an environmental sample collected from air, river, soil, or the like; a naturally occurring protein, a recombinant protein, a nucleic acid, an enzyme, a carbohydrate, an electrolyte; and a biological component such as blood, a body fluid, or a tissue.

[0125]

The step of (2) is a step of measuring the protease activity of the polypeptide of the present invention. This step is, for example, a step of measuring a marker substance released from a substrate for detection. In this step, since a marker substance in an amount (mole number) corresponding to the protease activity (total activity) of the polypeptide of the present invention is released from a substrate for detection, the protease activity of the polypeptide of the present invention can be measured by measuring a marker substance released from the substrate for detection. A marker substance released from the

substrate for detection can be measured using, for example, an optical instrument such as a spectrophotometer or a fluorophotometer. Furthermore, a marker substance released from a substrate for detection can be measured using, for example, an electrochemical measuring instrument such as a voltammeter or an amperometer. For example, the presence or absence of an endotoxin in a test sample can be determined by comparing the measured value shown in that step with a blank value (measured value obtained in a case in which an endotoxin-free test sample as an object of measurement).

[0126]

The measurement method of the present invention may further include another step in addition to the steps of (1) and (2). The measurement method of the present invention may include, for example, a step of determining the presence or absence of an endotoxin in a test sample by comparing the measurement obtainable in the step of (2) with a blank value. Furthermore, the measurement method of the present invention may also include, for example, a step of determining the presence or absence of an endotoxin in a test sample by determining the presence or absence of gelation of a mixed liquid. Furthermore, the measurement method of the present invention may include, for example, a step of converting the measured value obtainable in the

step of (2) into another value. Regarding the step of converting a measured value into another value, for example, a step of calculating the amount of an endotoxin based on the measured value may be mentioned as an example. Such a step is specifically, for example, a step of converting the measured value obtainable when a test sample is measured, into the amount of an endotoxin based on the relation (standard curve) between the measured value obtainable when a test sample is substituted with a standard substance at a known concentration, and the concentration of the standard substance.

[0127]

In regard to the measurement method of the present invention, it is preferable that the Limulus reaction is carried out in water or an aqueous solvent such as a buffer solution.

[0128]

<7> Reagent of present invention

The reagent of the present invention is a reagent for endotoxin measurement, including the polypeptide of the present invention as a constituent component. The reagent of the present invention can be suitably used in order to carry out the measurement method of the present invention.

[0129]

The reagent of the present invention may further

include another constituent component as long as the reagent includes the polypeptide of the present invention as a constituent component. Regarding the other constituent component as used herein, for example, horseshoe crab factor C, a horseshoe crab proclotting enzyme, a substrate for detection, a buffer agent, an alkali metal salt, an alkaline earth metal salt, and a surfactant.

[0130]

It is preferable that the reagent of the present invention includes horseshoe crab factor C as a constituent component in addition to the polypeptide of the present invention, and it is more preferable that the reagent of the present invention further includes a horseshoe crab proclotting enzyme as a constituent component. Furthermore, it is preferable that the reagent of the present invention is supplied as a freeze-dried product.

[0131]

<8> Kit of present invention

The kit of the present invention is a kit for endotoxin measurement, including the polypeptide of the present invention or the reagent of the present invention as a component part. The kit of the present invention can be suitably used in order to carry out the measurement method of the present invention.

[0132]

As long as the kit of the present invention includes the polypeptide of the present invention or the reagent of the present invention as a component part, the kit of the present invention may further include another component part. Examples of the other component part as used herein include horseshoe crab factor C, a horseshoe crab proclotting enzyme, a substrate for detection, a buffer solution, distilled water, an endotoxin standard product, a microplate, and an attached document with product information described therein.

[0133]

The kit of the present invention may include various component parts individually separately, or may include various component parts as a mixture formed by arbitrarily combining the component parts. The kit of the present invention may include, for example, various Limulus factors separately, or as an embodiment of having the various Limulus factors mixed in advance, or as an embodiment of further having a substrate for detection mixed with the various Limulus factors in advance.

Examples

[0134]

Hereinafter, an embodiment of the present invention

will be described specifically by way of Examples; however, the technical scope of the present invention is not intended to be limited to these Examples only.

[0135]

In the Examples of the present invention, the following abbreviations may be used.

- (a) TFC: Tachypleus tridentatus factor C
- (b) TFB: Tachypleus tridentatus factor B
- (c) Murasame-TFB: Tachypleus tridentatus factor B

variant

- (d) LFC: Limulus polyphemus factor C
- (e) LFB: Limulus polyphemus factor B
- (f) Murasame-LFB: Limulus polyphemus factor B

variant

[0136]

In the Examples of the present invention, production of an expression vector, production of a Limulus factor, and measurement of the activity of a Limulus factor can be carried out by referring to the methods described in the literature (Kobayashi, Y., Takahashi, T., Shibata, T., Ikeda, S., Koshiba, T., Mizumura, H., Oda, T., Kawabata, S. (2015) J. Biol. Chem. 290, 19379-86).

[0137]

In the Examples of the present invention, unless particularly stated otherwise, a PCR reaction was carried

out by using Phusion High-Fidelity DNA Polymerase (manufactured by New England Biolabs) and performing the operation according to the attached protocol.

[0138]

Purification of a DNA from a PCR reaction liquid in the Examples of the present invention, was carried out by using Wizard SV Gel and PCR Clean-Up System (manufactured by Promega Corporation) and performing the operation according to the attached protocol.

[0139]

Preparative separation of a DNA in the Examples of the present invention was carried out by subjecting a sample including a DNA to agarose gel electrophoresis, cutting out a desired DNA fragment with a scalpel, collecting the DNA fragment, and then performing the operation using Wizard SV Gel and PCR Clean-Up System (manufactured by Promega Corporation) according to the attached protocol.

[0140]

In the Examples of the present invention, unless particularly stated otherwise, the ligation reaction was carried out using T4DNA ligase (manufactured by New England Biolabs) and performing the operation according to the attached protocol.

[0141]

Amplification and purification of a vector in the Examples of the present invention were carried out by culturing *Escherichia coli* that had been transformed using a vector, and then performing the operations using Wizard Plus SV Minipreps DNA Purification System (manufactured by Promega Corporation) according to the attached protocol. Specifically, *Escherichia coli* DH5 α strain that had been transformed using a vector was applied on a plate of ampicillin-containing LB (LB/Amp) agar medium, the bacterial cells were subjected to static culture overnight, single colonies thus obtained were inoculated into ampicillin-containing LB medium, the bacterial cells were subjected to shaking culture overnight at 37°C to perform amplification of the vector, and the vector was purified from the bacterial cells of *Escherichia coli* in the culture fluid.

[0142]

Dephosphorylation of a DNA in the Examples of the present invention was carried out using Alkaline Phosphatase (*E. coli* C75) (manufactured by Takara Bio, Inc.) by performing the operation according to the attached protocol.

[0143]

Measurement of the protein concentration in the Examples of the present invention was carried out using

Micro BCA Protein Assay Kit (manufactured by Thermo Fisher Scientific) by performing the operation according to the attached protocol. Furthermore, the molar concentration of a Limulus factor in the Examples of the present invention was calculated by dividing the protein concentration obtainable by the above-mentioned measurement by the molecular weight of the Limulus factor.

[0144]

<Reference Example 1> Production of TFC

(1) Production of TFC expression vector

An expression vector for *Tachypleus tridentatus* factor C (TFC) was produced according to the following procedure.

[0145]

A DNA encoding full-length TFC excluding the signal sequence of the N-terminal (DNA having a base sequence represented by base numbers 76 to 3057 of SEQ ID NO: 11) was produced by a PCR reaction. As a template for the PCR reaction, a vector produced by inserting the TFC-encoding DNA into pSecTag2A vector (manufactured by Invitrogen) (Koshiba, T., Hashii, T., Kawabata, S. (2007) J. Biol. Chem. 282, 3962-7) was used. As primers for the PCR reaction, Primer 1 (SEQ ID NO: 21) and BGH reverse primer (SEQ ID NO: 28) were used.

[0146]

The DNA obtained by the PCR reaction was purified, and the DNA was subjected to limited digestion using restriction enzymes (Age I and Kpn I) and preparative separation. pHLsec vector (Aricescu, AR., Lu, W., Jones, EY. (2006) *Acta Crystallogr. D Biol. Crystallogr.* 62, 1243-50) was subjected to limited digestion using restriction enzymes and preparative separation in the same manner as described above, and then a ligation reaction between the vector and the above-mentioned DNA was carried out.

[0147]

Escherichia coli was transformed by using the ligation reaction liquid, and then amplification and purification of the vector were carried out. The vector was subjected to limited digestion using a restriction enzyme (EcoR I) and preparative separation, and DNA fragment 1 (DNA having a base sequence encoding the amino acid sequence of a pHLsec vector-derived secretion signal, and a base sequence represented by base numbers 76-2298 of SEQ ID NO: 11, the DNA having the sticky ends of EcoR I at the 5'-terminal and the 3'-terminal) was obtained. Furthermore, the vector was subjected to limited digestion using restriction enzymes (EcoR I and Xho I) and preparative separation, and DNA fragment 2 (DNA having a base sequence represented by base numbers 2299 to 2545 of SEQ ID NO: 11, the DNA having the sticky end of EcoR I at

the 5'-terminal and the sticky end of Xho I at the 3'-terminal) and DNA fragment 3 (DNA having a base sequence represented by base numbers 2546 to 3057 of SEQ ID NO: 11 and a base sequence encoding the amino acid sequence of a pHLsec vector-derived His tag, the DNA having the sticky ends of Xho I at the 5'-terminal and the 3'-terminal) were obtained.

[0148]

pCA7 vector (Takeda, M., Ohno, S., Seki, F., Nakatsu, Y., Tahara, M., Yanagi, Y. (2005) J. Virol. 79, 14346-54) was subjected to limited digestion using restriction enzymes (EcoR I and Xho I) and preparative separation, and then a ligation reaction between the vector and the DNA fragment 2 was carried out.

[0149]

Escherichia coli was transformed using the above-mentioned ligation reaction liquid, and then amplification and purification of the vector were carried out. The vector was subjected to limited digestion using a restriction enzyme (Xho I), dephosphorylation, and preparative separation, and then a ligation reaction between the vector and the DNA fragment 3 was carried out.

[0150]

Escherichia coli was transformed using the above-mentioned ligation reaction liquid, and then amplification

and purification of the vector were carried out. The vector was subjected to limited digestion using a restriction enzyme (EcoR I), dephosphorylation, and preparative separation, and then a ligation reaction between the vector and the DNA fragment 1 was carried out.

[0151]

Escherichia coli was transformed using the above-mentioned ligation reaction liquid, and then amplification and purification of the vector were carried out. In this manner, a vector encoding a TFC having a His tag at the C-terminal was obtained.

[0152]

A PCR reaction was carried out using the above-mentioned vector as a template and using Primer 8 (SEQ ID NO: 29) and Primer 2 (SEQ ID NO: 22), and the DNA thus amplified was purified. The DNA was subjected to limited digestion using a restriction enzyme (EcoR I) and preparative separation, and DNA fragment 1 was obtained. Furthermore, The DNA was subjected to limited digestion using restriction enzymes (EcoR I and Xho I) and preparative separation, and DNA fragment 2 and DNA fragment 4 (DNA having a base sequence represented by base numbers 2546 to 3057 of SEQ ID NO: 11, the DNA having the sticky ends of Xho I at the 5'-terminal and the 3'-terminal) were obtained.

[0153]

pCA7 vector was subjected to limited digestion using restriction enzymes (EcoR I and Xho I) and preparative separation, and then a ligation reaction between the pCA7 vector and the DNA fragment 2 was carried out.

[0154]

Escherichia coli was transformed using the above-mentioned ligation reaction liquid, and then amplification and purification of the vector were carried out. The vector was subjected to limited digestion using a restriction enzyme (Xho I), dephosphorylation, and preparative separation, and then a ligation reaction between the vector and the DNA fragment 4 was carried out.

[0155]

Escherichia coli was transformed using the above-mentioned ligation reaction liquid, and then amplification and purification of the vector were carried out. The vector was subjected to limited digestion using a restriction enzyme (EcoR I), dephosphorylation, and preparative separation, and then a ligation reaction between the vector and the DNA fragment 1 was carried out.

[0156]

Escherichia coli was transformed using the above-mentioned ligation reaction liquid, and then amplification and purification of the vector were carried out. In this

manner, a vector encoding a TFC that did not have a His tag at the C-terminal was obtained.

[0157]

The vector was subjected to limited digestion using a restriction enzyme (EcoR I), dephosphorylation, and preparative separation, and DNA fragment 5 (DNA having a base sequence represented by base numbers 2299 to 3057 of SEQ ID NO: 11 and the base sequence of pCA7 vector, the DNA having the sticky ends of EcoR I at the 5'-terminal and the 3'-terminal) was obtained.

[0158]

A PCR reaction was carried out using a vector produced by inserting a DNA encoding full-length TFC including the signal sequence of the N-terminal (DNA having a base sequence represented by base numbers 1 to 3057 of SEQ ID NO: 11) into pPSC8 vector (manufactured by Protein Sciences Corporation), as a template, and using Primer 3 (SEQ ID NO: 23) and Primer 2 (SEQ ID NO: 22), and thus the DNA thus amplified was purified. The DNA was subjected to limited digestion using a restriction enzyme (EcoR I) and preparative separation, and DNA fragment 6 (DNA having a base sequence represented by base numbers 1 to 2298 of SEQ ID NO: 11, the DNA having the sticky ends of EcoR I at the 5'-terminal and the 3'-terminal) was obtained.

Subsequently, a ligation reaction between the DNA fragment

6 and the DNA fragment 5 was carried out.

[0159]

Escherichia coli was transformed using the above-mentioned ligation reaction liquid, and then amplification and purification of the vector were carried out. In this manner, a vector encoding full-length TFC including the signal sequence of the N-terminal was obtained.

[0160]

An inverse PCR reaction was carried out using the above-mentioned vector as a template and phosphorylated primers (Primer 4 (SEQ ID NO: 24) and Primer 5 (SEQ ID NO: 25)). The inverse PCR reaction was carried out by using Q5 High-Fidelity DNA Polymerase (manufactured by New England Biolabs) and performing the operation according to the attached protocol. A restriction enzyme (Dpn I) was added to the PCR reaction liquid to degrade the template, and preparative separation of the DNA was carried out by performing phenol/chloroform extraction and ethanol precipitation. Subsequently, a ligation reaction (self-ligation) was carried out by using DNA Ligation Kit <Mighty Mix> (manufactured by Takara Bio, Inc.) and performing the operation according to the attached protocol.

[0161]

Escherichia coli was transformed using the above-mentioned ligation reaction liquid, and the amplification

and purification of the vector were carried out. In this manner, a vector encoding a TFC having the 6XHis tags and the amino acid sequence of factor Xa cleavage sequence (IEGR) inserted therein immediately after a signal sequence, was obtained.

[0162]

A PCR reaction was carried out using the above-mentioned vector as a template and using Primer 6 (SEQ ID NO: 26) and Primer 7 (SEQ ID NO: 27), and the DNA thus amplified was purified. The PCR reaction was carried out by using Q5 High-Fidelity DNA Polymerase and performing the operation according to the attached protocol. The DNA was subjected to limited digestion using restriction enzymes (Age I and Nhe I) and preparative separation, and DNA fragment 7 (DNA having a base sequence encoding the 6XHis tag, a base sequence encoding IEGR, and a base sequence represented by base numbers 76 to 2236 of SEQ ID NO: 11, the DNA having the sticky end of Age I at the 5'-terminal and the sticky end of Nhe I at the 3'-terminal) was obtained.

[0163]

The vector encoding a TFC that did not have a His tag at the C-terminal was subjected to limited digestion using restriction enzymes (Age I and Nhe I), dephosphorylation, and preparative separation, and DNA

fragment 8 (DNA having a base sequence represented by base numbers 2237 to 3057 of SEQ ID NO: 11 and the base sequence of pCA7 vector, the DNA having the sticky end of Nhe I at the 5'-terminal and the sticky end of Age I at the 3'-terminal) was obtained. Subsequently, a ligation reaction between the DNA fragment 8 and the DNA fragment 7 was carried out.

[0164]

Escherichia coli was transformed using the above-mentioned ligation reaction liquid, and then amplification and purification of the vector were carried out. The base sequence of the vector was subjected to a sequence analysis, and it was confirmed that there was no introduction of mutation caused by PCR error. In this manner, an expression vector for a polypeptide having the 6×His tag and the factor Xa cleavage sequence (IEGR) on the N-terminal side of a TFC sequence excluding the signal sequence of the N-terminal (hereinafter, referred to as "TFC/pCA7") was obtained.

[0165]

(2) Production of TFC

Tachypleus tridentatus factor C (TFC) was produced by a mammalian cell expression system according to the following procedure. Cell culture was carried out under the conditions of 37°C and 5% CO₂.

[0166]

HEK293S GnTI⁻ cells (ATCC: CRL-3022) were transformed using the expression vector for TFC (TFC/pCA7) obtained in the section (1). Specifically, to the cells that had reached a confluence of 80% to 90%, a transformation medium (DMEM medium including TFC/pCA7 (1.8 µg/mL), polyethyleneimine (2.7 µg/mL), a 1% penicillin-streptomycin-L-glutamine solution (manufactured by Wako Pure Chemical Industries, Ltd.), and 2% bovine fetal serum) was added to a concentration of 0.22 mL/cm², and benzamidine was added to the cell to a final concentration of 2 mM. The mixture was subjected to static culture for 120 hours. In this manner, a culture fluid including TFC that had been secreted from the cells was obtained.

[0167]

The culture fluid was centrifuged at 6,000×g for 30 minutes, and the supernatant was collected. The supernatant and a buffer solution (0.5 M NaH₂PO₄-NaOH (pH 8.0), 1.5 M NaCl, and 0.1 M imidazole) in a 0.1-fold amount were mixed, and then the mixture was applied to a nickel column (nickel-nitrilotriacetic acid agarose column, inner diameter: 1.0 cm × length: 5.0 cm). The column was washed with a washing solution (50 mM NaH₂PO₄-NaOH (pH 8.0), 150 mM NaCl, and 10 mM imidazole), and then TFC was eluted using an elution solution (50 mM NaH₂PO₄-NaOH (pH 8.0), 150

mM NaCl, and 20 to 200 mM imidazole). The eluted fraction of TFC was checked by gel electrophoresis (SDS-PAGE).

[0168]

The eluted fraction of TFC was collected, and the solution was replaced with a reaction solution (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 10 mM CaCl_2) by ultrafiltration. Subsequently, factor Xa was added to the solution to a concentration of 3.3 $\mu\text{g/mL}$, and the mixture was left to stand for 16 hours at 37°C. Thus, the His tag was released from the N-terminal of TFC.

[0169]

The above-mentioned reaction solution was applied to a sepharose column (benzamidine-Sepharose column, inner diameter: 0.25 cm $\times$ length: 1.5 cm), and factor Xa was eliminated by adsorbing factor Xa to the column. Subsequently, a flow-through fraction (flow-through) of the column was applied to a nickel column (nickel-nitrilotriacetic acid-agarose column, inner diameter: 0.25 cm $\times$ length: 1.5 cm), and the His tag released from TFC was eliminated by adsorbing the His tag to the column. The flow-through fraction of the column was used as TFC in the subsequent test.

[0170]

<Reference Example 2> Production of TFB

(1) Production of TFB expression vector

An expression vector for *Tachypleus tridentatus* factor B (TFB) was produced by the following procedure.

[0171]

A DNA encoding full-length TFB excluding the signal sequence of the N-terminal (DNA having a base sequence represented by base numbers 70 to 1200 of SEQ ID NO: 1) was produced by a PCR reaction. As a template for the PCR reaction, a vector produced by inserting a DNA encoding TFB into pPSC8 vector was used. Furthermore, as primers for the PCR reaction, Primer 9 (SEQ ID NO: 30) and Primer 10 (SEQ ID NO: 31) were used.

[0172]

The DNA obtained by the PCR reaction was purified, and the DNA was subjected to limited digestion using restriction enzymes (Age I and Xho I) and preparative separation were carried out. pCA7 vector was subjected to limited digestion using restriction enzymes (Age I and Xho I) and preparative separation, and then a ligation reaction between the pCA7 vector and the above-mentioned DNA was carried out. *Escherichia coli* was transformed using the above-mentioned ligation reaction liquid, and then amplification and purification of the vector were carried out. The base sequence of the vector was subjected to a sequence analysis, and it was confirmed that there was no introduction of mutation caused by PCR error. In this

manner, an expression vector for TFB excluding the signal sequence of the N-terminal (TFB/pCA7) was obtained.

[0173]

(2) Production of TFB

Tachypleus tridentatus factor B (TFB) was produced by a mammalian cell expression system by the following procedure. Cell culture was carried out under the conditions of 37°C and 5% CO₂.

[0174]

The same operation as that employed in "(2) Production of TFC" in <Reference Example 1> was carried out, except that the expression vector for TFB obtained in the section (1) (TFB/pCA7) was used instead of the expression vector for TFC (TFC/pCA7), and benzamidine was not added. Thus, a culture fluid including TFB that had been secreted from the cells was obtained.

[0175]

The culture fluid was centrifuged at 6,000×g for 30 minutes, and the supernatant was collected. 50 mM NaH₂PO₄-NaOH (pH 6.8) was added to the supernatant to dilute the supernatant by five times, and then the dilution was applied to an SP column (SP-Sepharose column, inner diameter: 1.0 cm × length: 10 cm). The column was washed with 50 mM NaH₂PO₄-NaOH (pH 6.8), and then TFB was eluted using an elution solution (50 mM NaH₂PO₄-NaOH (pH 6.8), and

50 to 500 mM NaCl). The elution fraction of TFB was checked by gel electrophoresis (SDS-PAGE).

[0176]

The eluted fraction of TFB was collected, and the solution was replaced with 20 mM Tris-HCl (pH 8.0) by ultrafiltration. The solution was applied to a DEAE column (DEAE-Sepharose column, inner diameter: 1.0 cm × length: 1.0 cm), and then TFB was eluted using an elution solution (20 mM Tris-HCl (pH 8.0), and 10 to 200 mM NaCl). The eluted fraction of TFB was checked by gel electrophoresis (SDS-PAGE). The eluted fraction of TFB was used as TFB in the subsequent test.

[0177]

<Example 1> Production of Murasame-TFB

An expression vector for *Tachypleus tridentatus* factor B variant (Murasame-TFB) was produced by the following procedure.

[0178]

(1) Production of Murasame-TFB expression vector

An inverse PCR reaction was carried out using the TFB/pCA7 as a template and using phosphorylated primers (Primer 11 (SEQ ID NO: 32) and Primer 12 (SEQ ID NO: 33)). The inverse PCR reaction was carried out by using Tks Gflex DNA polymerase (manufactured by Takara Bio, Inc.) and performing the operation according to the attached protocol.

A restriction enzyme (Dpn I) was added to the PCR reaction liquid, and the template was degraded. Preparative separation of the DNA was carried out by performing phenol/chloroform extraction and ethanol precipitation, and then a ligation reaction (self-ligation) was carried out using DNA Ligation Kit <Mighty Mix> and performing the operation according to the attached protocol.

[0179]

Escherichia coli was transformed using the above-mentioned ligation reaction liquid, and then amplification and purification of the vector were carried out. The base sequence of the vector was subjected to a sequence analysis, and it was confirmed that there was no introduction of mutation caused by PCR error. In this manner, an expression vector for Murasame-TFB (Murasame-TFB/pCA7) was obtained.

[0180]

(2) Production of Murasame-TFB

The *Tachypleus tridentatus* factor B variant (Murasame-TFB) was produced by a mammalian cell expression system according to the following procedure. Cell culture was carried out under the conditions of 37°C and 5% CO₂.

[0181]

The same operation as that employed in "(2) Production of TFC" in the section <Reference Example 1> was

carried out, except that the expression vector for Murasame-TFB (Murasame-TFB/pCA7) obtained in the section (1) was used instead of the expression vector for TFC (TFC/pCA7), and benzamidine was not added. Thus, a culture fluid including Murasame-TFB that had been secreted from the cells was obtained.

[0182]

The same operation as that employed in "(2) Production of TFB" in <Reference Example 2> was carried out, and an eluted fraction including Murasame-TFB was obtained. The eluted fraction of Murasame-TFB was used as Murasame-TFB in the subsequent test.

[0183]

<Reference Example 3> Measurement of protease activity (specific activity) of TFB

A solution of 160 nM TFC, 3.2 μ M LPS (derived from *Salmonella minnesota* R595, weight average molecular weight 1,700 Da, manufactured by List Biological Laboratories, Inc.), 20 mM Tris-HCl (pH 8.0), and 150 mM NaCl was produced, and the solution was left to stand at 37°C for 20 minutes. Thus, TFC was activated. Hereinafter, activated TFC will be referred to as " α -TFC".

[0184]

20 μ L of a solution including 50 nM TFB, 0.2 nM α -TFC, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 100 μ g/mL

BSA was produced, and the solution was left to stand for one hour at 37°C. To this solution, 5 µL of 2 mM Boc-Leu-Thr-Arg-MCA (manufactured by Peptide Institute, Inc.) dissolved in 20% DMF was added, and the mixture was left to stand for 5 minutes at 37°C. 0.6 M Acetic acid (75 µL) was added to the mixture to complete the enzyme reaction, and then the amount of MCA released from the peptide (properly proportional to the protease activity (total activity) of activated TFB) was measured with a fluorescence detector. The detection was carried out under the conditions of an excitation wavelength of 380 nm and a fluorescence wavelength of 440 nm.

[0185]

As a result, the protease activity (specific activity) of TFB was 31.07 ± 2.50 units/µmol.

[0186]

<Example 2> Measurement of protease activity
(specific activity) of Murasame-TFB

The same operation as that employed in <Reference Example 3> was carried out using Murasame-TFB instead of TFB, and the protease activity of Murasame-TFB was measured.

[0187]

As a result of the test, the protease activity (specific activity) of Murasame-TFB was 416.87 ± 20.50 units/µmol.

[0188]

The results of <Reference Example 3> and <Example 2> are presented in FIG. 1. From the results of the above-described test, it was found that Murasame-TFB has a protease activity (specific activity) that is 13.4 times the protease activity of TFB.

[0189]

<Reference Example 4> Evaluation of thermal stability of TFB

10 μ L of a solution of 100 nM TFB, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 100 μ g/mL BSA was produced, and the solution was left to stand for 2 minutes at a predetermined temperature (40°C, 50°C, 60°C, 70°C, 80°C, or 90°C). Subsequently, to this solution, 10 μ L of a solution of 0.4 nM α -TFC, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 100 μ g/mL BSA was added, and the mixture was left to stand for one hour at 37°C. Subsequently, the protease activity of TFB was measured by performing the same operation as that employed in <Reference Example 3>. The results of the above-described test are presented in Table 1.

[0190]

[Table 1]

Table 1

Temperature	Relative activity
40°C	103.60%
50°C	40.00%

60°C	0.0%
70°C	0.0%
80°C	0.0%
90°C	0.0%

[0191]

In Table 1, the "relative activity" is a numerical value (%) expressing, in percentage, a value obtained by dividing the protease activity (specific activity) of TFB that had been left to stand for 2 minutes at each of the various temperatures, by the protease activity (specific activity) of TFB shown in <Reference Example 3>.

[0192]

<Example 3> Evaluation of thermal stability of Murasame-TFB

The thermal stability of Murasame-TFB was evaluated by performing the same operation as that employed in <Reference Example 4> using Murasame-TFB instead of TFB. The results of the test are presented in Table 2.

[0193]

[Table 2]

Table 2

Temperature	Relative activity
40°C	99.64%
50°C	85.17%
60°C	72.63%
70°C	57.64%
80°C	50.12%
90°C	42.92%

[0194]

In Table 2, the relative activity is a numerical value (%) expressing, in percentage, a value obtained by dividing the protease activity (specific activity) of Murasame-TFB that had been left to stand for 2 minutes at each of the various temperatures, by the protease activity (specific activity) of Murasame-TFB shown in <Example 2>.

[0195]

The results of <Reference Example 4> and <Example 3> are presented in FIG. 2. From the results of the above-described test, it was found that TFB completely loses its protease activity (deactivated) when heated for 2 minutes at or above 60°C. Meanwhile, it was found that Murasame-TFB maintains 40% or more of its protease activity even when heated for 2 minutes at 90°C, and thus Murasame-TFB has higher thermal stability than TFB.

[0196]

<Reference Example 5> Production of LFC

(1) Production of LFC expression vector

An expression vector for *Limulus polyphemus* factor C (LFC) was produced according to the following procedure.

[0197]

A DNA encoding full-length LFC excluding the signal sequence of the N-terminal (DNA having a base sequence represented by base numbers 76 to 3060 of SEQ ID NO: 13) was produced by a PCR reaction using Tks Gflex DNA

polymerase (manufactured by Takara Bio, Inc.). As a template for the PCR reaction, a vector obtained by inserting a DNA encoding LFC into pBluescript II SK(+) (manufactured by Agilent Technologies, Inc.) was used. As primers for the PCR reaction, Primer 13 (SEQ ID NO: 34) and Primer 14 (SEQ ID NO: 35) were used.

[0198]

The DNA obtained by the above-described PCR was collected by performing phenol/chloroform extraction, and the DNA was subjected to limited digestion using restriction enzymes (Age I and Xho I) and preparative separation. TFC/pCA7 was subjected to limited digestion using restriction enzymes and preparative separation in the same manner as described above, and then a ligation reaction between the DNA and TFC/pCA7 was carried out.

[0199]

Escherichia coli was transformed using the above-mentioned ligation reaction liquid, and then amplification and purification of the vector were carried out. The base sequence of the vector was subjected to a sequence analysis, and it was checked that there was no introduction of mutation caused by PCR error. In this manner, an expression vector for a polypeptide having the 6×His tag and factor Xa cleavage sequence (IEGR) on the N-terminal side of the LFC sequence excluding the signal sequence of

the N-terminal (hereinafter, referred to as "LFC/pCA7") was obtained.

[0200]

(2) Production of LFC

Limulus polyphemus factor C (LFC) was produced by a mammalian cell expression system according to the following procedure. Cell culture was carried out under the conditions of 37°C and 5% CO₂.

[0201]

HEK293S GnTI⁻ cells (ATCC: CRL-3022) were transformed using the expression vector for LFC (LFC/pCA7) obtained in the section (1). Specifically, to the above-mentioned cells that had reached a confluence of 80% to 90%, a transformation medium (DMEM medium including LFC/pCA7 (1.8 µg/mL), polyethyleneimine (2.7 µg/mL), a 1% penicillin-streptomycin-L-glutamine solution (manufactured by Wako Pure Chemical Industries, Ltd.), and 2% bovine fetal serum) was added to a concentration of 0.22 mL/cm², and benzamidine was added to the cell to a final concentration of 2 mM. The mixture was subjected to static culture for 120 hours. In this manner, a culture fluid including LFC that had been secreted from the cells was obtained.

[0202]

The culture fluid was centrifuged at 6,000×g for 30

minutes, and the supernatant was collected. The supernatant and a buffer solution (0.5 M NaH_2PO_4 -NaOH (pH 8.0), 1.5 M NaCl, and 0.1 M imidazole) in a 0.1-fold amount were mixed, and then the mixture was applied to a nickel column (nickel-nitrilotriacetic acid agarose column, inner diameter: 1.0 cm $\times$ length: 2.0 cm). The column was washed with a washing solution (50 mM NaH_2PO_4 -NaOH (pH 8.0), 150 mM NaCl, and 10 mM imidazole), and then LFC was eluted using an elution solution (50 mM NaH_2PO_4 -NaOH (pH 8.0), 150 mM NaCl, and 20 to 200 mM imidazole). The eluted fraction of LFC was checked by gel electrophoresis (SDS-PAGE).

[0203]

The eluted fraction of LFC was collected, and the solution was replaced with a reaction solution (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 10 mM CaCl_2) by ultrafiltration. Subsequently, factor Xa was added to the solution to a concentration of 3.3 $\mu\text{g}/\text{mL}$, and the mixture was left to stand for two days at room temperature. Thus, the His tag was released from the N-terminal of LFC.

[0204]

The above-mentioned reaction solution was applied to a sepharose column (benzamidine-Sepharose column, inner diameter: 0.25 cm $\times$ length: 1.5 cm), and factor Xa was eliminated by adsorbing factor Xa to the column. Subsequently, a flow-through fraction (flow-through) of the

column was applied to a nickel column (nickel-nitrilotriacetic acid-agarose column, inner diameter: 0.25 cm × length: 1.5 cm), and the His tag released from LFC was eliminated by adsorbing the His tag to the column. The flow-through fraction of the column was used as LFC in the subsequent test.

[0205]

<Reference Example 6> Production of LFB

(1) Production of LFB expression vector

An expression vector for *Limulus polyphemus* factor B (LFB) was produced according to the following procedure.

[0206]

A DNA encoding the full-length sequence of LFB (DNA having a base sequence represented by base numbers 1 to 1200 of SEQ ID NO: 3) was produced by a PCR reaction using Tks Gflex DNA polymerase. As a template for the PCR reaction, a vector obtained by inserting a DNA encoding LFB into pBluescript II SK(+) was used. Furthermore, as primers for the PCR reaction, Primer 15 (SEQ ID NO: 36) and Primer 18 (SEQ ID NO: 39) were used.

[0207]

The DNA obtained by the above-described PCR reaction was purified, and the DNA was subjected to limited digestion using restriction enzymes (EcoR I and Xho I) and preparative separation. pCA7 vector was subjected to

limited digestion using restriction enzymes (EcoR I and Xho I) and preparative separation, and then a ligation reaction between the DNA and the pCA7 vector was carried out.

Escherichia coli was transformed using the above-mentioned ligation reaction liquid, and then amplification and purification of the vector were carried out. The base sequence of the vector was subjected to a sequence analysis, and it was confirmed that there was no introduction of mutation caused by PCR error. In this manner, an expression vector for the polypeptide having the full-length sequence of LFB was obtained.

[0208]

A PCR reaction was carried out using the above-mentioned vector as a template and using Primer 15 (SEQ ID NO: 36) and Primer 19 (SEQ ID NO: 40), and the DNA thus amplified was purified. The DNA was subjected to limited digestion using restriction enzymes (EcoR I and Xho I) and preparative separation, and thus a DNA fragment (DNA having a base sequence represented by base numbers 1 to 1200 of SEQ ID NO: 3 and a base sequence encoding the 6×His tag, the DNA having the sticky end of EcoR I at the 5'-terminal and the sticky end of Xho I at the 3'-terminal) was obtained.

[0209]

The pCA7 vector was subjected to limited digestion

using restriction enzymes (EcoR I and Xho I) and preparative separation, and then a ligation reaction between the pCA7 vector and the DNA was carried out. *Escherichia coli* was transformed using the above-mentioned ligation reaction, and amplification and purification of the vector were carried out. The base sequence of the vector was subjected to a sequence analysis, and it was confirmed that there was no introduction of mutation caused by PCR error. In this manner, an expression vector for a polypeptide having the 6XHis tag on the C-terminal side of the full-length sequence of LFB was obtained.

[0210]

A PCR reaction was carried out using the above-mentioned vector as a template and using Primer 20 (SEQ ID NO: 41) and Primer 21 (SEQ ID NO: 42), and an amplified DNA was purified. The DNA was subjected to limited digestion using restriction enzymes (Age I and Xho I) and preparative separation, and a DNA fragment (DNA having a base sequence represented by base numbers 70 to 1200 of SEQ ID NO: 3 and a base sequence encoding the 6XHis tag, the DNA having the sticky end of Age I at the 5'-terminal and the sticky end of Xho I at the 3'-terminal) was obtained.

[0211]

The pCA7 vector was subjected to limited digestion using restriction enzymes (Age I and Xho I) and preparative

separation, and then a ligation reaction between the pCA7 vector and the DNA was carried out. *Escherichia coli* was transformed using the above-mentioned ligation reaction liquid, and then amplification and purification of the vector were carried out. The base sequence of the vector was subjected to a sequence analysis, and it was confirmed that there was no introduction of mutation caused by PCR error. In this manner, an expression vector for a polypeptide having the 6×His tag on the C-terminal side of the LFB sequence excluding the signal sequence of the N-terminal (LFB/pCA7) was obtained.

[0212]

(2) Production of LFB

Limulus polyphemus factor B (LFB) was produced by a mammalian cell expression system according to the following procedure. Cell culture was carried out under the conditions of 37°C and 5% CO₂.

[0213]

The same operation as that employed in "(2) Production of LFC" in <Reference Example 5> was carried out, except that the expression vector for LFB (LFB/pCA7) obtained in the section (1) was used instead of the expression vector for LFC (LFC/pCA7), and benzamidine was not added. Thus, a culture fluid including LFB secreted from cells was obtained.

[0214]

The culture fluid was applied to a nickel column (nickel-nitrilotriacetic acid-agarose column, inner diameter: 1.0 cm × length: 1.5 cm), and an eluted fraction of LFB was obtained. Column purification was carried out according to the procedure described in "(2) Production of LFC") in <Reference Example 5>.

[0215]

An eluted fraction of LFB was collected, and the solution was substituted with a reaction solution (20 mM Tris-HCl (pH 8.0) and 300 mM NaCl) by ultrafiltration. The LFB thus obtained was used in the subsequent test.

[0216]

<Example 4> Production of Murasame-LFB

An expression vector for a *Limulus polyphemus* factor B variant (Murasame-LFB) was produced according to the following procedure.

[0217]

A DNA encoding the N-terminal side of Murasame-LFB (DNA having a base sequence represented by base numbers 1 to 594 of SEQ ID NO: 9; hereinafter, referred to as "DNA fragment 9") was produced by a PCR reaction using Tks Gflex DNA polymerase. As a template for the PCR reaction, a vector obtained by inserting a DNA encoding LFB into pCA7 was used. As primers for the PCR reaction, Primer 15 (SEQ

ID NO: 36) and Primer 16 (SEQ ID NO: 37) were used.

[0218]

A DNA encoding the C-terminal side of Murasame-LFB (DNA having a base sequence represented by base numbers 562 to 1200 of SEQ ID NO: 9; hereinafter, referred to as "DNA fragment 10") was produced by a PCR reaction in the same manner as described above. As primers for the PCR reaction, Primer 17 (SEQ ID NO: 38) and Primer 18 (SEQ ID NO: 39) were used.

[0219]

The DNA obtained by the PCR reaction was purified, and DNA fragment 9 and DNA fragment 10 were obtained.

[0220]

A DNA encoding the full-length sequence of Murasame-LFB (DNA having a base sequence represented by base numbers 1 to 1200 of SEQ ID NO: 9) was produced by a PCR reaction using Tks Gflex DNA polymerase. As a template for the PCR reaction, a mixture of DNA fragment 9 and DNA fragment 10 was used. Furthermore, as primers for the PCR reaction, Primer 15 (SEQ ID NO: 36) and Primer 18 (SEQ ID NO: 39) were used. Subsequently, the same operation as that employed in "(1) Production of LFB expression vector" in <Reference Example 6> was carried out. In this manner, an expression vector for a polypeptide having the 6×His tag on the C-terminal side of the Murasame-LFB sequence excluding

the signal sequence of the N-terminal (Murasame-LFB/pCA7) was obtained.

[0221]

(2) Production of Murasame-LFB

A *Limulus polyphemus* factor B variant (Murasame-LFB) was produced by a mammalian cell expression system according to the following procedure. Cell culture was carried out under the conditions of 37°C and 5% CO₂.

[0222]

The same operation as that employed in "(2) Production of LFB" in <Reference Example 6> was carried out using the expression vector for Murasame-LFB (Murasame-LFB/pCA7) obtained in the section (1) was used instead of the expression vector for LFB (LFB/pCA7). The Murasame-LFB thus obtained was used in the subsequent test.

[0223]

<Reference Example 7> Measurement of protease activity (specific activity) of LFB

A solution of 160 nM LFC, 3.2 μM LPS, 20 mM Tris-HCl (pH 8.0), and 150 mM NaCl was produced, and the solution was left to stand for 20 minutes at 37°C. Thus, LFC was activated. Hereinafter, the activated LFC will be referred to as "α-LFC".

[0224]

20 μL of a solution of 50 nM LFB, 2 nM α-LFC, 20 mM

Tris-HCl (pH 8.0), 150 mM NaCl, and 100 $\mu\text{g/mL}$ BSA was produced, and the solution was left to stand for one hour at 37°C. To this solution, 5 μL of 2 mM Boc-Leu-Thr-Arg-MCA dissolved in 20% DMF was added, and the mixture was left to stand for 5 minutes at 37°C. 0.6 M Acetic acid (75 μL) was added thereto to terminate the enzyme reaction, and then the amount of MCA released from the peptide (properly proportional to the protease activity (total activity) of activated LFB) was measured with a fluorescence detector. The detection was carried out under the conditions of an excitation wavelength of 380 nm and a fluorescence wavelength of 440 nm.

[0225]

As a result of the above-described detection, the protease activity (specific activity) of LFB was 44.67 ± 0.61 units/ μmol .

[0226]

<Example 5> Measurement of protease activity
(specific activity) of Murasame-LFB

The same operation as that employed in <Reference Example 7> was carried out using Murasame-LFB instead of LFB, and the protease activity of the Murasame-LFB was measured.

[0227]

As a result of the above-described test, the

protease activity (specific activity) of Murasame-LFB was 320.80 ± 9.56 units/ μmol .

[0228]

As a result of the above-described test, it was found that, similarly to Murasame-TFB, Murasame-LFB has higher protease activity (specific activity) than the polypeptide before modification (LFB).

[0229]

<Reference Example 8> Evaluation of thermal stability of LFB

10 μL of a solution of 100 nM LFB, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 100 $\mu\text{g/mL}$ BSA was produced, and the solution was left to stand for 2 minutes at a predetermined temperature (40°C, 50°C, 60°C, 70°C, 80°C, or 90°C). Subsequently, to this solution, 10 μL of a solution of 4 nM α -LFC, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 100 $\mu\text{g/mL}$ BSA was added, and the mixture was left to stand for one hour at 37°C. Subsequently, the same operation as that employed in <Reference Example 7> was carried out, and the protease activity of LFB was measured. The results of the above-described test are presented in Table 3.

[0230]

[Table 3]

Table 3

<u>Temperature</u>	<u>Relative activity</u>
--------------------	--------------------------

40°C	90.20%
50°C	69.19%
60°C	0.0%
70°C	0.0%
80°C	0.0%
90°C	0.0%

[0231]

The "relative activity" in Table 3 is a numerical value (%) expressing, in percentage, a value obtained by dividing the protease activity (specific activity) of LFB that had been left to stand for 2 minutes at each of the various temperatures, by the protease activity (specific activity) of LFB shown in <Reference Example 7>.

[0232]

<Example 6> Evaluation of thermal stability of Murasame-LFB

The same operation as that employed in <Reference Example 8> was carried out using Murasame-LFB instead of LFB, and the thermal stability of Murasame-LFB was evaluated. The results of the above-described test are presented in Table 4.

[0233]

[Table 4]

Table 4

Temperature	Relative activity
40°C	90.96%
50°C	84.37%
60°C	70.43%
70°C	50.00%

80°C	33.80%
90°C	26.41%

[0234]

The relative activity in Table 4 is a numerical value (%) expressing, in percentage, a value obtained by dividing the protease activity (specific activity) of Murasame-LFB that had been left to stand for 2 minutes at each of the various temperatures, by the protease activity (specific activity) of Murasame-LFB shown in <Example 5>.

[0235]

From the results of the above-described test, it was found that similarly to Murasame-TFB, Murasame-LFB has higher thermal stability than the polypeptide before modification (LFB).

Industrial Applicability

[0236]

According to the present invention, a polypeptide having a protease activity superior to that of naturally occurring horseshoe crab factor B can be produced. Therefore, the polypeptide provided by the present invention is expected to be a horseshoe crab factor B variant that can enhance the sensitivity of endotoxin measurement, compared to naturally occurring horseshoe crab factor B. Furthermore, according to the present invention, a polypeptide having a thermal stability superior to that

of naturally occurring horseshoe crab factor B can be produced. Therefore, the polypeptide provided by the present invention is expected to be a horseshoe crab factor B variant having excellent storage stability as a reagent, compared to naturally occurring horseshoe crab factor B.

[0237]

<Explanation of sequence listing>

SEQ ID NO: 1: Base sequence of cDNA of *Tachypleus tridentatus* factor B

SEQ ID NO: 2: Amino acid sequence of *Tachypleus tridentatus* factor B

SEQ ID NO: 3: Base sequence of cDNA of *Limulus polyphemus* factor B

SEQ ID NO: 4: Amino acid sequence of *Limulus polyphemus* factor B

SEQ ID NO: 5: Base sequence (1) of cDNA of *Tachypleus tridentatus* factor B variant

SEQ ID NO: 6: Base sequence (2) of cDNA of *Tachypleus tridentatus* factor B variant

SEQ ID NO: 7: Amino acid sequence of *Tachypleus tridentatus* factor B variant

SEQ ID NO: 8: Base sequence (1) of cDNA of *Limulus polyphemus* factor B variant

SEQ ID NO: 9: Base sequence (2) of cDNA of *Limulus polyphemus* factor B variant

SEQ ID NO: 10: Amino acid sequence of *Limulus*
polyphemus factor B variant

SEQ ID NO: 11: Base sequence of cDNA of *Tachypleus*
tridentatus factor C

SEQ ID NO: 12: Amino acid sequence of *Tachypleus*
tridentatus factor C

SEQ ID NO: 13: Base sequence of cDNA of *Limulus*
polyphemus factor C

SEQ ID NO: 14: Amino acid sequence of *Limulus*
polyphemus factor C

SEQ ID NO: 15: Base sequence of cDNA of
Carcinoscorpius rotundicauda factor C

SEQ ID NO: 16: Amino acid sequence of
Carcinoscorpius rotundicauda factor C

SEQ ID NO: 17: Base sequence of cDNA of *Tachypleus*
tridentatus proclotting enzyme

SEQ ID NO: 18: Amino acid sequence of *Tachypleus*
tridentatus proclotting enzyme

SEQ ID NO: 19: Base sequence of cDNA of *Limulus*
polyphemus proclotting enzyme

SEQ ID NO: 20: Amino acid sequence of cDNA of
Limulus polyphemus proclotting enzyme

SEQ ID NO: 21 to SEQ ID NO: 42: Primers

CLAIMS

1. A polypeptide having an amino acid sequence in which the amino acid residue at the 193-position in an amino acid sequence of a naturally occurring polypeptide of horseshoe crab factor B is substituted with a cysteine (Cys) residue.

2. A polypeptide having any one of amino acid sequence in the following (A) to (D), and having the function of horseshoe crab factor B:

(A) an amino acid sequence consisting of amino acid numbers 1 to 400 or amino acid numbers 24 to 400 of SEQ ID NO: 7,

(B) an amino acid sequence consisting of amino acid numbers 1 to 400 or amino acid numbers 24 to 400 of SEQ ID NO: 10,

(C) an amino acid sequence having at least one feature of the following (C1) and (C2):

(C1) a similarity of 99% or higher to at least one amino acid sequence in the items (A) and (B), provided that the cysteine (Cys) residue at the 193-position of SEQ ID:7 and SEQ ID: 10 is neither substituted nor deleted; and

(C2) an identity of 90% or higher to at least one amino acid sequence in the items (A) and (B), provided that the cysteine (Cys) residue at the 193-position of SEQ ID:7 and SEQ ID: 10 is neither substituted nor deleted
, and

(D) an amino acid sequence of a fusion polypeptide in which a peptide tag is added to the polypeptide according to any one of the items (A) to (C),

wherein the function of horseshoe crab factor B is a function of being changed to an activated form by contact with an activated factor C, cleaving a proclotting enzyme, and thereby changing the proclotting enzyme to a clotting enzyme.

3. A nucleic acid encoding the polypeptide according to claim 1 or 2.

4. A DNA having any one of the following (a), (b) and (d):

(a) a DNA having a base sequence according to any one of the following (a1) to (a4):

- (a1) a base sequence consisting of base numbers 1 to 1200 of SEQ ID NO: 5;
- (a2) a base sequence consisting of base numbers 70 to 1200 of SEQ ID NO: 5;
- (a3) a base sequence consisting of base numbers 1 to 1200 of SEQ ID NO: 6;
- and
- (a4) a base sequence consisting of base numbers 70 to 1200 of SEQ ID NO: 6,
- (b) a DNA having a base sequence according to any one of the following (b1) to (b4):
- (b1) a base sequence consisting of base numbers 1 to 1200 of SEQ ID NO: 8;
- (b2) a base sequence consisting of base numbers 70 to 1200 of SEQ ID NO: 8;
- (b3) a base sequence consisting of base numbers 1 to 1200 of SEQ ID NO: 9;
- and
- (b4) a base sequence consisting of base numbers 70 to 1200 of SEQ ID NO: 9,
- and
- (c) a DNA having the base sequence of a fusion DNA in which a peptide tag-encoding DNA is added to the DNA according to any one of the items (a) and (b), the DNA encoding a polypeptide having the function of horseshoe crab factor B.

5. A vector retaining the nucleic acid according to claim 3 and/or the DNA according to claim 4.

6. A cell retaining the nucleic acid according to claim 3, the DNA according to claim 4, and/or the vector according to claim 5.

7. A method for producing a polypeptide, the method comprising a step of producing a polypeptide having the function of horseshoe crab factor B using the cell according to claim 6.

8. A polypeptide obtainable by the method according to claim 7.

9. A method for measuring an endotoxin, the method comprising steps of the following (1) and (2):

(1) a step of mixing the polypeptide according to claim 1, 2, or 8 with horseshoe crab factor C and a test sample; and

(2) a step of measuring protease activity of the polypeptide.

10. A reagent for endotoxin measurement, the reagent comprising the polypeptide according to claim 1, 2, or 8 as a constituent component.

11. A kit for endotoxin measurement, the kit comprising the polypeptide according to claim 1, 2, or 8, or the reagent according to claim 10, as a component part.