

DESCRIPTION

Title of Invention

PRODUCTION METHOD FOR INSOLUBLE RECOMBINANT PROTEIN AGGREGATE

5 Technical Field

[0001] The present invention relates to a method for producing an insoluble recombinant protein aggregate by a method of separating insoluble bodies of a recombinant protein from a recombinant cell expressing the insoluble recombinant protein, and an insoluble
10 recombinant protein aggregate obtained by the method.

Background Art

[0002] Industrial-scale production of a target protein has been made possible by using a genetic recombinant host cell. Many methods for isolating and purifying a recombinant protein produced by a
15 recombinant cell have been reported.

[0003] In the case where the recombinant protein as insoluble bodies is compactly produced as insoluble granules in the recombinant cell, it is possible to isolate the insoluble granules in relatively high yield and high purity by centrifuging a suspension containing a component such
20 as a protein derived from a host cell. For example, a method of isolating a target protein from insoluble recombinant cells solubilized with a metal hydroxide such as sodium hydroxide (Patent Literature 1), and the like have been reported.

[0004] On the other hand, for example, the following purification
25 methods have been reported in the case where it is difficult to separate the recombinant protein by centrifugation even in the case where the

recombinant protein is in a solubilized state or insoluble bodies in recombinant cells, unlike compact insoluble granules. That is, for example, a method in which a protein derived from a host cell is hydrolyzed with an organic acid such as formic acid or propionic acid, insoluble bodies derived from a host cell are removed by centrifugation or the like, and then the target recombinant protein is recovered in an undenatured state and purified by a technique such as chromatography (Patent Literature 2) has been reported. In this report, the target protein remains in an undenatured state even in the case where the organic acid is added, and does not aggregate.

[0005] Not a target recombinant protein is necessarily produced as insoluble granules in a recombinant cell. It is known that the state of formation of insoluble granules greatly changes depending on the nature of the target recombinant protein itself or various parameters of the culturing process such as medium composition at the time of production, culture temperature, and production rate. Therefore, studies are directed to the modification of a recombinant protein or the development of an efficient production method thereof so as to produce large insoluble granules that can be centrifuged as easily as possible.

[0006] On the other hand, in the case where there is a method which is capable of easily separating fine insoluble bodies or insoluble granules that are difficult or very time consuming for centrifugation, by centrifugation, filtration, or the like, it is very industrially useful, but such a method is not known.

Citation List

Patent Literature

[0007] [Patent Literature 1] Japanese Unexamined Patent
Publication No. 2013-523665

[Patent Literature 2] Japanese Unexamined Patent
Publication No. 2004-503204

5 **Summary of Invention**

Technical Problem

[0008] An object of the present invention is to provide a method for
efficiently separating insoluble bodies of a recombinant protein from a
recombinant cell expressing a target recombinant protein as insoluble
10 bodies in the cell, and a method for producing a recombinant protein
aggregate by the separation method.

Solution to Problem

[0009] As a result of extensive studies on a method which is capable
of easily separating fine insoluble bodies or insoluble granules of a
recombinant protein that are difficult or very time consuming for
15 centrifugation, the present inventors have found that the recombinant
protein can be easily separated by efficiently aggregating the insoluble
bodies or insoluble granules to be enlarged. The present invention has
been completed based on these findings.

20 [0010] That is, the present invention relates to, for example, each of
the following inventions.

[1] A method for producing a recombinant protein aggregate by
separating insoluble bodies of a recombinant protein as an aggregate
from a recombinant cell expressing the recombinant protein as insoluble
25 bodies in the cell, including disrupting the recombinant cell, aggregating
the insoluble bodies of the recombinant protein, and separating the

resulting aggregate.

[2] The method for producing a recombinant protein aggregate according to [1], further including separating the recombinant protein aggregate by a centrifugal force of $10,000 \times g$ or less.

5 [3] The method for producing a recombinant protein aggregate according to [1] or [2], further including separating the recombinant protein aggregate by using a centrifuge selected from the group consisting of a separation plate type centrifuge, a basket type centrifuge, and a decanter type centrifuge.

10 [4] The method for producing a recombinant protein aggregate according to [1], further including separating the recombinant protein aggregate by spontaneous sedimentation or filtration.

[5] The method for producing a recombinant protein aggregate according to any one of [1] to [4], in which aggregation of the insoluble
15 bodies of the recombinant protein is carried out by adding one or more selected from the group consisting of a metal salt, an acid, and an anionic flocculant.

[6] A method for producing a recombinant protein aggregate, including the following steps (A) to (C):

20 a step (A) of disrupting a recombinant cell expressing a target recombinant protein as insoluble bodies in the cell to obtain a disrupted suspension containing the insoluble bodies of the recombinant protein;

a step (B) of adding one or more selected from the group consisting of a metal salt, an acid, and an anionic flocculant to the
25 disrupted suspension obtained in the step (A), and aggregating the insoluble bodies of the recombinant protein to obtain the recombinant

protein aggregate; and

a step (C) of separating the aggregate obtained in the step (B) from the suspension.

5 [7] The method for producing a recombinant protein aggregate according to [6], further including heating in the step (B).

[8] The method for producing a recombinant protein aggregate according to claim 7, further including stirring in the step (B).

10 [9] The method for producing a recombinant protein aggregate according to any one of [5] to [8], in which the metal salt is a metal salt selected from the group consisting of an alkaline earth metal salt and an earth metal salt.

[10] The method for producing a recombinant protein aggregate according to [9], in which the metal salt is a metal salt selected from the group consisting of an alkaline earth metal halide, an alkaline earth metal nitrate, an alkaline earth metal sulfate, an earth metal halide, an earth metal nitrate, and an earth metal sulfate.

[11] The method for producing a recombinant protein aggregate according to any one of [5] to [10], in which the acid is an oxo acid.

20 [12] The method for producing a recombinant protein aggregate according to [11], in which the oxo acid is an oxo acid selected from the group consisting of acetic acid, sulfuric acid, and citric acid.

25 [13] The method for producing a recombinant protein aggregate according to any one of [5] to [12], in which the anionic flocculant is an anionic flocculant selected from the group consisting of

a polyacrylate, an anionic polyacrylamide, and an acrylamide-acrylate copolymer.

5 [14] The method for producing a recombinant protein aggregate according to any one of [1] to [13], in which the disruption of the recombinant cell is mechanical disruption.

 [15] The method for producing a recombinant protein aggregate according to any one of [1] and [6] to [14], in which the separation of the recombinant protein aggregate is carried out by filtration.

10 [16] The method for producing a recombinant protein aggregate according to any one of [1] to [15], in which the recombinant cell is a recombinant cell transformed with a host selected from the group consisting of a bacterium, a yeast, a filamentous fungus, an insect cell, a plant cell, and an animal cell.

15 [17] The method for producing a recombinant protein aggregate according to any one of [1] to [16], in which the recombinant protein is a structural protein.

 [18] The method for producing a recombinant protein aggregate according to [17], in which the structural protein is a protein
20 derived from a protein selected from the group consisting of keratin, collagen, elastin, resilin, silkworm silk, and spider silk.

 [19] The method for producing a recombinant protein aggregate according to any one of [1] to [18], in which the resulting recombinant protein aggregate has a particle size of 4 μm or more and
25 50 μm or less as measured by an electrical sensing zone method.

 [20] A recombinant protein aggregate obtained by the method

for producing a recombinant protein aggregate according to any one of [1] to [18], which has a particle size of 4 μm or more and 50 μm or less as measured by an electrical sensing zone method.

5 [21] A method for separating insoluble bodies of a recombinant protein from a recombinant cell expressing the recombinant protein as insoluble bodies in the cell, including disrupting the recombinant cell, aggregating the insoluble bodies of the recombinant protein, and separating the resulting aggregate.

10 [22] The method for separating a recombinant protein according to [21], further including separating the recombinant protein aggregate by a centrifugal force of $10,000 \times g$ or less.

15 [23] The method for separating a recombinant protein according to [21] or [22], further including separating the recombinant protein aggregate by using a centrifuge selected from the group consisting of a separation plate type centrifuge, a basket type centrifuge, and a decanter type centrifuge.

[24] The method for separating a recombinant protein according to [21], further including separating the recombinant protein aggregate by spontaneous sedimentation or filtration.

20 [25] The method for separating a recombinant protein according to any one of [21] to [24], in which aggregation of the insoluble bodies of the recombinant protein is carried out by adding one or more selected from the group consisting of a metal salt, an acid, and an anionic flocculant.

25 [26] A method for separating a recombinant protein, including the following steps (A) to (C):

a step (A) of disrupting a recombinant cell expressing a target recombinant protein as insoluble bodies in the cell to obtain a disrupted suspension containing the insoluble bodies of the recombinant protein;

5 a step (B) of adding one or more selected from the group consisting of a metal salt, an acid, and an anionic flocculant to the disrupted suspension obtained in the step (A), and aggregating the insoluble bodies of the recombinant protein to obtain a recombinant protein aggregate; and

10 a step (C) of separating the aggregate obtained in the step (B) from the suspension.

[27] The method for separating a recombinant protein according to [26], further including heating in the step (B).

[28] The method for separating a recombinant protein according to [27], further including stirring in the step (B).

15 [29] The method for separating a recombinant protein according to any one of [25] to [28], in which the metal salt is a metal salt selected from the group consisting of an alkaline earth metal salt and an earth metal salt.

20 [30] The method for separating a recombinant protein according to [29], in which the metal salt is a metal salt selected from the group consisting of an alkaline earth metal halide, an alkaline earth metal nitrate, an alkaline earth metal sulfate, an earth metal halide, an earth metal nitrate, and an earth metal sulfate.

25 [31] The method for separating a recombinant protein according to any one of [25] to [30], in which the acid is an oxo acid.

[32] The method for separating a recombinant protein

according to [31], in which the oxo acid is an oxo acid selected from the group consisting of acetic acid, sulfuric acid, and citric acid.

5 [33] The method for separating a recombinant protein according to any one of [25] to [32], in which the anionic flocculant is an anionic flocculant selected from the group consisting of a polyacrylate, an anionic polyacrylamide, and an acrylamide-acrylate copolymer.

10 [34] The method for separating a recombinant protein according to any one of [21] to [33], in which the disruption of the recombinant cell is mechanical disruption.

[35] The method for separating a recombinant protein according to any one of [21] and [26] to [34], in which the separation of the recombinant protein aggregate is carried out by filtration.

15 [36] The method for separating a recombinant protein according to any one of [21] to [35], in which the recombinant cell is a recombinant cell transformed with a host selected from the group consisting of a bacterium, a yeast, a filamentous fungus, an insect cell, a plant cell, and an animal cell.

20 [37] The method for separating a recombinant protein according to any one of [21] to [36], in which the recombinant protein is a structural protein.

25 [38] The method for separating a recombinant protein according to [37], in which the structural protein is a protein derived from a protein selected from the group consisting of keratin, collagen, elastin, resilin, silkworm silk, and spider silk.

[39] A method for producing a recombinant protein aggregate

using the separation method according to any one of [1] to [38], in which the recombinant protein aggregate obtained by the separation method has a particle size of 4 μm or more and 50 μm or less as measured by an electrical sensing zone method.

5 **Advantageous Effects of Invention**

[0011] According to the method for producing a recombinant protein aggregate of the present invention, since insoluble bodies can be aggregated and enlarged, the recombinant protein aggregate can be produced by efficiently separating insoluble bodies of the recombinant protein from a recombinant cell expressing the target recombinant protein as insoluble bodies in the cell by, for example, spontaneous sedimentation, centrifugation, or filtration. Further, not only insoluble bodies or insoluble granules of the recombinant protein, which could not be readily separated by centrifugation, filtration, or the like, can be easily separated, but also the purity of the separated recombinant protein can be improved. According to the present invention, such an unexpected effect is exerted.

15 **Brief Description of Drawings**

[0012] Fig. 1 is a photograph showing the results of studying an aggregation effect of insoluble bodies by adding a metal salt in Example 1.

Fig. 2 is a photograph showing the results of examining the aggregation effect of the insoluble bodies by adding the metal salt in Example 2.

25 Fig. 3 is a photograph showing the results of examining the aggregation effect of insoluble bodies of proteins having different

hydropathy indices by adding the metal salt in Example 3.

Fig. 4 is a photograph showing the results of examining the aggregation effect of insoluble bodies of proteins having different hydropathy indices by adding the metal salt in Example 4.

5 Fig. 5 is a photograph showing the results of polyacrylamide gel electrophoresis (SDS-PAGE) analysis on improvement of the purity of a target recombinant protein based on metal salt addition aggregation in Example 4. A is a photograph showing the results of staining with an Oriole (trademark) fluorescent gel stain (manufactured by Bio-Rad
10 Laboratories, Inc.) capable of staining all proteins after the electrophoresis, and B is a photograph showing the results of staining with an InVision (trademark) His-tag In-gel Stain (manufactured by Thermo Fisher Scientific, Inc.) reacting to a His tag region of PRT410, after the electrophoresis.

15 Fig. 6 is a photograph showing the results of examining the aggregation effect of insoluble bodies by adding an acid in Example 5.

Fig. 7 is a photograph showing the results of examining the aggregation effect of insoluble bodies by adding an acid in the insoluble bodies after washing in Example 5.

20 Fig. 8 is a photograph showing the results of examining the aggregation effect of insoluble bodies of proteins having different hydropathy indices by adding an acid in Example 6.

Fig. 9 is a photograph showing the results of SDS-PAGE analysis on improvement of the purity of a target recombinant protein
25 based on acid addition aggregation in Example 6.

Fig. 10 is a photograph showing the results of examining the

aggregation effect of insoluble bodies by adding a flocculant in Example 8.

Fig. 11 is a photograph showing the results of SDS-PAGE analysis on improvement of the purity of a target recombinant protein based on anionic flocculant addition aggregation in Example 9.

Fig. 12 is a diagram showing frequency distribution and cumulative distribution of particle sizes for confirming the aggregation effect in Example 10.

Fig. 13 is a photograph showing the results of SDS-PAGE analysis on degradation of contaminating proteins based on heating in Example 12.

Fig. 14 is a photograph showing the results of SDS-PAGE analysis on improvement of the purity of a target recombinant protein based on heating in Example 12.

Fig. 15 is a diagram showing the frequency distribution and the cumulative distribution of particle sizes of Samples C, X, 1, 2, and 3 for confirming the aggregation effect by continuous heating in Example 13.

Fig. 16 is a diagram showing the frequency distribution and the cumulative distribution of particle sizes of Samples 4, 5, 6, 7, and 8 for confirming the aggregation effect by continuous heating in Example 13.

Description of Embodiments

[0013] Hereinafter, embodiments for carrying out the present invention will be described in detail. However, the present invention is not limited to the following embodiments.

[0014] The method for producing a recombinant protein aggregate according to an embodiment is a method for producing a recombinant

protein aggregate by separating insoluble bodies of a recombinant protein as an aggregate from a recombinant cell expressing the recombinant protein as insoluble bodies in the cell, including disrupting the recombinant cell, aggregating the insoluble bodies of the recombinant protein, and separating the resulting aggregate. In the present production method, aggregation of insoluble bodies of the recombinant protein is preferably carried out by adding one or more selected from the group consisting of a metal salt, an acid, and an anionic flocculant.

[0015] The method for producing a recombinant protein aggregate according to another embodiment includes the following steps (A) to (C):

a step (A) of disrupting a recombinant cell expressing a target recombinant protein as insoluble bodies in the cell to obtain a disrupted suspension containing the insoluble bodies of the recombinant protein;

a step (B) of adding one or more selected from the group consisting of a metal salt, an acid, and an anionic flocculant to the disrupted suspension obtained in the step (A), and aggregating the insoluble bodies of the recombinant protein to obtain the recombinant protein aggregate; and

a step (C) of separating the aggregate obtained in the step (B) from the suspension.

[0016] (Recombinant protein)

The insoluble recombinant protein (sometimes referred to as "target protein" in the present specification) to be separated by the method for producing a recombinant protein aggregate according to the

present embodiment is expressed as insoluble bodies in a recombinant cell which will be described hereinafter. The recombinant protein may be any insoluble protein which is preferably produced on an industrial scale, and examples thereof include a protein that can be used for industrial use, a protein that can be used for medical use, and a structural protein. Specific examples of the protein that can be used for industrial or medical use include an enzyme, a regulatory protein, a receptor, a peptide hormone, a cytokine, a membrane and a transport protein, an antigen used for vaccination, a vaccine, an antigen binding protein, an immunostimulatory protein, an allergen, a full-length antibody and an antibody fragment, and derivatives thereof. Specific examples of the structural protein include keratin, collagen, elastin, resilin, silkworm silk and spider silk, and proteins derived therefrom.

[0017] The protein derived from spider silk or silkworm silk which is a fibroin-like protein may be, for example, a protein containing a domain sequence represented by Formula 1: $[(A)_n \text{ motif-REP}]_m$ (in which, in Formula 1, $(A)_n$ motif represents an amino acid sequence consisting of 4 to 20 amino acid residues and the number of alanine residues to the total number of amino acid residues in the $(A)_n$ motif is 80% or more; REP represents an amino acid sequence consisting of 10 to 200 amino acid residues; m represents an integer of 8 to 300; a plurality of $(A)_n$ motifs may be the same amino acid sequence or different amino acid sequences; and a plurality of REP's may be the same amino acid sequence or different amino acid sequences.).

Specific examples thereof include proteins containing the amino acid sequences represented by SEQ ID NO: 1 (PRT410), SEQ ID NO: 2

(PRT853), SEQ ID NO: 3 (PRT647), SEQ ID NO: 4 (PRT699), and SEQ ID NO: 5 (PRT698). The hydropathy indices of these proteins are -0.81, -0.68, 0.04, 0.17, and 0.43, respectively. The value of the hydropathy index is a value calculated according to the method described in International Publication No. WO2014/103846.

[0018] The protein derived from collagen may be, for example, a protein containing a domain sequence represented by Formula 2: [REP2]_o (in which, in Formula 2, o represents an integer of 5 to 300; REP2 represents an amino acid sequence consisting of Gly-X-Y Gly where X and Y each represent any amino acid residue other than Gly, and a plurality of REP2's may be the same amino acid sequence or different amino acid sequences). A specific example thereof may be a protein containing the amino acid sequence represented by SEQ ID NO: 6 (Collagen-type 4-Kai). The amino acid sequence represented by SEQ ID NO: 6 is an amino acid sequence in which an amino acid sequence (tag sequence and hinge sequence) represented by SEQ ID NO: 10 has been added to the N-terminus of the amino acid sequence from the 301st residue to the 540th residue corresponding to a repeat portion and a motif of a partial sequence of human collagen type 4 (NCBI GenBank Accession No.: CAA 56335. 1, GI: 3702452) obtained from the NCBI database. The Collagen-type 4-Kai has a hydropathy index of -0.75.

[0019] The protein derived from resilin may be, for example, a protein containing a domain sequence represented by Formula 3: [REP3]_p (in which, in Formula 3, p represents an integer of 4 to 300; REP3 represents an amino acid sequence consisting of Ser-J-J-Tyr-Gly-U-Pro

where J represents any amino acid residue and is particularly preferably
 an amino acid residue selected from the group consisting of Asp, Ser,
 and Thr, and U represents any amino acid residue and is particularly
 preferably an amino acid residue selected from the group consisting of
 5 Pro, Ala, Thr, and Ser; and a plurality of REP3's may be the same amino
 acid sequence or different amino acid sequences.). A specific example
 thereof may be a protein containing the amino acid sequence
 represented by SEQ ID NO: 7. The amino acid sequence represented
 by SEQ ID NO: 7 is an amino acid sequence in which an amino acid
 10 sequence (tag sequence and hinge sequence) represented by SEQ ID
 NO: 10 has been added to the N-terminus of the amino acid sequence
 from the 19th residue to the 321st residue of the sequence in which Thr
 at the 87th residue is substituted with Ser and Asn at the 95th residue is
 substituted with Asp, in the amino acid sequence of resilin (NCBI
 15 GenBank Accession No. NP_611157. 1, GI : 24654243). The
 Resilin-Kai (SEQ ID NO: 7) has a hydropathy index of -1.22.
 [0020] Examples of the protein derived from elastin include proteins
 having amino acid sequences such as NCBI GenBank Accession Nos.
 AAC98395 (human), I47076 (sheep), and NP786966 (bovine).
 20 Specifically, a protein containing the amino acid sequence represented
 by SEQ ID NO: 8 can be mentioned. The amino acid sequence
 represented by SEQ ID NO: 8 is an amino acid sequence in which an
 amino acid sequence (tag sequence and hinge sequence) represented by
 SEQ ID NO: 10 has been added to the N-terminus of the amino acid
 25 sequence from the 121th residue to the 390th residue of the amino acid
 sequence of NCBI GenBank Accession No. AAC98395. The elastin

short (SEQ ID NO: 8) has a hydropathy index of 0.42.

[0021] The protein derived from keratin may be, for example, type I keratin of *Capra hircus*. A specific example thereof may be a protein containing the amino acid sequence represented by SEQ ID NO: 9 (amino acid sequence of NCBI GenBank Accession No. ACY30466). The type I keratin 26 (SEQ ID NO: 9) has a hydropathy index of -0.53.

[0022] (Recombinant cell)

The recombinant cell in the present embodiment is a recombinant cell expressing a recombinant protein as insoluble bodies in the cell and can be obtained by a general method using genetic engineering techniques.

[0023] The recombinant cell can be obtained, for example, by transforming a host (host cell) with an expression vector having a nucleic acid sequence encoding a target protein and one or a plurality of regulatory sequences operably linked to the nucleic acid sequence.

[0024] The regulatory sequence is a sequence that controls the expression of a recombinant protein in a host (for example, a promoter, an enhancer, a ribosome binding sequence, or a transcription termination sequence), and can be appropriately selected depending on the type of the host. The type of the expression vector such as a plasmid vector, a viral vector, a cosmid vector, a fosmid vector, or an artificial chromosome vector can be appropriately selected depending on the type of the host.

[0025] Both prokaryotes and eukaryotes such as yeast, filamentous fungi, insect cells, animal cells, and plant cells can be suitably used as hosts. More preferred are bacteria, yeast, filamentous fungi, insect

cells, plant cells, and animal cells. Preferred examples of prokaryotes include *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas*, *Corynebacterium*, and *Lactococcus*, among which more preferred are *Escherichia coli* cells.

5 [0026] An expression vector which can autonomously replicate in a host cell or can be incorporated into a chromosome of a host and which contains a promoter at a position capable of transcribing a nucleic acid encoding a target protein is suitably used as the expression vector. A ribosome binding sequence, a transcription termination sequence, or a
10 gene sequence that controls a promoter may be included in the expression vector.

[0027] The promoter may be any inducible promoter which functions in a host cell and is capable of inducing the expression of a target protein. The inducible promoter is a promoter capable of controlling
15 transcription due to the presence of an inducer (expression inducer), the absence of a repressor molecule, and physical factors such as an increase or decrease in temperature, osmotic pressure, or pH value.

[0028] Examples of prokaryotic hosts include microorganisms belonging to the genus *Escherichia*, *Brevibacillus*, *Serratia*, *Bacillus*,
20 *Microbacterium*, *Brevibacterium*, *Corynebacterium* and *Pseudomonas*.

[0029] Examples of microorganisms belonging to the genus *Escherichia* include *Escherichia coli* BL21 (Novagen, Inc.), *Escherichia coli* BL21 (DE3) (Life Technologies Corporation), *Escherichia coli* BLR(DE3) (Merck KGaA), *Escherichia coli* DH1,
25 *Escherichia coli* GI698, *Escherichia coli* HB101, *Escherichia coli* JM109, *Escherichia coli* K5 (ATCC 23506), *Escherichia coli* KY3276,

Escherichia coli MC1000, *Escherichia coli* MG1655 (ATCC 47076),
Escherichia coli No. 49, *Escherichia coli* Rosetta (DE3) (Novagen,
Inc.), *Escherichia coli* TB1, *Escherichia coli* Tuner (Novagen, Inc.),
Escherichia coli Tuner (DE3) (Novagen, Inc.), *Escherichia coli* W1485,
5 *Escherichia coli* W3110 (ATCC 27325), *Escherichia coli* XL1-Blue,
and *Escherichia coli* XL2-Blue.

[0030] Examples of microorganisms belonging to the genus
Brevibacillus include *Brevibacillus agri*, *Brevibacillus borstelensis*,
Brevibacillus centrosporus, *Brevibacillus formosus*, *Brevibacillus*
10 *invocatus*, *Brevibacillus laterosporus*, *Brevibacillus limnophilus*,
Brevibacillus parabrevis, *Brevibacillus reuszeri*, *Brevibacillus*
thermoruber, *Brevibacillus brevis* 47 (FERM BP-1223), *Brevibacillus*
brevis 47K (FERM BP-2308), *Brevibacillus brevis* 47-5 (FERM
BP-1664), *Brevibacillus brevis* 47-5Q (JCM 8975), *Brevibacillus*
15 *choshinensis* HPD31 (FERM BP-1087), *Brevibacillus choshinensis*
HPD31-S (FERM BP-6623), *Brevibacillus choshinensis* HPD31-OK
(FERM BP-4573), and *Brevibacillus choshinensis* SP3 strain
(manufactured by Takara Bio, Inc.).

[0031] Examples of microorganisms belonging to the genus *Serratia*
20 include *Serratia liquefacience* ATCC 14460, *Serratia entomophila*,
Serratia ficaria, *Serratia fonticola*, *Serratia grimesii*, *Serratia*
proteamaculans, *Serratia odorifera*, *Serratia plymuthica*, and *Serratia*
rubidaea.

[0032] Examples of microorganisms belonging to the genus *Bacillus*
25 include *Bacillus subtilis* and *Bacillus amyloliquefaciens*.

[0033] Examples of microorganisms belonging to the genus

Microbacterium include *Microbacterium ammoniophilum* ATCC 15354.

[0034] Examples of microorganisms belonging to the genus *Brevibacterium* include *Brevibacterium divaricatum* (*Corynebacterium glutamicum*) ATCC 14020, *Brevibacterium flavum* (*Corynebacterium glutamicum* ATCC 14067) ATCC 13826, ATCC 14067, *Brevibacterium immariophilum* ATCC 14068, *Brevibacterium lactofermentum* (*Corynebacterium glutamicum* ATCC 13869) ATCC 13665, ATCC 13869, *Brevibacterium roseum* ATCC 13825, *Brevibacterium saccharolyticum* ATCC 14066, *Brevibacterium tiogenitalis* ATCC 19240, *Brevibacterium album* ATCC 15111, and *Brevibacterium cerinum* ATCC 15112.

[0035] Examples of microorganisms belonging to the genus *Corynebacterium* include *Corynebacterium ammoniagenes* ATCC 6871, ATCC 6872, *Corynebacterium glutamicum* ATCC 13032, *Corynebacterium glutamicum* ATCC 14067, *Corynebacterium acetoacidophilum* ATCC 13870, *Corynebacterium acetoglutamicum* ATCC 15806, *Corynebacterium alkanolyticum* ATCC 21511, *Corynebacterium callunae* ATCC 15991, *Corynebacterium glutamicum* ATCC 13020, ATCC 13032, ATCC 13060, *Corynebacterium lilium* ATCC 15990, *Corynebacterium melassecola* ATCC 17965, *Corynebacterium thermoaminogenes* AJ12340 (FERM BP-1539), and *Corynebacterium herculis* ATCC 13868.

[0036] Examples of microorganisms belonging to the genus *Pseudomonas* include *Pseudomonas putida*, *Pseudomonas fluorescens*, *Pseudomonas brassicacearum*, *Pseudomonas fulva*, and *Pseudomonas* sp. D-0110.

[0037] As a method for introducing an expression vector into the foregoing prokaryotic host cell, any method can be used as long as it introduces DNA into the host cell. Examples thereof include a method using calcium ions [Proc. Natl. Acad. Sci. USA, 69, 2110 (1972)], a
5 protoplast method (Japanese Unexamined Patent Publication No. S63-248394), or a method described in Gene, 17, 107 (1982) or Molecular & General Genetics, 168, 111 (1979).

[0038] Transformation of microorganisms belonging to the genus *Brevibacillus* can be carried out, for example, by the method of
10 Takahashi et al. (J. Bacteriol., 1983, 156: 1130-1134), the method of Takagi et al. (Agric. Biol. Chem., 1989, 53: 3099-3100), or the method of Okamoto et al. (Biosci. Biotechnol. Biochem., 1997, 61: 202-203).

[0039] Examples of the vector into which a nucleic acid encoding a target protein is introduced (hereinafter, simply referred to as "vector")
15 include pBTrp2, pBTac1, and pBTac2 (all commercially available from Boehringer Mannheim GmbH), pKK233-2 (manufactured by Pharmacia Corporation), pSE280 (manufactured by Invitrogen Corporation), pGEMEX-1 (manufactured by Promega Corporation), pQE-8 (manufactured by QIAGEN Corporation), pKYP10 (Japanese
20 Unexamined Patent Publication No. S58-110600), pKYP200 [Agric. Biol. Chem., 48, 669 (1984)], pLSA1 [Agric. Biol. Chem., 53, 277 (1989)], pGEL1 [Proc. Natl. Acad. Sci. USA, 82, 4306 (1985)], pBluescript II SK(-) (manufactured by Stratagene Corporation), pTrs30 [constructed from *Escherichia coli* JM109/pTrS30 (FERM BP-5407)],
25 pTrs32 [constructed from *Escherichia coli* JM109/pTrS32 (FERM BP-5408)], pGHA2 [constructed from *Escherichia coli* IGHA2 (FERM

B-400), Japanese Unexamined Patent Publication No. S60-221091], pGKA2 [constructed from *Escherichia coli* IGKA2 (FERM BP-6798), Japanese Unexamined Patent Publication No. S60-221091], pTerm2 (US 4686191, US 4939094, US 5160735), pSupex, pUB110, pTP5, pC194, pEG400 [J. Bacteriol., 172, 2392 (1990)], pGEX (manufactured by Pharmacia Corporation), and pET systems (manufactured by Novagen, Inc.).

[0040] In the case where *Escherichia coli* is used as a host, pUC18, pBluescriptII, pSupex, pET22b, pCold, or the like can be mentioned as a suitable vector.

[0041] Specific examples of vectors suitable for microorganisms belonging to the genus *Brevibacillus* include pUB110 or pHY500 (Japanese Unexamined Patent Publication No. H2-31682), pNY700 (Japanese Unexamined Patent Publication No. H4-278091), pHY4831 (J. Bacteriol., 1987, 1239-1245), pNU200 (UDAKA Shigezou, Journal of the Agricultural Chemical Society of Japan, 1987, 61: 669-676), pNU100 (Appl. Microbiol. Biotechnol., 1989, 30: 75-80), pNU211 (J. Biochem., 1992, 112: 488-491), pNU211R2L5 (Japanese Unexamined Patent Publication No. H7-170984), pNH301 (Appl. Environ. Microbiol., 1992, 58: 525-531), pNH326, pNH400 (J. Bacteriol., 1995, 177: 745-749), and pHT210 (Japanese Unexamined Patent Publication No. H6-133782), pHT110R2L5 (Appl. Microbiol. Biotechnol., 1994, 42: 358-363), which are known as *Bacillus subtilis* vectors; and pNCO2 (Japanese Unexamined Patent Publication No. 2002-238569) which is a shuttle vector between *Escherichia coli* and a microorganism belonging to the genus *Brevibacillus*.

[0042] The promoter in the case where a prokaryote is used as a host is not limited as long as it functions in the host cell. Examples thereof include promoters derived from *Escherichia coli* or phage such as a trp promoter (Ptrp), a lac promoter, a PL promoter, a PR promoter, and a T7 promoter. Also, promoters artificially designed and modified, such as a promoter (Ptrp×2) in which two Ptrp are connected in series, a tac promoter, a lacT7 promoter, and a let I promoter, can also be used. It is preferable to use a plasmid in which the distance between the Shine-Dalgarno sequence, which is a ribosome binding sequence, and the initiation codon is adjusted to an appropriate distance (for example, 6 to 18 bases). In the above expression vector, a transcription termination sequence is not always necessary for the expression of the nucleic acid, but it is preferable to arrange the transcription termination sequence directly under the nucleic acid encoding the target protein.

[0043] Examples of eukaryotic hosts include yeast, filamentous fungi (mold and the like), and insect cells.

[0044] Examples of the yeast include yeasts belonging to the genus *Saccharomyces*, *Schizosaccharomyces*, *Kluyveromyces*, *Trichosporon*, *Schwanniomyces*, *Pichia*, *Candida*, *Yarrowia*, *Hansenula*, and the like. More specific examples of the yeast include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Trichosporon pullulans*, *Schwanniomyces alluvius*, *Schwanniomyces occidentalis*, *Candida utilis*, *Pichia pastoris*, *Pichia angusta*, *Pichia methanolica*, *Pichia polymorpha*, *Pichia stipitis*, *Yarrowia lipolytica*, and *Hansenula polymorpha*.

[0045] It is preferred that the expression vector in the case where yeast

is used as a host cell usually include an origin of replication (in the case where amplification in a host is required), a selection marker for propagation of the vector in *Escherichia coli*, a promoter and a terminator for recombinant protein expression in yeast, and a selection marker for yeast.

[0046] In the case where the expression vector is a non-integrating vector, it is preferable to further include an autonomously replicating sequence (ARS). This makes it possible to improve the stability of the expression vectors in cells (Myers, A. M., et al. (1986) Gene 45: 299-310).

[0047] Examples of the vector in the case where yeast is used as a host include YEP13 (ATCC 37115), YEp24 (ATCC 37051), YCp50 (ATCC 37419), YIp, pHS19, pHS15, pA0804, pHIL3Ol, pHIL-S1, pPIC9K, pPICZ α , pGAPZ α , and pPICZ B.

[0048] A specific example of the promoter in the case where yeast is used as a host is not limited as long as it can be expressed in the yeast. Examples of the promoter include a promoter of glycolytic genes such as hexose kinase, a PHO5 promoter, a PGK promoter, a GAP promoter, an ADH promoter, a gal 1 promoter, a gal 10 promoter, a heat shock polypeptide promoter, an MF α 1 promoter, a CUP 1 promoter, a pGAP promoter, a pGCW14 promoter, an AOX1 promoter, and an MOX promoter.

[0049] As a method for introducing an expression vector into yeast, any method can be used as long as it introduces DNA into yeast. Examples thereof include an electroporation method (Methods Enzymol., 194, 182 (1990)), a spheroplast method (Proc. Natl. Acad.

Sci., USA, 81, 4889 (1984)), a lithium acetate method (J. Bacteriol., 153, 163 (1983)), and a method described in Proc. Natl. Acad. Sci. USA, 75, 1929 (1978).

5 [0050] Examples of filamentous fungi include fungi belonging to the genus *Acremonium*, *Aspergillus*, *Ustilago*, *Trichoderma*, *Neurospora*, *Fusarium*, *Humicola*, *Penicillium*, *Myceliophthora*, *Botrytis*, *Magnaporthe*, *Mucor*, *Metarhizium*, *Monascus*, *Rhizopus*, and *Rhizomucor*.

10 [0051] Specific examples of filamentous fungi include *Acremonium alabamense*, *Acremonium cellulolyticus*, *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus oryzae*, *Aspergillus sake*, *Aspergillus sojae*, *Aspergillus tubigenensis*, *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus parasiticus*, *Aspergillus ficuum*, *Aspergillus phoenicis*, *Aspergillus foetidus*, *Aspergillus flavus*, *Aspergillus fumigatus*,
15 *Aspergillus japonicus*, *Trichoderma viride*, *Trichoderma harzianum*, *Trichoderma reseei*, *Chrysosporium lucknowense*, *Thermoascus*, *Sporotrichum*, *Sporotrichum cellulophilum*, *Talaromyces*, *Thielavia terrestris*, *Thielavia*, *Neurospora crassa*, *Fusarium oxysporus*, *Fusarium graminearum*, *Fusarium venenatum*, *Humicola insolens*,
20 *Penicillium chrysogenum*, *Penicillium camemberti*, *Penicillium canescens*, *Penicillium emersonii*, *Penicillium funiculosum*, *Penicillium griseoroseum*, *Penicillium purpurogenum*, *Penicillium roqueforti*, *Myceliophthora thermophilum*, *Mucor ambiguus*, *Mucor circinelloides*, *Mucor fragilis*, *Mucor hiemalis*, *Mucor inaequisporus*, *Mucor oblongiellipticus*,
25 *Mucor racemosus*, *Mucor recurvus*, *Mucor saturninus*, *Mucor subtilissimus*, *Ogataea polymorpha*, *Phanerochaete*

chrysosporium, *Rhizomucor miehei*, *Rhizomucor pusillus*, and *Rhizopus arrhizus*.

5 [0052] A specific example of the promoter in the case where a filamentous fungus is used as a host may be any one of a gene related to a glycolytic system, a gene related to constitutive expression, an enzyme gene related to hydrolysis, and the like. Specific examples thereof include amyB, glaA, agdA, glaB, TEF1, xynF1 tannase gene, No. 8AN, gpdA, pgkA, enoA, melO, sodM, catA, and catB.

10 [0053] Introduction of the expression vector into filamentous fungi can be carried out by a conventionally known method. Examples thereof include the method of Cohen et al. (calcium chloride method) [Proc. Natl. Acad. Sci. USA, 69: 2110 (1972)], a protoplast method [Mol. Gen. Genet., 168:111 (1979)], a competent method [J. Mol. Biol., 56: 209 (1971)], and an electroporation method.

15 [0054] Insect cells include, for example, lepidopteran insect cells, more specifically insect cells derived from *Spodoptera frugiperda* such as Sf9 and Sf21, and insect cells derived from *Trichoplusia ni* such as High 5.

20 [0055] Examples of the vector in the case where an insect cell is used as a host include baculoviruses such as *Autographa californica* nuclear polyhedrosis virus which is a virus that infects insects belonging to the family *Noctuidae* (Baculovirus Expression Vectors, A Laboratory Manual, W. H. Freeman and Company, New York (1992)).

25 [0056] In the case where an insect cell is used as a host, a polypeptide can be expressed by the method described in, for example, Current Protocols in Molecular Biology, Baculovirus Expression Vectors, A

Laboratory Manual, W. H. Freeman and Company, New York (1992), or Bio/Technology, 6, 47 (1988). That is, a recombinant gene transfer vector and a baculovirus are co-introduced into an insect cell to obtain a recombinant virus (expression vector) in an insect cell culture supernatant, and then the recombinant virus is further infected into an insect cell, whereby the polypeptide can be expressed. Examples of the gene transfer vector used in the above method include pVL1392, pVL1393, and pBlueBacIII (all manufactured by Invitrogen Corporation).

[0057] As a method for co-introducing a recombinant gene transfer vector and a baculovirus into an insect cell for constructing the recombinant virus, for example, a calcium phosphate method (Japanese Unexamined Patent Publication No. H2-227075), a lipofection method (Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)), or the like can be mentioned.

[0058] The recombinant vector preferably further contains a selection marker gene for selecting a transformant. For example, in *Escherichia coli*, resistance genes for various drugs such as tetracycline, ampicillin, and kanamycin can be used as selection marker genes. A recessive selection marker capable of complementing a genetic mutation involved in auxotrophy can also be used. In yeast, a resistance gene for geneticin can be used as a selection marker gene, and a gene complementing a genetic mutation involved in auxotrophy, or a selection marker such as LEU2, URA3, TRP1, or HIS3 can also be used. Examples of the selection marker gene for filamentous fungi include a marker gene selected from the group consisting of niaD

(Biosci. Biotechnol. Biochem., 59, 1795-1797 (1995)), argB (Enzyme Microbiol Technol, 6, 386-389, (1984)), sC (Gene, 84, 329-334, (1989)), ptrA (BiosciBiotechnol Biochem, 64, 1416-1421, (2000)), pyrG (BiochemBiophys Res Commun, 112, 284-289, (1983)), amdS (Gene, 26, 205-221, (1983)), aureobasidin resistance gene (Mol Gen Genet, 261, 290-296, (1999)), benomyl resistance gene (Proc Natl Acad Sci USA, 83, 4869-4873, (1986)) and hygromycin resistance gene (Gene, 57, 21-26, (1987)), and a leucine auxotrophy-complementing gene. Further, in the case where the host is an auxotrophic mutant strain, a wild-type gene complementing the auxotrophy can also be used as a selection marker gene.

[0059] The selection of the host transformed with the foregoing expression vector can be carried out by plaque hybridization, colony hybridization, or the like using a probe that selectively binds to the foregoing nucleic acid. As the probe, it is possible to use a probe obtained by modifying a partial DNA fragment amplified by a PCR method based on sequence information of the foregoing nucleic acid with a radioisotope or digoxigenin.

[0060] (Expression of recombinant protein)

In a recombinant cell transformed with the expression vector for expressing a target protein, the recombinant protein is expressed as insoluble bodies in the cell. The recombinant protein can be expressed by culturing recombinant cells in a culture medium. The method of culturing the recombinant cells in the culture medium can be carried out according to a method commonly used for culturing a host.

[0061] In the case where the host is a prokaryote such as *Escherichia*

coli or a eukaryote such as yeast, any of a natural medium and a synthetic medium may be used as a culture medium as long as it contains a carbon source, a nitrogen source, inorganic salts, and the like which can be assimilated by the host and it is capable of efficiently culturing the host.

[0062] As the carbon source, any carbon source that can be assimilated by the host may be used. Examples of the carbon source that can be used include carbohydrates such as glucose, fructose, sucrose, and molasses, starch and starch hydrolyzates containing them, organic acids such as acetic acid and propionic acid, and alcohols such as ethanol and propanol.

[0063] Examples of the nitrogen source that can be used include ammonium salts of inorganic or organic acids such as ammonia, ammonium chloride, ammonium sulfate, ammonium acetate and ammonium phosphate, other nitrogen-containing compounds, peptone, meat extract, yeast extract, corn steep liquor, casein hydrolyzate, soybean cake and soybean cake hydrolyzate, various fermented bacterial cells and digested products thereof.

[0064] Examples of the inorganic salt that can be used include potassium dihydrogen phosphate, dipotassium phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, and calcium carbonate.

[0065] Culture of a prokaryote such as *Escherichia coli* or a eukaryote such as yeast can be carried out under aerobic conditions such as shaking culture or deep aeration stirring culture. The culture temperature is, for example, 15°C to 40°C. The culture time is usually

16 hours to 7 days. It is preferable to maintain the pH of the culture medium during the culture at 3.0 to 9.0. The pH of the culture medium can be adjusted using an inorganic acid, an organic acid, an alkali solution, urea, calcium carbonate, ammonia, or the like.

5 [0066] In addition, antibiotics such as ampicillin and tetracycline may be added to the culture medium as necessary during the culture. In the case of culturing a microorganism transformed with an expression vector using an inducible promoter as a promoter, an inducer may be added to the medium as necessary. For example, in the case of
10 culturing a microorganism transformed with an expression vector using a lac promoter, isopropyl- β -D-thiogalactopyranoside or the like is used, and in the case of culturing a microorganism transformed with an expression vector using a trp promoter, indoleacrylic acid or the like may be added to the medium.

15 [0067] As a culture medium for insect cells, commonly used TNM-FH medium (manufactured by Pharmingen Inc.), Sf-900 II SFM medium (manufactured by Life Technologies Corporation), ExCell 400 and ExCell 405 (both manufactured by JRH Biosciences Inc.), Grace's Insect Medium (Nature, 195, 788 (1962)), and the like can be used.

20 [0068] Culture of insect cells can be carried out, for example, for a culture time of 1 to 5 days under conditions such as pH 6 to 7 of culture medium and culture temperature 25°C to 30°C. In addition, an antibiotic such as gentamicin may be added to the culture medium as necessary during the culture.

25 [0069] In the case where the host is a plant cell, the transformed plant cell may be directly cultured, or it may be differentiated into a plant

organ and then cultured. As the culture medium for culturing a plant cell, for example, commonly used Murashige and Skoog (MS) medium, White medium, or a medium in which a plant hormone such as auxin or cytokinin is added to these media can be used.

5 [0070] Culture of animal cells can be carried out, for example, for a culture time of 3 to 60 days under conditions such as pH 5 to 9 of the culture medium and culture temperature 20°C to 40°C. In addition, an antibiotic such as kanamycin or hygromycin may be added to the medium as necessary during the culture.

10 [0071] According to the foregoing method, the target protein can be expressed as insoluble bodies in a recombinant cell.

[0072] Step (A) of disrupting recombinant cell

The step (A) is a step of disrupting a recombinant cell expressing a target recombinant protein as insoluble bodies in the cell to
15 obtain a disrupted suspension containing the insoluble bodies of the recombinant protein.

[0073] Disruption of a recombinant cell can be carried out according to a known method. That is, the disruption of a recombinant cell can be carried out by cell disruption by treatment with an enzyme such as
20 lysozyme, mutanolysin, lyticase, or zymolyase; cell disruption by contact with an organic solvent or the like; cell disruption using osmotic pressure; physical/mechanical cell disruption by a ball mill, a French press, a high pressure homogenizer, ultrasonic treatment, or the like; and a combination thereof.

25 [0074] For the disruption of the recombinant cell, the culture solution obtained by the above-mentioned culture can be used as such, but a

suspension of the washed recombinant cells is preferably used in order to improve the purity of the recombinant protein to be obtained later.

[0075] The suspension of washed recombinant cells can be prepared by the following method. That is, the recombinant cells are separated from the culture solution by centrifugation, filtration, or the like. Considering the subsequent steps, it is preferable to wash the recombinant cells with water, and it is also preferable to further wash the recombinant cells with water after washing with a buffered aqueous solution or the like. The suspension of washed recombinant cells can be prepared by suspending the obtained recombinant cells in a solution suitable for the above disruption method so as to obtain a suitable concentration.

[0076] Alternatively, it is also possible to use a suspension obtained in such a manner that the recombinant cells obtained from the culture solution are treated with an organic solvent or the like, the soluble fraction such as protein derived from the host of the recombinant cells is removed, and then the insoluble fraction is added to a solution suitable for the above disruption method so as to obtain a suitable concentration. At this time, in the case where the bacterial cells are disrupted by treatment with an organic solvent or the like, the treatment with an organic solvent or the like (contact with an organic solvent or the like) can be regarded as the above disruption treatment. That is, after the treatment with an organic solvent or the like, the following aggregation step (B) of recombinant protein insoluble bodies can be carried out.

[0077] Examples of the suitable solution include water such as industrial water, deionized water, or reverse osmosis (RO) water, and a

buffered aqueous solution. The buffered aqueous solution may be, for example, a Tris/HCl buffer solution.

[0078] The resulting disrupted suspension contains insoluble bodies of the recombinant protein. In the present specification, the term "insoluble body" refers to a protein insoluble in a solution (suspension), which may form insoluble granules in some cases.

[0079] In addition, the disrupted suspension contains cell fragments that can be easily centrifuged, and the following aggregation step may be carried out using the suspension after removing these cell fragments.

[0080] In addition, insoluble granules in the disrupted suspension may involve impurities and may be separable by centrifugation. In such a case, centrifugation may be carried out, and the precipitate fraction containing the insoluble granules thus obtained may be resuspended in the buffered aqueous solution to proceed to the following aggregation step.

[0081] Aggregation step (B) of recombinant protein insoluble bodies

In the step (B), one or more selected from the group consisting of a metal salt, an acid, and an anionic flocculant are added to the disrupted suspension obtained in the step (A), and the insoluble bodies of the recombinant protein are aggregated to obtain a recombinant protein aggregate. In the step (B), heating and/or stirring may be carried out as necessary.

[0082] Examples of the metal salt include an alkaline earth metal salt and an earth metal salt. Specific examples thereof include an alkaline earth metal halide, an alkaline earth metal nitrate, an alkaline earth metal sulfate, an earth metal halide, an earth metal nitrate, and an earth

metal sulfate. The metal salt is preferably a divalent or higher polyvalent metal salt.

5 [0083] Examples of the alkaline earth metal halide include calcium chloride, magnesium chloride, magnesium bromide, calcium bromide, magnesium iodide, and calcium iodide.

[0084] Examples of the alkaline earth metal nitrate include calcium nitrate, magnesium nitrate, strontium nitrate, and barium nitrate.

[0085] Examples of the alkaline earth metal sulfate include calcium sulfate, magnesium sulfate, strontium sulfate, and barium sulfate.

10 [0086] Examples of the earth metal halide include aluminum trichloride and gallium trichloride.

[0087] Examples of the earth metal nitrate include aluminum nitrate and gallium nitrate.

15 [0088] Examples of the earth metal sulfate include aluminum sulfate and gallium sulfate.

[0089] These metal salts may be used alone or in combination of two or more thereof.

20 [0090] Suitable examples of the metal salt include an alkali metal halide and an alkaline earth metal halide, and specific suitable examples of the metal salt include lithium chloride and calcium chloride.

[0091] In the case where the recombinant protein forms compact insoluble granules in the disrupted suspension, the metal salt is effective even with addition of a small amount. For example, the metal salt may be added in an amount of 0.01 to 20 mM and preferably 1 to 10 mM.

25 In the case of insoluble bodies that do not form insoluble granules or insoluble granules that take time to precipitate by centrifugation, the

metal salts may be added in an amount of 2 to 50 mM and preferably 5 to 10 mM.

[0092] Any of an inorganic acid and an organic acid can be used as the acid. A suitable acid may be, for example, oxo acid.

5 [0093] Examples of the oxo acid of inorganic acid include sulfuric acid, nitric acid, and phosphoric acid. Examples of the oxo acid of organic acid include formic acid, acetic acid, citric acid, and tartaric acid. The oxo acid is preferably acetic acid, sulfuric acid, or citric acid, and more preferably citric acid.

10 [0094] In the case where the recombinant protein forms compact insoluble granules in the disrupted suspension, the acid is effective even with addition of a small amount. For example, the acid may be added in an amount of 0.01 to 20 mM, preferably 1 to 20 mM, and more preferably 5 to 20 mM. In the case of insoluble bodies that do not
15 form insoluble granules or insoluble granules that take time to precipitate by centrifugation, the acid may be added in an amount of 2 to 50 mM and preferably 10 to 30 mM.

[0095] These acids may be used alone or in combination of two or more thereof.

20 [0096] In the present specification, the term "anionic flocculant" refers to a polymer flocculant (polymer) having an organic anion group. Examples of the anionic flocculant include a polyacrylate-based anionic flocculant, an anionic polyacrylamide-based anionic flocculant, and an acrylamide-acrylate copolymer-based anionic flocculant. Specific
25 examples of the anionic flocculant include KURIFARM PA series (PA-923, PA-896, PA-895, PA-893, PA-865, PA-823, PA-813, PA-804,

PA-465, PA-404, PA-402, PA-265, and the like) manufactured by Kurita Water Industries Ltd., ACCOFLOC (A-95 to A-100, A-110 to A-150, A-190, A-235 H to A-250, and the like) and SUMIFLOC (FA-40 to FA-70) manufactured by MT Aquapolymer, Inc., DIAFLOC AP series (AP335B, AP741B, AP825C, and the like) manufactured by Mitsubishi Rayon Co., Ltd., TAKIFLOC A series (A-102~A-106, A-108, A-142, and A-162) manufactured by Taki Chemical Co., Ltd., and TOGAMIFLOC (TA-089, TA-104, TA-109, TA-124, TA-144, TAE-2325, TAE-2335, TAE-2644, and the like) manufactured by Togami Electric Mfg. Co. Ltd.

[0097] Among these flocculants, there are cases where the flocculants have an action of aggregating the host cells themselves. Therefore, in the case where a flocculant is used, it is preferable to use a disrupted suspension from which the cell fragments are removed in advance. Regarding an amount of the anionic flocculant to be added, the anionic flocculant is added so that the recombinant protein becomes 0.001 to 0.1% and preferably 0.01 to 0.05% in the disrupted suspension.

[0098] Adding a metal salt, an acid, or an anionic flocculant in combination with each other rather than adding each thereof alone provides an effect by adding a low concentration.

[0099] In the aggregation step (B), one or more selected from the group consisting of a metal salt, an acid, and an anionic flocculant may be added, followed by heating so that aggregation is promoted and the aggregate becomes larger. Means for heating is not particularly limited. The heating temperature (peak temperature) is not particularly limited, but from the viewpoint of efficiently obtaining insoluble bodies or

insoluble granules and from the viewpoint of killing bacterial cells, the heating temperature is, for example, 60°C or higher, preferably 70°C or higher, and more preferably 80°C or higher, depending on the type of target recombinant protein. In addition, from the viewpoint of suppressing the decomposition of the target protein and improving the purity of the target protein, the heating temperature is, for example, 130°C or lower, preferably 110°C or lower, and more preferably 90°C or lower, depending on the type of the target protein.

[0100] The heating time (the time for maintaining the heating temperature) is not particularly limited, but from the viewpoint of efficiently obtaining insoluble bodies or insoluble granules and from the viewpoint of killing bacterial cells, the heating time is, for example, 0.5 hours or more, preferably 1 hour or more, and more preferably 2 hours or more, depending on the type of target recombinant protein. In addition, from the viewpoint of suppressing the decomposition of the target protein and improving the working efficiency, the heating time is, for example, 15 hours or less, preferably 10 hours or less, and more preferably 5 hours or less, depending on the type of the target protein.

[0101] The heating time for obtaining insoluble bodies or insoluble granules can be greatly shortened by continuously heating the disrupted suspension. The temperature in the case of continuously heating the disrupted suspension is not particularly limited, but from the viewpoint of efficiently obtaining insoluble bodies or insoluble granules and from the viewpoint of killing bacterial cells, the heating temperature is, for example, 70°C or higher, preferably 80°C or higher, and more preferably 90°C or higher, depending on the type of target recombinant

protein. In addition, from the viewpoint of suppressing the decomposition of the target protein and improving the purity of the target protein, the heating temperature is, for example, 140°C or lower, preferably 120°C or lower, and more preferably 100°C or lower, depending on the type of the target protein.

[0102] The heating time for obtaining insoluble bodies or insoluble granules in the case of continuously heating the disrupted suspension liquid is not particularly limited, but from the viewpoint of efficiently obtaining insoluble bodies or insoluble granules and from the viewpoint of killing the bacterial cells, the heating time is, for example, 1 second or more, preferably 10 seconds or more, and more preferably 30 seconds or more, depending on the type of target recombinant protein. In addition, from the viewpoint of suppressing the degradation of the target protein and improving the working efficiency, the heating time is, for example, 120 seconds or less, preferably 90 seconds or less, and more preferably 60 seconds or less, depending on the type of the target protein.

[0103] There is no particular limitation on the method of continuously heating the disrupted suspension, and it is sufficient that the insoluble bodies or insoluble granules can be heated to 70°C or higher and 140°C or lower and the temperature after heating can be held within 120 seconds. Use of a continuous liquid sterilizer or the like can be mentioned. Particularly, a continuous liquid sterilizer MINI UHT T-20 (manufactured by Powerpoint International Ltd.) can be mentioned.

[0104] In the aggregation step (B), in addition to heating, further stirring may be carried out so that the aggregate becomes larger. The

stirring means is not particularly limited. The stirring speed is not particularly limited, but from the viewpoint of efficiently obtaining insoluble granules, the speed at which the aggregate of insoluble bodies in the solution does not precipitate is preferable, for example, 70 rpm or more, preferably 150 rpm or more, and more preferably 300 rpm or more. From the viewpoint of suppressing the disruption of the formed insoluble bodies, the stirring speed is 1500 rpm or less, preferably 1000 rpm or less, and more preferably 500 rpm or less. The stirring may be carried out at any time during the aggregation step (B). In the case of heating, the stirring is preferably carried out together with heating.

[0105] Step (C) of separating recombinant protein aggregate

The step (C) is a step of separating the aggregate obtained in the step (B) from the suspension. Aggregation of the recombinant protein insoluble bodies is started at the same time as the addition of one or more selected from the group consisting of a metal salt, an acid, and an anionic flocculant to the disrupted suspension, and then appropriate separation means such as spontaneous sedimentation, centrifugation, or filtration can be used to separate the aggregate. After the addition of one or more selected from the group consisting of a metal salt, an acid, and an anionic flocculant, heating and optionally further stirring promote aggregation and makes the aggregate larger, which makes separation easier.

[0106] In one example, the aggregate can be recovered by centrifugation at $2,500 \times g$ for 5 to 30 minutes. In the case where the recombinant protein forms insoluble granules which are originally centrifugeable in the disrupted suspension, one or more selected from

the group consisting of a metal salt, an acid, and an anionic flocculant are added, followed by heating and optionally stirring so that the insoluble granules can be further enlarged and therefore spontaneously settled. A recombinant protein insoluble bodies which are difficult to separate by centrifugation can also be separated by centrifugation, filtration, or the like, by adding one or more selected from the group consisting of a metal salt, an acid, and an anionic flocculant, followed by heating and optionally stirring so that insoluble granules can be further enlarged.

[0107] There has been no report so far that sedimentation can be carried out with a low centrifugal force like $2,500 \times g$ in the centrifugation of insoluble granules, and a cylindrical centrifuge with a high centrifugal force of usually $12,000 \times g$ or more has been used. However, according to the present invention, an insoluble recombinant protein can be precipitated as an aggregate with a lower centrifugal force, which therefore means that a separation plate type (disk type) centrifuge and a decanter type centrifuge, such as WESTFALIA, CLARIFIER, and ALFA LAVAL, which could only be used for the separation of bacterial cells until now, furthermore for example, a basket type centrifuge can be used for separating insoluble recombinant proteins. These separation plate type and decanter type centrifuges can be a very useful means in industrial production due to having a centrifugal force of $10,000 \times g$ or less and capability of continuously separating a large amount of suspension.

[0108] In addition, in the case where the above-described aggregation step is not carried out, even large insoluble granules that can be easily

sedimented by ordinary centrifugation are highly likely to cause clogging in membrane filtration, but performing this aggregation step makes it possible to easily separate those large insoluble granules by the membrane. Particularly, after one or more selected from the group
5 consisting of a metal salt, an acid, and an anionic flocculant are added, heating and optionally stirring are carried out, so that membrane separation of those large insoluble granules becomes easier.

[0109] Further, host cell-derived impurities separable from the recombinant protein insoluble bodies can be removed by separation
10 operation alone, and therefore the purity of the recombinant protein can be improved. In addition, in the case where the separated recombinant protein insoluble bodies are resuspended and then aggregated and separated again, the purity of the recombinant protein can be further improved.

[0110] The particle size of the recombinant protein aggregate obtained by the step (C) of separating a recombinant protein aggregate can be measured by, for example, an electrical sensing zone method. The particle size of the recombinant protein aggregate is, for example, 4 μm or more, preferably 5 μm or more, more preferably 10 μm or more, and
15 still more preferably 15 μm or more, from the viewpoint of improving filterability. The upper limit of the particle size of the aggregate is not particularly limited, but it may be 50 μm or less, 40 μm or less, 30 μm or less, or 20 μm or less.

[0111] As an electrical sensing zone method, a particle size
25 distribution measurement method according to JIS Z 8832 can be mentioned. In particular, a measurement method using a particle size

analyzer CDA-1000 (Sysmex Corporation) can be mentioned.

[0112] The recombinant protein aggregate obtained by the separation can be further purified using the method described in, for example, Japanese Unexamined Patent Publication No. 2013-523665 to improve the purity thereof.

Examples

[0113] Hereinafter, the present invention will be described more specifically with reference to Examples. However, the present invention is not limited to the following Examples.

10 [0114] (1) Construction of target protein-expressing strain (recombinant cell)

GEN495, GEN971, GEN740, GEN797, and GEN796, which are nucleic acids encoding fibroins having spider silk-derived sequences having amino acid sequences represented by SEQ ID NO: 1 (PRT410),
15 SEQ ID NO: 2 (PRT853), SEQ ID NO: 3 (PRT647), SEQ ID NO: 4 (PRT699), and SEQ ID NO: 5 (PRT698), were synthesized, respectively. To each of the nucleic acid, an NdeI site was added at the 5' end and an EcoRI site was added at the downstream of the termination codon. The hydropathy index (HI) and molecular weight of each protein are as
20 shown in Table 1.

[0115] [Table 1]

SEQ ID NO	Protein name	Nucleic acid name	Hydropathy index (HI)	Molecular weight (kDa)
1	PRT410	GEN495	-0.81	53.6
2	PRT853	GEN971	-0.68	218.3
3	PRT647	GEN740	0.04	54.1
4	PRT699	GEN797	0.17	48.8
5	PRT698	GEN796	0.43	48.5

[0116] These five types of nucleic acids were each cloned into a cloning vector (pUC118). Thereafter, the same nucleic acids were each excised with restriction enzymes NdeI and EcoRI and then recombined into a protein expression vector pET-22 b(+) to obtain expression vectors. *Escherichia coli* BLR(DE3) was transformed with each of the five expression vectors to obtain transformed *Escherichia coli* (recombinant cells) expressing the target protein.

[0117] (2) Expression of target protein

The above transformed *Escherichia coli* was cultured in 2 mL of an LB medium containing ampicillin for 15 hours. This culture solution was added to 100 mL of a seed culture medium (Table 2) containing ampicillin so that the OD₆₀₀ was 0.005. The temperature of the culture solution was maintained at 30°C and flask culture was carried out (about 15 hours) until the OD 600 was 5, whereby a seed culture solution was obtained.

[0118] [Table 2]

Seed culture medium

Reagent	Concentration (g/L)
Glucose	5.0
KH ₂ PO ₄	4.0
K ₂ HPO ₄	9.3
Yeast Extract	6.0
Ampicillin	0.1

[0119] The seed culture solution was added to a jar fermenter to which

500 mL of a production medium (Table 3) had been added so that the OD_{600} was 0.05. The culture was carried out with constant control at pH 6.9 while the temperature of the culture solution was maintained at 37°C. Further, the concentration of dissolved oxygen in the culture solution was maintained at 20% of the dissolved oxygen saturation concentration.

[0120] [Table 3]

Production medium	
Reagent	Concentration (g/L)
Glucose	12.0
KH ₂ PO ₄	9.0
MgSO ₄ ·7H ₂ O	2.4
Yeast Extract	15
FeSO ₄ ·7H ₂ O	0.04
MnSO ₄ ·5H ₂ O	0.04
CaCl ₂ ·2H ₂ O	0.04
ADECANOL (Adeka Corporation, LG-295S)	0.1(mL/L)

[0121] Immediately after glucose in the production medium was completely consumed, a feed solution (455 g/1 L glucose, 120 g/1 L yeast extract) was added at a rate of 1 mL/min. The culture was carried out with constant control at pH 6.9 while the temperature of the culture solution was maintained at 37°C. Further, the culture was carried out for 20 hours while the concentration of dissolved oxygen in the culture solution was maintained at 20% of the dissolved oxygen saturation concentration. Thereafter, 1 M isopropyl-β-thiogalactopyranoside (IPTG) was added to the culture solution at a final concentration of 1 mM to induce the expression of the target protein. Twenty hours after IPTG addition, the culture solution was centrifuged to recover the bacterial cells. SDS-PAGE was carried out using the bacterial cells prepared from the culture solution before addition of IPTG and after addition of IPTG, and it was confirmed that the target protein was expressed as insoluble bodies due to the appearance of the band of the target protein size depending on IPTG addition.

[0122] [Example 1: Effect of adding metal salt - part 1]

1.8 $\mu\text{g/g}$ wet bacterial cells of DNase (Sigma-Aldrich Co. LLC) and 164 $\mu\text{g/g}$ wet bacterial cells of Lysozyme (Thermo Fisher Scientific, Inc.) were added to an RO water suspension of *Escherichia coli* BLR(DE3) expressing PRT853 (HI: -0.68) as insoluble bodies, which was then treated four times with a high pressure homogenizer (GEA, PANDA PLUS) at room temperature at a pressure of 600 bar to disrupt the bacterial cells. After disrupting, insoluble bodies were obtained using a centrifuge (TOMY MX-305) at $11,000 \times g$ for 5 minutes. The insoluble bodies were thus relatively small insoluble granules which need to be centrifuged over a considerable amount of time to obtain. After suspending the insoluble granules in water, the metal salts shown in Table 4 were added at a concentration of 0.5 M. Fig. 1 is a photograph of each sample in the case of being centrifuged at $2,680 \times g$ for 10 seconds after addition of metal salts.

[0123] By adding a polyvalent metal salt, it was confirmed that the insoluble bodies could be precipitated by centrifugation at $2,680 \times g$ for 10 seconds.

[0124] [Table 4]

No.	Metal salt
(1)	Not added
(2)	0.5 M sodium chloride
(3)	0.5 M magnesium chloride
(4)	0.5 M potassium chloride
(5)	0.5M calcium chloride
(6)	0.5M lithium chloride
(7)	0.5 M magnesium sulfate
(8)	0.5 M potassium sulfate
(9)	0.5 M sodium nitrate
(10)	0.5 M magnesium nitrate
(11)	0.5 M potassium nitrate
(12)	0.5 M lithium nitrate
(13)	0.5 M sodium acetate
(14)	0.5 M lithium acetate
(15)	0.5 M sodium carbonate
(16)	0.5 M trisodium citrate

[0125] [Example 2: Effect of adding metal salt - part 2]

For the metal salts (magnesium chloride, calcium chloride, magnesium sulfate, and magnesium nitrate) having excellent aggregation effect in Example 1, the aggregation effect at low concentration was confirmed using insoluble bodies of PRT853 (see Table 5). Fig. 2 is a photograph of each sample in the case of being centrifuged at $2,680 \times g$ for 10 seconds after addition of metal salts.

[0126] Aggregation effect was observed with any metal salt at a concentration of 1 mM, but remarkable aggregation effect was observed at a concentration of 5 mM or more.

[0127] [Table 5]

No.	Metal salt
(1)	Not added
(2)	10 mM magnesium chloride
(3)	5 mM magnesium chloride
(4)	1 mM magnesium chloride
(5)	10 mM calcium chloride
(6)	5 mM calcium chloride
(7)	1 mM calcium chloride
(8)	10 mM magnesium sulfate
(9)	5 mM magnesium sulfate
(10)	1 mM magnesium sulfate
(11)	10 mM magnesium nitrate
(12)	5 mM magnesium nitrate
(13)	1 mM magnesium nitrate

[0128] [Example 3: Effect in proteins having different HI]

The effect of adding metal salts in proteins with different hydrophobicity was confirmed. For four insoluble bodies of PRT410 (HI: -0.81), PRT647 (HI: 0.04), PRT699 (HI: 0.17), and PRT698 (HI: 0.43), the aggregation effect by addition of metal salts (calcium chloride and magnesium chloride) was confirmed in the same manner as in Example 1.

[0129] 1.8 $\mu\text{g/g}$ wet bacterial cells of DNase and 164 $\mu\text{g/g}$ wet bacterial cells of Lysozyme were added to an RO water suspension of *Escherichia coli* BLR(DE3) expressing each insoluble body, which was then treated four times with a high pressure homogenizer at room temperature and at a pressure of 600 bar to disrupt the bacterial cells. After disrupting, calcium chloride or magnesium chloride was added to the disrupted suspension at a concentration of 10 to 150 mM, followed by centrifugation at $2,680 \times g$ for 10 seconds to confirm the state of aggregation. Fig. 3 is a photograph of each sample in the case of being centrifuged at $2,680 \times g$ for 10 seconds after addition of metal salts.

[0130] Through this centrifugation operation, the insoluble bodies of PRT410 and PRT699 could be precipitated without adding metal salts (calcium chloride and magnesium chloride), but it was confirmed that even a small amount could precipitate the insoluble bodies more compactly by addition of metal salts. On the other hand, PRT647 and PRT698 could not be precipitated under the present centrifugation conditions unless metal salts were added, but could be aggregated and precipitated by addition of metal salts. Particularly, the higher concentration of metal salts can result in more compact aggregation and precipitation of the insoluble bodies (see Fig. 3).

[0131] Since the addition effect of metal salts was observed in proteins with different hydrophobicity, it is considered that the present metal salt addition method can be applied to aggregation of insoluble bodies of various proteins.

[0132] [Example 4: Improvement of purification purity]

Four insoluble bodies of PRT410, PRT647, PRT699, and PRT698 could be precipitated in 5 minutes in the case of being centrifuged at $11,000 \times g$, 20°C . The precipitate fractions obtained by centrifugation were each suspended again in RO water, and the effect of adding metal salts to the present suspensions (centrifugal resuspensions) was confirmed.

[0133] For the insoluble bodies resuspended in RO water, the effect of adding the metal salts was confirmed in the same manner as in Example 3. Fig. 4 is a photograph of each sample in the case of being centrifuged at $2,680 \times g$ for 10 seconds after addition of metal salts. As shown in Fig. 4, a metal salt addition effect was observed as in

Example 3. In addition, as will be described below, the purity of the insoluble bodies can be improved by this resuspension.

[0134] Fig. 5 and Table 6 show the results of improved purity of insoluble bodies by the metal salt addition and the centrifugal resuspension operation. Fig. 5 is a photograph showing the results (electrophoresis results) of SDS-PAGE analysis of each treatment liquid of PRT410 obtained in Example 3 and Example 4. In A and B of Fig. 5, a suspension (disrupted suspension) of PRT410 immediately after disrupting the bacterial cells with a high pressure homogenizer was applied to lane 1; the precipitate fraction obtained by adding calcium chloride to the disrupted suspension at a concentration of 10 mM, followed by precipitation and centrifugation at $2,500 \times g$ for 5 minutes was applied to lane 2; the precipitate fraction obtained by adding magnesium chloride to the disrupted suspension at a concentration of 10 mM, followed by precipitation and centrifugation at $2,500 \times g$ for 5 minutes was applied to lane 3; and a molecular weight marker protein was applied to lane M.

[0135] A of Fig. 5 is a photograph showing the results of staining with an Oriole (trademark) fluorescent gel stain (manufactured by Bio-Rad Laboratories, Inc.) capable of staining all proteins after the electrophoresis, and B of Fig. 5 is a photograph showing the results of staining with an InVision (trademark) His-tag In-gel Stain (manufactured by Thermo Fisher Scientific, Inc.) reacting to a His tag region of PRT410, after the electrophoresis. PRT410 with a theoretical molecular weight of 53.6 kDa was detected as a band near the molecular weight marker of 60 kDa.

[0136] Using the Gel Doc (trademark) EZ Gel Imager (manufactured by Bio-Rad Laboratories, Inc.), the electrophoretic band of Oriole-stained gel was analyzed to calculate the purified purity of PRT410 in each treatment liquid. The results are shown in Table 6 (immediately after disrupting).

[0137] The purity of the precipitate fraction obtained without adding metal salts was 10.5%, but since more compact aggregation and precipitation could be made by addition of metal salts, the purity could be improved up to 30.7% by adding calcium chloride and 34.1% by adding magnesium chloride (see lanes 1 to 3 in Fig. 5, and Table 6 (immediately after disrupting)).

[0138] Further, in A and B of Fig. 5, a suspension (centrifugal resuspension) obtained by precipitating the disrupted suspension (lane 1) by centrifugation at $11,000 \times g$ at 20°C for 5 minutes, and suspending the resulting precipitate fraction again in RO water was applied to lane 4; the precipitate fraction obtained by adding calcium chloride to the centrifugal resuspension at a concentration of 10 mM, followed by low speed centrifugation at $2,500 \times g$ for 5 minutes was applied to lane 5; and the precipitate fraction obtained by adding magnesium chloride to the centrifugal resuspension at a concentration of 10 mM, followed by low speed centrifugation at $2,500 \times g$ for 5 minutes was applied to lane 6. The purity could be improved from 10.5% to 34.8% (see lane 4 in Fig. 5, and Table 6 (centrifuged and resuspended)) by precipitating the suspension immediately after disrupting the bacterial cells with a high pressure homogenizer by centrifugation at $11,000 \times g$ at 20°C for 5 minutes, and resuspending the resulting precipitate fraction again in RO

water (centrifugal resuspension), but by adding calcium chloride or magnesium chloride to the centrifugal resuspension at a concentration of 10 mM, and aggregating the insoluble bodies, the separation could be made by low speed centrifugation at $2,500 \times g$ for 5 minutes, and the purity of the resulting precipitate fraction was improved to 48.6% and 50.1%, respectively (see lanes 5 and 6 in Fig. 5, and Table 6 (centrifuged and resuspended)). It was thus possible to significantly improve the purity by adding metal salts.

[0139] It was confirmed that this metal salt addition method is not only effective for aggregation of insoluble bodies and is but also an effective means for removal of impurities derived from host cells.

[0140] [Table 6]

	Purity (%)		
	Not added	10 mM calcium chloride	10 mM magnesium chloride
Immediately after disrupting	10.5	30.7	34.1
Centrifuged and resuspended	34.8	48.6	50.1

[0141] In addition, 50 mM metal salt was added to each of the centrifugal resuspensions and disrupted suspensions of four insoluble bodies of PRT410, PRT647, PRT699, and PRT698, followed by aggregation and centrifugation at $2,680 \times g$ for 10 seconds. For the precipitate fraction obtained in each case, the recovery rate of the protein was determined. The results are shown in Table 7.

[0142] The recovery rate was calculated by measuring the absorbance at 595 nm with a microplate reader (TECAN, Infinite F200), assuming that the numerical value of the absorbance before centrifugation is 0% and the numerical value of the absorbance of the supernatant after

treatment of $11,000 \times g$ for 10 minutes is 100 %.

[0143] [Table 7]

Protein name	Recovery rate (%)		
	Not added	50 mM calcium chloride	50 mM magnesium chloride
PRT410	18	98	97
PRT647	0	100	97
PRT699	15	99	98
PRT698	2	97	94

[0144] As is apparent from Table 7, insoluble bodies could be recovered with very high yield by adding metal salts.

5 [0145] It was confirmed that this metal salt addition method is not limited to the type of protein as long as it is an insoluble protein, is a very effective means for aggregation of insoluble bodies and removal of impurities derived from host cells, irrespective of whether the form of insoluble body is a compact insoluble granule or not, and is also an
10 excellent method capable of recovering insoluble bodies with very high yield.

[0146] [Example 5: Effect of adding acid - part 1]

1.8 $\mu\text{g/g}$ wet bacterial cells of DNase (Sigma-Aldrich Co. LLC) and 164 $\mu\text{g/g}$ wet bacterial cells of Lysozyme (Thermo Fisher Scientific, Inc.) were added to an RO water suspension of *Escherichia coli* BLR(DE3) expressing PRT853 (HI: -0.68) as insoluble bodies,
15 which was then treated four times with a high pressure homogenizer (GEA, PANDA PLUS) at room temperature at a pressure of 600 bar to obtain a disrupted suspension of the bacterial cells.

20 [0147] Acetic acid, citric acid, or sulfuric acid was added to the disrupted suspension at a concentration of 10 to 100 mM (for the

relationship between sample number and acid concentration, see Table 8), followed by centrifugation at $2,500 \times g$ for 30 seconds to confirm the state of aggregation of insoluble bodies. Fig. 6 is a photograph of each sample after centrifugation. With no addition of an acid (Sample 1),
5 insoluble bodies could not be precipitated and obtained under these low speed centrifugation conditions. However, with addition of an acid, an aggregate of insoluble bodies could be obtained under these low speed centrifugation conditions, even at any concentration of the acid shown in Table 8 (Samples 2 to 10).

10 [0148] The effect of adding an acid in the insoluble bodies after resuspension in RO water (centrifugal resuspension) was also examined. That is, the disrupted suspension of the bacterial cells was treated with a centrifuge (TOMY MX-305) at $11,000 \times g$ for 5 minutes to obtain insoluble bodies. Such insoluble bodies are thus relatively small
15 insoluble granules which need to be centrifuged at considerable centrifugal force and time in order to obtain without adding an acid. After suspending the insoluble bodies in RO water (centrifugal resuspension), an acid having the concentration shown in Table 8 was added as above, followed by centrifugation at $2,500 \times g$ for 30 seconds
20 to confirm the state of aggregation of insoluble bodies. Fig. 7 is a photograph of each sample after centrifugation. It was confirmed that, even in the centrifugal resuspension resuspended in RO water, aggregation of insoluble bodies can be effectively achieved with an acid.

25 In addition, as a result of resuspension in RO water, the granules are washed and therefore the medium and components derived from the bacterial cells are removed, so that it was possible to obtain pure

insoluble bodies even in the eyes, as compared with the insoluble bodies which were not resuspended in RO water.

[0149] [Table 8]

No.	Acid
1	Not added
2	0.10 M acetic acid
3	0.05 M acetic acid
4	0.01 M acetic acid
5	0.10 M citric acid
6	0.05 M citric acid
7	0.01 M citric acid
8	0.10 M sulfuric acid
9	0.05 M sulfuric acid
10	0.01 M sulfuric acid

[0150] [Example 6: Effect of adding acid in proteins having different HI]

For four insoluble bodies of PRT410 (HI: -0.81), PRT647 (HI: 0.04), PRT699 (HI: 0.17), and PRT698 (HI: 0.43) with different hydrophobicity, in the same manner as in Example 5, 5 to 30 mM citric acid was added and the aggregation effect of the insoluble bodies was confirmed as follows.

[0151] 1.8 $\mu\text{g/g}$ wet bacterial cells of DNase and 164 $\mu\text{g/g}$ wet bacterial cells of Lysozyme were added to the RO water suspension of *Escherichia coli* BLR(DE3) expressing each insoluble body, which was then treated four times with a high pressure homogenizer at room temperature and at a pressure of 600 bar to disrupt the bacterial cells. After disrupting, citric acid was added to the disrupted suspension at a concentration of 5 to 30 mM, followed by centrifugation at $2,500 \times g$ for 30 seconds to confirm the state of aggregation. Fig. 8 is a

photograph of each sample after centrifugation.

[0152] In Fig. 8, 0, 5, 10, 20, and 30 indicate the concentration (mM) of citric acid added (0 mM: citric acid not added). H is a sample obtained by centrifuging a suspension with no addition of citric acid at 11,000 × g for 5 minutes.

[0153] The insoluble bodies of any protein could not be obtained unless the centrifugation conditions were 11,000 × g for 5 minutes, in the case where an acid was not added, but insoluble bodies could be obtained by low speed centrifugation in any protein by adding citric acid of 10 mM or more. Aggregation was also observed with the addition of 5 mM citric acid, but sedimentation was incomplete in a short time of 30 seconds (see Fig. 8).

[0154] Since the acid addition effect was observed in proteins with different hydrophobicity, it is considered that this acid addition method can be applied to aggregation of insoluble bodies of various proteins.

[0155] For each of the four precipitate fractions of PRT410, PRT647, PRT699, and PRT698 recovered by low speed centrifugation, following addition of 10 mM citric acid, the protein recovery rate was determined in the same manner as in Example 4. The results are shown in Table 9.

[0156] [Table 9]

Protein	Recovery rate (%)
PRT410	101
PRT647	103
PRT699	104
PRT698	103

[0157] As is apparent from Table 9, insoluble bodies could be recovered without loss by addition of acid in any protein.

[0158] Next, the purity of the recovered PRT410 insoluble bodies was analyzed by SDS-PAGE. Fig. 9 is a photograph showing the results (electrophoresis results) of SDS-PAGE analysis of each treatment liquid of PRT410. In Fig. 9, a molecular weight marker protein was applied to lane M; the disrupted suspension without addition of an acid was applied to lane 1; and insoluble bodies recovered following addition of 10 mM citric acid to the disrupted suspension were applied to lane 2 so that the protein concentration would be each 1.5 µg. For the staining after electrophoresis, two staining reagents of Oriole™ fluorescent gel stain (manufactured by Bio-Rad Laboratories, Inc.) capable of staining all proteins and InVision™ His-tag In-gel Stain (manufactured by Thermo Fisher Scientific, Inc.) which reacts with the His tag region of PRT410 were used. PRT410 with a theoretical molecular weight of 53.6 kDa was detected as a band near the molecular weight marker of 60 kDa.

[0159] Using the Gel Doc (trademark) EZ Gel Imager (manufactured by Bio-Rad Laboratories, Inc.), the electrophoretic band of Oriole-stained gel was analyzed to calculate the purified purity of PRT410 in each treatment liquid. The results are shown in Table 10.

[0160] [Table 10]

Not added (%)	10 mM citric acid (%)
11.6	23.7

[0161] The purity of the precipitate fraction obtained without adding an acid was 11.6%, but since compact aggregation and precipitation could be made by addition of an acid, the purity could be improved up to 23.7% (see Fig. 9).

[0162] It was confirmed that this acid addition method is not only very effective for aggregation of insoluble bodies, but also an effective means for removal of impurities derived from host cells.

[0163] It was confirmed that this acid addition method is not limited to the type of protein as long as it is an insoluble protein, is a very effective means for aggregation of insoluble bodies and removal of impurities derived from host cells, irrespective of whether the form of insoluble body is a compact insoluble granule or not, and is also an excellent method capable of recovering insoluble bodies with very high yield.

[0164] [Example 7: Effect of removing lipopolysaccharide (LPS)]

Escherichia coli used as a host cell has LPS called a cell wall-derived endotoxin unique to Gram-negative bacteria. In the case where the endotoxin is present in excess, it is known to have effects such as fever, multiple organ failure, and tachycardia. It is thus preferable to reduce the endotoxin. The LPS content in the insoluble bodies aggregated by the metal salt or acid addition of the present invention was measured to confirm the effect of reducing LPS.

[0165] Insoluble bodies (1) not subjected to the metal salt or acid addition were obtained by the following method.

That is, 1.8 µg/g wet bacterial cells of DNase (Sigma-Aldrich Co. LLC) and 164 µg/g wet bacterial cells of Lysozyme (Thermo Fisher Scientific, Inc.) were added to the RO water suspension of *Escherichia coli* BLR(DE3) expressing PRT853, which was then treated four times with a high pressure homogenizer (GEA, PANDA PLUS) at room temperature and at a pressure of 600 bar to disrupt the bacterial cells.

Disruption was followed by treatment with a centrifuge (TOMY MX-305) at $11,000 \times g$ for 20 minutes. The precipitate fraction was suspended again in RO water and treated at $11,000 \times g$ for 30 minutes. This washing operation was carried out twice. The resulting precipitate fraction was suspended again in RO water and treated at $11,000 \times g$ for 60 minutes to obtain insoluble bodies (1) as a precipitate fraction. The insoluble bodies (1) were obtained at 20°C.

[0166] The insoluble bodies (2) aggregated by addition of a metal salt were obtained by the following method.

That is, the treatment was carried out four times with a high pressure homogenizer, and until the bacterial cells were disrupted, the same procedure as above was carried out. After disrupting, 10 mM calcium chloride was added to aggregate the insoluble bodies which were then treated at $2,500 \times g$ for 10 minutes. The resulting precipitate fraction was suspended again in RO water and treated at $2,500 \times g$ for 10 minutes. This washing operation was carried out twice to obtain insoluble bodies (2) by addition of a metal salt as a precipitate fraction.

[0167] The insoluble bodies (3) aggregated by adding an acid were obtained by the following method.

That is, insoluble bodies (3) by addition of an acid were obtained in the same manner as in obtaining the insoluble bodies (2) with addition of a metal salt, except that 10 mM citric acid was added instead of adding a metal salt.

[0168] The LPS content in these three types of insoluble bodies was measured by the following method.

(A) Preparation of measuring sample

About 75 mg (insoluble bodies (1): 75.5 mg, insoluble bodies (2): 75.1 mg, and insoluble bodies (3): 75.0 mg) of each of the above three types of insoluble body samples was weighed and distilled water for injection (Otsuka Pharmaceutical Factory, Inc.) was added thereto at a concentration of 50 mg/mL to prepare a suspension. After stirring with a vortex mixer, the pH was checked, and a 5 N sodium hydroxide aqueous solution (Wako Pure Chemical Industries, Ltd.) was added to adjust to neutrality. The insoluble body sample was heat-treated at 90°C for 20 minutes using a block heater. After heat release, centrifugation was carried out at 10,000 rpm for 10 minutes, and the supernatant was recovered and used as a measurement stock solution.

[0169] (A) Measurement of LPS content

Using a Limulus ES-il single test Wako (Wako Pure Chemical Industries, Ltd.) according to the attached explanatory material, a turbidimetric time analysis using a toxinometer (ET-6000/J, Wako Pure Chemical Industries, Ltd.) was carried out. For the measurement, CSE (*E. coli* UKT-B) attached to the kit was used as a standard endotoxin. For each specimen, firstly, the measurement stock solution was diluted 1,000-fold and measured. The measured values were obtained by 1,000-fold dilution for the insoluble bodies (1) and the insoluble bodies (2), but since the insoluble bodies (3) were below the detection limit (<0.01 EU/mL), the measured value could be obtained at 10-fold dilution by changing the dilution ratio. The results are shown in Table 11.

[0170] [Table 11]

Sample	LPS content (μg/g)
Insoluble bodies (1)	3.10
Insoluble bodies (2)	1.19
Insoluble bodies (3)	0.008

[0171] It was confirmed that the LPS content was reduced in the insoluble bodies (2) and (3) aggregated by adding a metal salt or an acid, as compared with the insoluble bodies (1) obtained without adding a metal salt or an acid. In particular, the LPS content was very low in the insoluble bodies (3) obtained by aggregation with an acid. The method of aggregating and obtaining insoluble bodies by adding a metal salt or an acid was an excellent method capable of also reducing the LPS content.

[0172] [Example 8: Aggregation effect by anionic flocculant]

1.8 μg/g wet bacterial cells of DNase and 164 μg/g wet bacterial cells of Lysozyme were added to an RO water suspension of *Escherichia coli* BLR(DE3) expressing PRT853 (HI: -0.68) as insoluble granules, which was then treated four times with a high pressure homogenizer at room temperature at a pressure of 600 bar to disrupt the bacterial cells to obtain a disrupted suspension. After disrupting, the flocculant shown in Table 12 was added to the disrupted suspension at a concentration of 0.05%, and the sedimentation state of the insoluble granules was confirmed by standing still or centrifugation (2,680 × g for 10 seconds). The results are shown in Fig. 10.

Only an anionic polyacrylate-based flocculant (KURIFARM PA-896) was able to effectively precipitate the insoluble granules in both static and centrifugal separations.

[0173] [Table 12]

No.	Flocculant
1	KURIFARM PA-896 (anionic flocculant)
2	KURIFARM PN-901 (nonionic flocculant)
3	KURIFARM PC-601 (cationic flocculant)
4	KURIFARM PC-668 (cationic flocculant)
5	KURIFARM PC-696 (cationic flocculant)
6	KURIFARM PC-702 (cationic flocculant)
7	KURIFARM PC-797 (cationic flocculant)
8	KURIFUTURE PF-512 (amphoteric flocculant)
9	KURIFUTURE PF-833 (amphoteric flocculant)
10	PRESS AID111 (cationic flocculant)

[0174] [Example 9: Effect of improving purity by anionic flocculant]

The effect of adding the anionic flocculant KURIFARM PA-896, whose aggregation effect was confirmed in Example 8, to the disrupted suspension and the centrifugal resuspension of the bacterial cells was confirmed. This centrifugal resuspension is a suspension in which the disrupted suspension is centrifuged at $11,000 \times g$ at 20°C for 5 minutes and the resulting precipitate fraction is resuspended again in RO water. A disrupted suspension of the bacterial cells was obtained in the same manner as in Example 8, except that *Escherichia coli* BLR(DE3) expressing PRT410 (HI: -0.81) as insoluble granules was used.

[0175] After addition of the flocculant, centrifugation was carried out at $2,500 \times g$ for 5 minutes, and the purity of the insoluble bodies in the resulting precipitate fraction was analyzed by SDS-PAGE. The results are shown in Fig. 11. In Fig. 11, a suspension (disrupted suspension) of PRT410 immediately after disrupting the bacterial cells with a high pressure homogenizer was applied to lane 1; the precipitate fraction obtained by adding KURIFARM PA-896 to the disrupted suspension at

a concentration of 0.01%, followed by precipitation and centrifugation at $2,500 \times g$ for 5 minutes was applied to lane 2; a suspension (centrifugal resuspension) obtained by precipitating the disrupted suspension (lane 1) by centrifugation at $11,000 \times g$ at 20°C for 5 minutes, and suspending the resulting precipitate fraction again in RO water was applied to lane 3; and the precipitate fraction obtained by adding KURIFARM PA-896 to the centrifugal resuspension at a concentration of 0.01%, followed by low speed centrifugation at $2,500 \times g$ for 5 minutes was applied to lane 4. For the staining after electrophoresis, two staining reagents of Oriole™ fluorescent gel stain (manufactured by Bio-Rad Laboratories, Inc.) capable of staining all proteins and InVision™ His-tag In-gel Stain (manufactured by Thermo Fisher Scientific, Inc.) which reacts with the His tag region of PRT410 were used. PRT410 with a theoretical molecular weight of 53.6 kDa was detected as a band near the molecular weight marker of 60 kDa.

[0176] In the case of adding KURIFARM PA-896 to the disrupted suspension at a concentration of 0.01% and aggregating the insoluble bodies, the separation could be made by low speed centrifugation at $2,500 \times g$ for 5 minutes, and the purity of the resulting precipitate fraction was improved from 12.2% to 53.5% (see lanes 1 and 2 in Fig. 11, and Table 13 (immediately after disrupting)). The purity of the precipitate fraction could be improved from 12.2% to 36.3% (see lanes 1 and 3 in Figure 11, and Table 13 (centrifuged and resuspended)) by precipitating the suspension immediately after disrupting bacterial cells with a high pressure homogenizer by centrifugation at $11,000 \times g$ at 20°C for 5 minutes, and resuspending the resulting precipitate fraction

again in RO water (centrifugal resuspension), but by adding KURIFARM PA-896 to the centrifugal resuspension at a concentration of 0.01% and aggregating the insoluble bodies, the separation could be made by low speed centrifugation at $2,500 \times g$ for 5 minutes, and the
5 purity of the resulting precipitate fraction was improved again from 36.3% to 51.8% (see lanes 3 and 4 in Fig. 11, and Table 13 (centrifuged and resuspended)). It was thus possible to significantly improve the purity by adding an anionic flocculant.

[0177] [Table 13]

	Purity (%)	
	Not added	Flocculant added
Immediately after disrupting	12.2	53.5
Centrifuged and resuspended	36.3	51.8

[0178] [Example 10: Effect by acid addition, heating, and stirring]

The aggregation effect by heating was confirmed using the insoluble bodies of PRT410. 1.8 µg/g wet bacterial cells of DNase and 164 µg/g wet bacterial cells of Lysozyme were added to the RO water suspension of *Escherichia coli* BLR(DE3) expressing PRT410, which was then treated four times with a high pressure homogenizer at room temperature at a pressure of 600 bar to disrupt bacterial cells. Disruption was followed by centrifugation at $2,500 \times g$ for 10 minutes, and the supernatant was discarded to adjust to 2.5-fold concentration. Then, the concentrate was 2.5-fold diluted with RO water. Citric acid was added to the disrupted suspension at a concentration of 20 mM, and then heating and stirring were carried out as needed to obtain an aggregate. Each sample was treated under the conditions described in Table 14. The heating was carried out using a hot bath and the heating time was the keeping time from the time in the case where the hot bath reached 80°C. The stirring was carried out at 200 rpm. With respect to the resulting aggregate, the particle concentration and the median diameter were measured using a particle size analyzer CDA-1000 (Sysmex Corporation). The results are shown in Table 14. Fig. 12 shows the frequency distribution and cumulative distribution of the median diameters.

[0179] Sample X that was made without adding an acid and without stirring, Sample 1 that was made with only heating and without adding an acid, and Sample 2 that was made without adding an acid and with heating and stirring exhibited almost no aggregation effect. On the other hand, Sample 3 that was made with only adding an acid and without heating/stirring exhibited an increase in particle size, as compared with Samples X, 1 and 2. Further, in Sample 7 heated at 80°C for 2 hours, an increase in particle size was observed as compared with Sample 3. Samples 4 to 6, which had different heating times and were further stirred, were found to have an effect of increasing the particle size more by stirring. The aggregation depends on the treatment time, but the effect was observed from at least 0.5 hour. This trend is proved by Fig. 12. Since the filterability improves as the peak of the particle size distribution becomes sharper, it was confirmed from Fig. 12 that addition of an acid, heating, and stirring improve the filterability.

[0180] [Table 14]

No.	pH	Stirring	Treatment temperature (°C)	Treatment time (h)	Particle concentration (particles/ml)	Median diameter (μm)		
						d10%	d50%	d90%
X	Not adjusted (6.0)	Not stirred	22	-	4.68×10^{10}	0.90	2.72	5.08
1	Not adjusted (6.0)	Not stirred	80	2	2.57×10^{10}	1.37	3.23	6.40
2	Not adjusted (6.0)	Stirred	80	2	8.04×10^9	1.381	5.679	7.505
3	3.75	Not stirred	22	-	4.62×10^9	2.359	3.955	7.472
4	3.75	Stirred	80	0.5	7.09×10^{10}	4.37	7.51	9.90
5	3.75	Stirred	80	1	4.44×10^8	4.75	8.62	12.28
6	3.75	Stirred	80	2	2.09×10^8	7.67	10.39	12.37
7	3.75	Not stirred	80	2	9.98×10^8	3.84	5.62	8.57

[0181] [Example 11: Effect of improving filterability by type of acid]

The effect of improving filterability by adding an acid was confirmed using the insoluble bodies of PRT410. Since it was confirmed that filtration can be carried out by adding an acid, the effect of improving filterability depending on the type of acid was compared for three types of acids: citric acid, hydrochloric acid, and sulfuric acid. Experimental method was the same as in Example 10, except that the acid is different. With respect to acid treatment, Tables 15 and 16 show examples of the results concerning the relationship between the maximum filtration amount and the filtration time and the permeation flux after heating and stirring at 80°C for 2 hours. As a result of the comparison, it was confirmed that citric acid was industrially superior because citric acid exhibited the largest maximum filtration amount and a stable permeation flux.

[0182] [Table 15]

	Filtration area (cm ²)	Maximum filtration amount (m ³ /m ²)
Not added	45.3	Non-filterable
Citric acid	45.3	0.276
Hydrochloric acid	45.3	0.100
Sulfuric acid	45.3	0.092

[0183] [Table 16]

pH	Heating temperature (°C)	Heating time (h)	Particle concentration (particles/ml)	Median diameter (μm)		
				d10%	d50%	d90%
3.75 (Citric acid)	80	2	1.34×10^8	7.928	9.980	12.365
3.75 (Hydrochloric acid)	80	2	1.88×10^8	7.374	9.641	12.755
3.75 (Sulfuric acid)	80	2	2.49×10^8	7.283	9.430	11.835

[0184] [Example 12: Effect of improving protein purity by heating]

Purity of insoluble bodies of heated PRT799 (SEQ ID NO: 11, 200 kDa) and PRT587 (SEQ ID NO: 12, 100 kDa) was analyzed by SDS-PAGE. Figs. 13 and 14 are photographs showing the results (electrophoresis results) of SDS-PAGE analysis of each treatment liquid of PRT799 and PRT587. Citric acid was added to each treatment liquid, and the pH was adjusted to 3.75. In Fig. 13, a disrupted suspension heated at 80°C for 3 hours was applied to lane 1; and a disrupted suspension not heated was applied to lane 2. It was confirmed in the disrupted suspension of lane 1 that contaminating proteins were decomposed by heating. In Fig. 14, a disrupted suspension not heated was applied to lane 1; and a disrupted suspension heated at 80°C for 2 hours was applied to lane 2. It was confirmed that

the band near the molecular marker of 40 kDa was decomposed, and improvement in the purity of the target protein by heating was confirmed since the detected intensity of the band (target protein) detected near the 100 kDa molecular marker in lane 2 was 1.2-fold higher as compared with the band of lane 1 not heated.

[0185] [Example 13: Effect by continuous heating]

The aggregation effect by continuous heating was confirmed using the insoluble bodies of PRT799. 1.8 µg/g wet bacterial cells of DNase and 164 µg/g wet bacterial cells of Lysozyme were added to the RO water suspension of *Escherichia coli* BLR(DE3) expressing PRT799, which was then treated four times with a high pressure homogenizer at room temperature at a pressure of 600 bar to disrupt bacterial cells. Disruption was followed by centrifugation with a centrifuge (TOMY MX-305) at $2,500 \times g$ for 10 minutes, and the supernatant was discarded to adjust to 2.5-fold concentration. Then, the concentrate was 2.5-fold diluted with RO water. Citric acid was added to the disrupted suspension at a concentration of 20 mM, and then heating was carried out to obtain an aggregate. Each sample was treated under the conditions described in Table 17. The heating was carried out using a continuous liquid sterilizer MINI UHT T-20 (manufactured by Powerpoint International Ltd.). The heating temperature was 80°C, 85°C, 90°C, or 95°C, and the heating time of the disrupted suspension was 30 seconds or 60 seconds. With respect to the resulting aggregate, the particle concentration and the median diameter were measured using a particle size analyzer CDA-1000 (Sysmex Corporation). The results are shown in Table 17. Figs. 14

and 15 show the frequency distribution and cumulative distribution of the median diameters.

[0186] [Table 17]

No.	pH	Treatment temperature (°C)	Treatment time	Particle concentration (particles/ml)	Median diameter (µm)		
					d10%	d50%	d90%
C	Before heating (3.75)	25	Not heated	1.52×10^{10}	0.959	2.940	4.861
X	3.75	80	2 hours	5.08×10^8	4.001	6.412	8.685
1	3.75	80	30 seconds	1.32×10^9	3.987	7.824	10.275
2	3.75	80	60 seconds	1.09×10^9	3.951	8.163	11.415
3	3.75	85	30 seconds	6.48×10^8	5.166	8.778	11.415
4	3.75	85	60 seconds	4.19×10^8	4.865	9.070	12.525
5	3.75	90	30 seconds	1.44×10^8	6.773	11.045	13.825
6	3.75	90	60 seconds	2.51×10^8	5.434	10.845	13.810
7	3.75	95	30 seconds	8.27×10^7	5.664	11.825	14.510
8	3.75	95	60 seconds	1.02×10^8	3.035	11.535	14.475

[0187] From the results in Table 17, in the case where the aggregate of Sample X obtained by heating using a hot bath at a heating temperature of 80°C for a heating time of 2 hours was compared with the aggregate of Sample 1, 2, 3, 4, 5, 6, 7, or 8 obtained using a continuous liquid sterilizer MINI UHT T-20 (manufactured by Powerpoint International Ltd.) at a heating temperature of 80°C, 85°C, 90°C, or 95°C for a heating time of 30 seconds or 60 seconds, the aggregate obtained using a continuous liquid sterilizer MINI UHT T-20 (manufactured by Powerpoint International Ltd.) had a particle size equal to or larger than that of the aggregate obtained by heating in a hot bath.

[0188] It was confirmed from Figs. 15 and 16 that the aggregate could be efficiently enlarged by heating the disrupted suspension at a high temperature for a short period of time.

[0189] [Example 14: Effect of improving filterability by continuous heating]

The results of filtration area and maximum filtration amount of Samples X, 5, 6, 7, and 8 are shown in Table 18. According to Table 18, the filterability in the case of heating at a high temperature for a short period of time by using a continuous liquid sterilizer was about the same or about the same as or higher than that obtained by heating using a hot bath for 2 hours, and it was confirmed that filterability was improved in the case where the disrupted suspension was heated at a high temperature for a short period of time.

[0190] [Table 18]

No.	pH	Treatment temperature (°C)	Treatment time	Filtration area (cm ²)	Maximum filtration amount (m ³ /m ²)
X	3.75	80	2 hours	45.3	0.078
5	3.75	90	30 seconds	45.3	0.099
6	3.75	90	60 seconds	45.3	0.101
7	3.75	95	30 seconds	45.3	0.103
8	3.75	95	60 seconds	45.3	0.099

CLAIMS

1. A method for producing a recombinant protein aggregate comprising the following steps (A) to (C):

a step (A) of disrupting a recombinant cell expressing a target recombinant protein as insoluble bodies in the cell to obtain a disrupted suspension containing the insoluble bodies of the recombinant protein;

a step (B) of adding one or more selected from the group consisting of a metal salt, an acid, and an anionic flocculant to the disrupted suspension obtained in the step (A), and aggregating the insoluble bodies of the recombinant protein to obtain the recombinant protein aggregate; and

a step (C) of separating the aggregate obtained in the step (B) from the suspension, wherein the recombinant protein is not substantially dissolved in the disrupted suspension.

2. The method for producing a recombinant protein aggregate according to claim 1, further comprising:

separating the recombinant protein aggregate by a centrifugal force of $10,000 \times g$ or less.

3. The method for producing a recombinant protein aggregate according to claim 1, further comprising:

separating the recombinant protein aggregate by using a centrifuge selected from the group consisting of a separation plate type centrifuge, a basket type centrifuge, and a decanter type centrifuge.

4. The method for producing a recombinant protein aggregate according to claim 1, further comprising:

separating the recombinant protein aggregate by spontaneous

sedimentation or filtration.

5. The method for producing a recombinant protein aggregate according to claim 1, further comprising:

heating in the step (B).

6. The method for producing a recombinant protein aggregate according to claim 5, further comprising:

stirring in the step (B).

7. The method for producing a recombinant protein aggregate according to claim 1, wherein the metal salt is a metal salt selected from the group consisting of an alkaline earth metal salt and an earth metal salt.

8. The method for producing a recombinant protein aggregate according to claim 7, wherein the metal salt is a metal salt selected from the group consisting of an alkaline earth metal halide, an alkaline earth metal nitrate, an alkaline earth metal sulfate, an earth metal halide, an earth metal nitrate, and an earth metal sulfate.

9. The method for producing a recombinant protein aggregate according to claim 1, wherein the acid is an oxo acid.

10. The method for producing a recombinant protein aggregate according to claim 9, wherein the oxo acid is an oxo acid selected from the group consisting of acetic acid, sulfuric acid, and citric acid.

11. The method for producing a recombinant protein aggregate according to claim 1, wherein the anionic flocculant is an anionic flocculant selected from the group consisting of a polyacrylate, an anionic polyacrylamide, and an acrylamide-acrylate copolymer.

12. The method for producing a recombinant protein aggregate according to claim 1, wherein the disruption of the recombinant cell is mechanical disruption.

13. The method for producing a recombinant protein aggregate according to claim 1, wherein the separation of the recombinant protein aggregate is carried out by filtration.

14. The method for producing a recombinant protein aggregate according to claim 1, wherein the recombinant cell is a recombinant cell transformed with a host selected from the group consisting of a bacterium, a yeast, a filamentous fungus, an insect cell, a plant cell, and an animal cell.

15. The method for producing a recombinant protein aggregate according to claim 1, wherein the recombinant protein is a structural protein.

16. The method for producing a recombinant protein aggregate according to claim 15, wherein the structural protein is a protein derived from a protein selected from the group consisting of keratin, collagen, elastin, resilin, silkworm silk, and spider silk.

17. The method for producing a recombinant protein aggregate according to claim 1, wherein the resulting recombinant protein aggregate has a particle size of 4 μm to 50 μm as measured by an electrical sensing zone method.

SEQUENCE LISTING

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KOJIMA INDUSTRIES CORPORATION

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Gln	Gly	Pro	Gly	Gln	Gln	Gly	Pro	Tyr	Gly	Pro	Gly	Ala	Ser	Gly
1625						1630					1635			
Pro	Gly	Gln	Gln	Gly	Pro	Tyr	Gly	Pro	Gly	Ala	Ser	Ala	Ala	Ala
1640						1645					1650			
Ala	Ala	Ala	Gly	Pro	Gly	Gln	Tyr	Gly	Pro	Gly	Gln	Gln	Gly	Pro
1655						1660					1665			
Ser	Ala	Ser	Ala	Ala	Ala	Ala	Ala	Ala	Gly	Gln	Tyr	Gly	Ser	Gly
1670						1675					1680			
Pro	Gly	Gln	Tyr	Gly	Pro	Tyr	Gly	Pro	Gly	Gln	Ser	Gly	Pro	Gly
1685						1690					1695			

Ser Gly Gln Gln Gly Gln Gly Pro Tyr Gly Pro Gly Ala Ser Ala
 1700 1705 1710
 Ala Ala Ala Ala Ala Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro
 1715 1720 1725
 Tyr Gly Pro Gly Gln Ser Ala Ala Ala Ala Ala Ala Gly Pro Gly
 1730 1735 1740
 Ser Gly Gln Tyr Gly Pro Gly Ala Ser Gly Gln Asn Gly Pro Gly
 1745 1750 1755
 Ser Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro Gly Gln Ser Ala
 1760 1765 1770
 Ala Ala Ala Ala Ala Gly Gln Tyr Gln Gln Gly Pro Gly Gln Gln
 1775 1780 1785
 Gly Pro Tyr Gly Pro Gly Ala Ser Ala Ala Ala Ala Ala Ala Gly
 1790 1795 1800
 Gln Tyr Gly Ser Gly Pro Gly Gln Gln Gly Pro Tyr Gly Pro Gly
 1805 1810 1815
 Gln Ser Gly Ser Gly Gln Gln Gly Pro Gly Gln Gln Gly Pro Tyr
 1820 1825 1830
 Ala Ser Ala Ala Ala Ala Ala Ala Gly Pro Gly Ser Gly Gln Gln
 1835 1840 1845
 Gly Pro Gly Ala Ser Ala Ala Ala Ala Ala Ala Ala Lys Leu Gly
 1850 1855 1860
 Gln Gln Gly Pro Tyr Gly Pro Gly Ala Ser Ala Ala Ala Ala Ala
 1865 1870 1875
 Ala Gly Gln Asn Gly Pro Gly Ser Gly Gln Gln Gly Pro Gly Gln
 1880 1885 1890
 Ser Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln Gly

1895	1900	1905
Pro Gly 1910	Ser Ser Ala Ala 1915	Ala Ala Ala Gly Pro Gly Gln Tyr 1920
Gly Pro 1925	Gly Gln Gln Gly Pro 1930	Ser Ala Ser Ala Ala Ala Ala 1935
Ala Gly 1940	Pro Gly Ser Gly Gln 1945	Gln Gly Pro Gly Ala Ser Gly Gln 1950
Tyr Gly 1955	Pro Gly Gln Gln Gly 1960	Pro Gly Gln Gln Gly Pro Gly Ser 1965
Ser Ala 1970	Ala Ala Ala Ala Ala 1975	Ala Gly Gln Tyr Gly Ser Gly Pro Gly 1980
Gln Gln 1985	Gly Pro Tyr Gly Ser 1990	Ala Ala Ala Ala Ala Ala Gly Pro 1995
Gly Ser 2000	Gly Gln Tyr Gly Gln 2005	Gly Pro Tyr Gly Pro Gly Ala Ser 2010
Gly Pro 2015	Gly Gln Tyr Gly Pro 2020	Gly Gln Gln Gly Pro Ser Ala Ser 2025
Ala Ala 2030	Ala Ala Ala Ala Gly 2035	Ser Gly Gln Gln Gly Pro Gly Gln 2040
Tyr Gly 2045	Pro Tyr Ala Ser Ala 2050	Ala Ala Ala Ala Ala Gly Gln Tyr 2055
Gly Ser 2060	Gly Pro Gly Gln Gln 2065	Gly Pro Tyr Gly Pro Gly Gln Ser 2070
Gly Ser 2075	Gly Gln Gln Gly Pro 2080	Gly Gln Gln Gly Pro Tyr Ala Ser 2085
Ala Ala 2090	Ala Ala Ala Ala Gly 2095	Pro Gly Gln Gln Gly Pro Tyr Gly 2100

Pro	Gly	Ser	Ser	Ala	Ala	Ala	Ala	Ala	Ala	Gly	Gln	Tyr	Gly	Tyr
	2105					2110					2115			
Gly	Pro	Gly	Gln	Gln	Gly	Pro	Tyr	Gly	Pro	Gly	Ala	Ser	Gly	Gln
	2120					2125					2130			
Asn	Gly	Pro	Gly	Ser	Gly	Gln	Tyr	Gly	Pro	Gly	Gln	Gln	Gly	Pro
	2135					2140					2145			
Gly	Gln	Ser	Ala	Ala	Ala	Ala	Ala	Ala	Gly	Pro	Gly	Gln	Gln	Gly
	2150					2155					2160			
Pro	Tyr	Gly	Pro	Gly	Ala	Ser	Ala	Ala	Ala	Ala	Ala	Ala	Gly	Gln
	2165					2170					2175			
Tyr	Gly	Pro	Gly	Gln	Gln	Gly	Pro	Gly	Gln	Tyr	Gly	Pro	Gly	Ser
	2180					2185					2190			
Ser	Gly	Pro	Gly	Gln	Gln	Gly	Pro	Tyr	Gly	Pro	Gly	Ser	Ser	Ala
	2195					2200					2205			
Ala	Ala	Ala	Ala	Ala	Gly	Gln	Tyr	Gly	Pro	Gly	Gln	Gln	Gly	Pro
	2210					2215					2220			
Tyr	Gly	Pro	Gly	Gln	Ser	Ala	Ala	Ala	Ala	Ala	Ala	Gly	Gln	Tyr
	2225					2230					2235			
Gln	Gln	Gly	Pro	Gly	Gln	Gln	Gly	Pro	Tyr	Gly	Pro	Gly	Ala	Ser
	2240					2245					2250			
Gly	Pro	Gly	Gln	Gln	Gly	Pro	Tyr	Gly	Pro	Gly	Ala	Ser	Ala	Ala
	2255					2260					2265			
Ala	Ala	Ala	Ala	Gly	Pro	Gly	Gln	Tyr	Gly	Pro	Gly	Gln	Gln	Gly
	2270					2275					2280			
Pro	Ser	Ala	Ser	Ala	Ala	Ala	Ala	Ala	Ala	Gly	Gln	Tyr	Gly	Ser
	2285					2290					2295			

Gly Pro Gly Gln Tyr Gly Pro Tyr Gly Pro Gly Gln Ser Gly Pro
2300 2305 2310

Gly Ser Gly Gln Gln Gly Gln Gly Pro Tyr Gly Pro Gly Ala Ser
2315 2320 2325

Ala Ala Ala Ala Ala Ala Gly Gln Tyr Gly Pro Gly Gln Gln Gly
2330 2335 2340

Pro Tyr Gly Pro Gly Gln Ser Ala Ala Ala Ala Ala Ala Gly Pro
2345 2350 2355

Gly Ser Gly Gln Tyr Gly Pro Gly Ala Ser Gly Gln Asn Gly Pro
2360 2365 2370

Gly Ser Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro Gly Gln Ser
2375 2380 2385

Ala Ala Ala Ala Ala Ala Gly Gln Tyr Gln Gln Gly Pro Gly Gln
2390 2395 2400

Gln Gly Pro Tyr Gly Pro Gly Ala Ser Ala Ala Ala Ala Ala Ala
2405 2410 2415

Gly Gln Tyr Gly Ser Gly Pro Gly Gln Gln Gly Pro Tyr Gly Pro
2420 2425 2430

Gly Gln Ser Gly Ser Gly Gln Gln Gly Pro Gly Gln Gln Gly Pro
2435 2440 2445

Tyr Ala Ser Ala Ala Ala Ala Ala Ala Ala Gly Pro Gly Ser Gly Gln
2450 2455 2460

Gln Gly Pro Gly Ala Ser Ala Ala Ala Ala Ala Ala Ala
2465 2470 2475

<210> 3
<211> 595
<212> PRT
<213> Artificial Sequence

<220>

<223> PRT647

<400> 3

Met His His His His His His Ser Ser Gly Ser Ser Thr Thr Met Asn
1 5 10 15

Trp Ser Thr Arg Leu Val Leu Ser Ile Leu Val Val Leu Cys Thr Gln
20 25 30

Ser Leu Cys Ala Leu Gly Gln Ala Asn Thr Pro Trp Ser Ser Lys Glu
35 40 45

Asn Ala Asp Ala Phe Ile Gly Ala Phe Met Asn Ala Ala Ser Gln Ser
50 55 60

Gly Ala Phe Ser Ser Asp Gln Ile Asp Asp Met Ser Val Ile Ser Asn
65 70 75 80

Thr Leu Met Ala Ala Met Asp Asn Met Gly Gly Arg Ile Thr Gln Ser
85 90 95

Lys Leu Gln Ala Leu Asp Met Ala Phe Ala Ser Ser Val Ala Glu Ile
100 105 110

Ala Val Ala Asp Gly Gln Asn Val Gly Ala Ala Thr Asn Ala Ile Ser
115 120 125

Asp Ala Leu Arg Ser Ala Phe Tyr Gln Thr Thr Gly Val Val Asn Asn
130 135 140

Gln Phe Ile Thr Gly Ile Ser Ser Leu Ile Gly Met Phe Ala Gln Val
145 150 155 160

Ser Gly Asn Glu Val Ser Tyr Ser Ser Ala Gly Ser Ser Ser Ala Ala
165 170 175

Ala Ser Glu Ala Val Ser Ala Gly Gln Gly Pro Ala Ala Gln Pro Val
180 185 190

Tyr Ala Pro Ser Ala Ser Ala Ala Ala Ala Ala Ala Ser Gly Ala Ala
195 200 205

Pro Ala Ile Gln Gln Ala Tyr Glu Arg Gly Gly Ser Gly Ser Ala Ala
210 215 220

Ala Ala Ala Gly Ser Gly Pro Ser Gly Tyr Gly Gln Gly Ala Gly Gly
225 230 235 240

Pro Gly Gly Ala Gly Ala Ala Ala Gly Ala Ala Ala Ala Gly Gly Ser
245 250 255

Gly Pro Gly Gly Tyr Gly Gln Gly Pro Ala Ala Tyr Gly Pro Ser Gly
260 265 270

Pro Ser Gly Gln Gln Gly Tyr Gly Pro Gly Gly Ser Gly Ala Ala Ala
275 280 285

Ala Ala Ala Ala Ala Ala Gly Ser Gly Pro Ser Gly Tyr Gly Pro Gly
290 295 300

Ala Gly Gly Pro Gly Gly Ala Gly Ala Ala Ala Ala Ala Ala Ala Ala
305 310 315 320

Gly Gly Ser Gly Pro Gly Gly Tyr Gly Gln Gly Gln Ala Ser Tyr Gly
325 330 335

Pro Ser Gly Pro Ser Gly Gln Gln Gly Tyr Gly Pro Gly Gly Ser Gly
340 345 350

Ala Ala Ala Ala Ala Ala Ala Ala Ala Gly Gly Pro Gly Phe Gly Gly
355 360 365

Gln Gln Gly Tyr Gly Pro Gly Gly Ser Gly Ala Ala Ala Ala Ala Ala
370 375 380

Ala Gly Gly Ala Gly Pro Gly Arg Gln Gln Ala Tyr Gly Pro Gly Gly
385 390 395 400

Ser Gly Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Gly Ser Gly Pro Ser
405 410 415

Gly Tyr Gly Pro Ser Ala Ala Gly Pro Ser Gly Pro Gly Gly Ser Gly
420 425 430

Ala Ala Gly Gly Ser Gly Pro Gly Gly Phe Gly Gln Gly Pro Ala Gly
435 440 445

Tyr Gly Pro Ser Gly Pro Gly Gly Gln Gln Gly Tyr Gly Pro Gly Ala
450 455 460

Ser Gly Ala Ala Ala Ala Ala Ala Ala Ala Ser Gly Ser Gly Gly Tyr Gly
465 470 475 480

Pro Ser Gln Tyr Val Pro Ser Ser Val Ala Ser Ser Ala Ala Ser Ala
485 490 495

Ala Ser Ala Leu Ser Ser Pro Thr Thr His Ala Arg Ile Ser Ser His
500 505 510

Ala Ser Thr Leu Leu Ser Ser Gly Pro Thr Asn Ala Ala Ala Leu Ser
515 520 525

Asn Val Ile Ser Asn Ala Val Ser Gln Val Ser Ala Ser Asn Pro Gly
530 535 540

Ser Ser Ser Cys Asp Val Leu Val Gln Ala Leu Leu Glu Ile Ile Thr
545 550 555 560

Ala Leu Ile Ser Ile Leu Asp Ser Ser Ser Val Gly Gln Val Asn Tyr
565 570 575

Gly Ser Ser Gly Gln Tyr Ala Gln Ile Val Gly Gln Ser Met Gln Gln
580 585 590

Ala Met Gly
595

<210> 4

<211> 576
<212> PRT
<213> Artificial Sequence

<220>
<223> PRT699

<400> 4

Met His His His His His His Ser Ser Gly Ser Ser Gly Pro Gly Val
1 5 10 15

Leu Gly Pro Tyr Gly Pro Gly Ala Ser Ala Ala Ala Ala Ala Ala Ala
20 25 30

Gly Ser Asn Gly Pro Gly Ser Gly Val Leu Gly Pro Gly Gln Ser Gly
35 40 45

Gln Tyr Gly Pro Gly Val Leu Gly Pro Gly Val Leu Gly Pro Gly Ser
50 55 60

Ser Ala Ala Ala Ala Ala Ala Ala Gly Pro Gly Gln Tyr Gly Pro Gly
65 70 75 80

Val Leu Gly Pro Ser Ala Ser Ala Ala Ala Ala Ala Ala Gly Pro
85 90 95

Gly Ser Gly Val Leu Gly Pro Gly Ala Ser Gly Gln Tyr Gly Pro Gly
100 105 110

Val Leu Gly Pro Gly Val Leu Gly Pro Gly Ser Ser Ala Ala Ala Ala
115 120 125

Ala Ala Ala Gly Ser Tyr Gly Ser Gly Pro Gly Val Leu Gly Pro Tyr
130 135 140

Gly Ser Ala Ala Ala Ala Ala Ala Ala Gly Pro Gly Ser Gly Gln Tyr
145 150 155 160

Gly Gln Gly Pro Tyr Gly Pro Gly Ala Ser Gly Pro Gly Gln Tyr Gly
165 170 175

Pro Gly Val Leu Gly Pro Ser Ala Ser Ala Ala Ala Ala Ala Ala Ala
180 185 190

Gly Ser Gly Val Leu Gly Pro Gly Gln Tyr Gly Pro Tyr Ala Ser Ala
195 200 205

Ala Ala Ala Ala Ala Ala Gly Ser Tyr Gly Ser Gly Pro Gly Val Leu
210 215 220

Gly Pro Tyr Gly Pro Gly Gln Ser Gly Ser Gly Val Leu Gly Pro Gly
225 230 235 240

Val Leu Gly Pro Tyr Ala Ser Ala Ala Ala Ala Ala Ala Ala Gly Pro
245 250 255

Gly Val Leu Gly Pro Tyr Gly Pro Gly Ser Ser Ala Ala Ala Ala Ala
260 265 270

Ala Ala Gly Ser Tyr Gly Tyr Gly Pro Gly Val Leu Gly Pro Tyr Gly
275 280 285

Pro Gly Ala Ser Gly Gln Asn Gly Pro Gly Ser Gly Gln Tyr Gly Pro
290 295 300

Gly Val Leu Gly Pro Gly Pro Ser Ala Ala Ala Ala Ala Ala Ala Gly
305 310 315 320

Pro Gly Val Leu Gly Pro Tyr Gly Pro Gly Ala Ser Ala Ala Ala Ala
325 330 335

Ala Ala Ala Gly Ser Tyr Gly Pro Gly Val Leu Gly Pro Gly Gln Tyr
340 345 350

Gly Pro Gly Ser Ser Gly Pro Gly Val Leu Gly Pro Tyr Gly Pro Gly
355 360 365

Ser Ser Ala Ala Ala Ala Ala Ala Ala Gly Ser Tyr Gly Pro Gly Val
370 375 380

Leu Gly Pro Tyr Gly Pro Gly Pro Ser Ala Ala Ala Ala Ala Ala Ala

385		390		395		400									
Gly	Ser	Tyr	Val	Leu	Gly	Pro	Gly	Val	Leu	Gly	Pro	Tyr	Gly	Pro	Gly
			405						410					415	
Ala	Ser	Gly	Pro	Gly	Val	Leu	Gly	Pro	Tyr	Gly	Pro	Gly	Ala	Ser	Ala
			420					425					430		
Ala	Ala	Ala	Ala	Ala	Ala	Gly	Pro	Gly	Gln	Tyr	Gly	Pro	Gly	Val	Leu
			435					440				445			
Gly	Pro	Ser	Ala	Ser	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Gly	Ser	Tyr	Gly
	450						455					460			
Ser	Gly	Pro	Gly	Gln	Tyr	Gly	Pro	Tyr	Gly	Pro	Gly	Gln	Ser	Gly	Pro
465					470					475					480
Gly	Ser	Gly	Val	Leu	Gly	Gln	Gly	Pro	Tyr	Gly	Pro	Gly	Ala	Ser	Ala
			485						490					495	
Ala	Ala	Ala	Ala	Ala	Ala	Gly	Ser	Tyr	Gly	Pro	Gly	Val	Leu	Gly	Pro
			500						505				510		
Tyr	Gly	Pro	Gly	Pro	Ser	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Gly	Pro	Gly
		515						520					525		
Ser	Gly	Gln	Tyr	Gly	Pro	Gly	Ala	Ser	Gly	Gln	Asn	Gly	Pro	Gly	Ser
	530						535				540				
Gly	Gln	Tyr	Gly	Pro	Gly	Val	Leu	Gly	Pro	Gly	Pro	Ser	Ala	Ala	Ala
545						550				555					560
Ala	Ala	Ala	Ala	Gly	Pro	Gly	Ser	Gly	Val	Leu	Gly	Pro	Gly	Ala	Ser
				565						570				575	

<210> 5
 <211> 576
 <212> PRT
 <213> Artificial Sequence

<220>

<223> PRT698

<400> 5

Met His His His His His His Ser Ser Gly Ser Ser Gly Pro Gly Val
1 5 10 15

Leu Gly Pro Tyr Gly Pro Gly Ala Ser Ala Ala Ala Ala Ala Ala Ala
20 25 30

Gly Ser Asn Gly Pro Gly Ser Gly Val Leu Gly Pro Gly Ile Ser Gly
35 40 45

Ile Tyr Gly Pro Gly Val Leu Gly Pro Gly Val Leu Gly Pro Gly Ser
50 55 60

Ser Ala Ala Ala Ala Ala Ala Ala Gly Pro Gly Ile Tyr Gly Pro Gly
65 70 75 80

Val Leu Gly Pro Ser Ala Ser Ala Ala Ala Ala Ala Ala Gly Pro
85 90 95

Gly Ser Gly Val Leu Gly Pro Gly Ala Ser Gly Ile Tyr Gly Pro Gly
100 105 110

Val Leu Gly Pro Gly Val Leu Gly Pro Gly Ser Ser Ala Ala Ala Ala
115 120 125

Ala Ala Ala Gly Ser Tyr Gly Ser Gly Pro Gly Val Leu Gly Pro Tyr
130 135 140

Gly Ser Ala Ala Ala Ala Ala Ala Gly Pro Gly Ser Gly Ile Tyr
145 150 155 160

Gly Ile Gly Pro Tyr Gly Pro Gly Ala Ser Gly Pro Gly Ile Tyr Gly
165 170 175

Pro Gly Val Leu Gly Pro Ser Ala Ser Ala Ala Ala Ala Ala Ala Ala
180 185 190

Gly Ser Gly Val Leu Gly Pro Gly Ile Tyr Gly Pro Tyr Ala Ser Ala

195						200						205					
Ala	Ala	Ala	Ala	Ala	Ala	Gly	Ser	Tyr	Gly	Ser	Gly	Pro	Gly	Val	Leu		
210						215					220						
Gly	Pro	Tyr	Gly	Pro	Gly	Ile	Ser	Gly	Ser	Gly	Val	Leu	Gly	Pro	Gly		
225					230					235					240		
Val	Leu	Gly	Pro	Tyr	Ala	Ser	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Gly	Pro		
				245					250					255			
Gly	Val	Leu	Gly	Pro	Tyr	Gly	Pro	Gly	Ser	Ser	Ala	Ala	Ala	Ala	Ala		
			260					265					270				
Ala	Ala	Gly	Ser	Tyr	Gly	Tyr	Gly	Pro	Gly	Val	Leu	Gly	Pro	Tyr	Gly		
		275					280					285					
Pro	Gly	Ala	Ser	Gly	Ile	Asn	Gly	Pro	Gly	Ser	Gly	Ile	Tyr	Gly	Pro		
	290					295					300						
Gly	Val	Leu	Gly	Pro	Gly	Pro	Ser	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Gly		
305					310				315						320		
Pro	Gly	Val	Leu	Gly	Pro	Tyr	Gly	Pro	Gly	Ala	Ser	Ala	Ala	Ala	Ala		
				325					330					335			
Ala	Ala	Ala	Gly	Ser	Tyr	Gly	Pro	Gly	Val	Leu	Gly	Pro	Gly	Ile	Tyr		
			340					345					350				
Gly	Pro	Gly	Ser	Ser	Gly	Pro	Gly	Val	Leu	Gly	Pro	Tyr	Gly	Pro	Gly		
		355					360					365					
Ser	Ser	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Gly	Ser	Tyr	Gly	Pro	Gly	Val		
		370					375					380					
Leu	Gly	Pro	Tyr	Gly	Pro	Gly	Pro	Ser	Ala	Ala	Ala	Ala	Ala	Ala	Ala		
385					390				395						400		
Gly	Ser	Tyr	Val	Leu	Gly	Pro	Gly	Val	Leu	Gly	Pro	Tyr	Gly	Pro	Gly		
				405					410					415			

Ala Ser Gly Pro Gly Val Leu Gly Pro Tyr Gly Pro Gly Ala Ser Ala
420 425 430

Ala Ala Ala Ala Ala Ala Gly Pro Gly Ile Tyr Gly Pro Gly Val Leu
435 440 445

Gly Pro Ser Ala Ser Ala Ala Ala Ala Ala Ala Gly Ser Tyr Gly
450 455 460

Ser Gly Pro Gly Ile Tyr Gly Pro Tyr Gly Pro Gly Ile Ser Gly Pro
465 470 475 480

Gly Ser Gly Val Leu Gly Ile Gly Pro Tyr Gly Pro Gly Ala Ser Ala
485 490 495

Ala Ala Ala Ala Ala Ala Gly Ser Tyr Gly Pro Gly Val Leu Gly Pro
500 505 510

Tyr Gly Pro Gly Pro Ser Ala Ala Ala Ala Ala Ala Gly Pro Gly
515 520 525

Ser Gly Ile Tyr Gly Pro Gly Ala Ser Gly Ile Asn Gly Pro Gly Ser
530 535 540

Gly Ile Tyr Gly Pro Gly Val Leu Gly Pro Gly Pro Ser Ala Ala Ala
545 550 555 560

Ala Ala Ala Ala Gly Pro Gly Ser Gly Val Leu Gly Pro Gly Ala Ser
565 570 575

<210> 6
<211> 252
<212> PRT
<213> Artificial Sequence

<220>
<223> Collagen-type4-Kai

<400> 6

Met His His His His His His Ser Ser Gly Ser Ser Lys Asp Gly Val

1					5					10					15				
Pro	Gly	Phe	Pro	Gly	Ser	Glu	Gly	Val	Lys	Gly	Asn	Arg	Gly	Phe	Pro				
			20							25				30					
Gly	Leu	Met	Gly	Glu	Asp	Gly	Ile	Lys	Gly	Gln	Lys	Gly	Asp	Ile	Gly				
			35							40				45					
Pro	Pro	Gly	Phe	Arg	Gly	Pro	Thr	Glu	Tyr	Tyr	Asp	Thr	Tyr	Gln	Glu				
			50				55							60					
Lys	Gly	Asp	Glu	Gly	Thr	Pro	Gly	Pro	Pro	Gly	Pro	Arg	Gly	Ala	Arg				
65							70				75							80	
Gly	Pro	Gln	Gly	Pro	Ser	Gly	Pro	Pro	Gly	Val	Pro	Gly	Ser	Pro	Gly				
			85							90							95		
Ser	Ser	Arg	Pro	Gly	Leu	Arg	Gly	Ala	Pro	Gly	Trp	Pro	Gly	Leu	Lys				
			100							105				110					
Gly	Ser	Lys	Gly	Glu	Arg	Gly	Arg	Pro	Gly	Lys	Asp	Ala	Met	Gly	Thr				
			115							120				125					
Pro	Gly	Ser	Pro	Gly	Cys	Ala	Gly	Ser	Pro	Gly	Leu	Pro	Gly	Ser	Pro				
			130				135							140					
Gly	Pro	Pro	Gly	Pro	Pro	Gly	Asp	Ile	Val	Phe	Arg	Lys	Gly	Pro	Pro				
145							150				155							160	
Gly	Asp	His	Gly	Leu	Pro	Gly	Tyr	Leu	Gly	Ser	Pro	Gly	Ile	Pro	Gly				
			165							170							175		
Val	Asp	Gly	Pro	Lys	Gly	Glu	Pro	Gly	Leu	Leu	Cys	Thr	Gln	Cys	Pro				
			180							185							190		
Tyr	Ile	Pro	Gly	Pro	Pro	Gly	Leu	Pro	Gly	Leu	Pro	Gly	Leu	His	Gly				
			195							200							205		
Val	Lys	Gly	Ile	Pro	Gly	Arg	Gln	Gly	Ala	Ala	Gly	Leu	Lys	Gly	Ser				
			210				215							220					

Pro Gly Ser Pro Gly Asn Thr Gly Leu Pro Gly Phe Pro Gly Phe Pro
 225 230 235 240

Gly Ala Gln Gly Asp Pro Gly Leu Lys Gly Glu Lys
 245 250

<210> 7
 <211> 315
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Resilin-Kai

<400> 7

Met His His His His His His Ser Ser Gly Ser Ser Pro Glu Pro Pro
 1 5 10 15

Val Asn Ser Tyr Leu Pro Pro Ser Asp Ser Tyr Gly Ala Pro Gly Gln
 20 25 30

Ser Gly Pro Gly Gly Arg Pro Ser Asp Ser Tyr Gly Ala Pro Gly Gly
 35 40 45

Gly Asn Gly Gly Arg Pro Ser Asp Ser Tyr Gly Ala Pro Gly Gln Gly
 50 55 60

Gln Gly Gln Gly Gln Gly Gln Gly Gly Tyr Ala Gly Lys Pro Ser Asp
 65 70 75 80

Ser Tyr Gly Ala Pro Gly Gly Gly Asp Gly Asn Gly Gly Arg Pro Ser
 85 90 95

Ser Ser Tyr Gly Ala Pro Gly Gly Gly Asn Gly Gly Arg Pro Ser Asp
 100 105 110

Thr Tyr Gly Ala Pro Gly Gly Gly Asn Gly Gly Arg Pro Ser Asp Thr
 115 120 125

Tyr Gly Ala Pro Gly Gly Gly Gly Asn Gly Asn Gly Gly Arg Pro Ser

130

135

140

Ser Ser Tyr Gly Ala Pro Gly Gln Gly Gln Gly Asn Gly Asn Gly Gly
 145 150 155 160

Arg Pro Ser Ser Ser Tyr Gly Ala Pro Gly Gly Gly Asn Gly Gly Arg
 165 170 175

Pro Ser Asp Thr Tyr Gly Ala Pro Gly Gly Gly Asn Gly Gly Arg Pro
 180 185 190

Ser Asp Thr Tyr Gly Ala Pro Gly Gly Gly Asn Asn Gly Gly Arg Pro
 195 200 205

Ser Ser Ser Tyr Gly Ala Pro Gly Gly Gly Asn Gly Gly Arg Pro Ser
 210 215 220

Asp Thr Tyr Gly Ala Pro Gly Gly Gly Asn Gly Asn Gly Ser Gly Gly
 225 230 235 240

Arg Pro Ser Ser Ser Tyr Gly Ala Pro Gly Gln Gly Gln Gly Gly Phe
 245 250 255

Gly Gly Arg Pro Ser Asp Ser Tyr Gly Ala Pro Gly Gln Asn Gln Lys
 260 265 270

Pro Ser Asp Ser Tyr Gly Ala Pro Gly Ser Gly Asn Gly Asn Gly Gly
 275 280 285

Arg Pro Ser Ser Ser Tyr Gly Ala Pro Gly Ser Gly Pro Gly Gly Arg
 290 295 300

Pro Ser Asp Ser Tyr Gly Pro Pro Ala Ser Gly
 305 310 315

<210> 8

<211> 282

<212> PRT

<213> Artificial Sequence

<220>

<223> elastin short

<400> 8

Met His His His His His His Ser Ser Gly Ser Ser Leu Gly Val Ser
1 5 10 15

Ala Gly Ala Val Val Pro Gln Pro Gly Ala Gly Val Lys Pro Gly Lys
20 25 30

Val Pro Gly Val Gly Leu Pro Gly Val Tyr Pro Gly Gly Val Leu Pro
35 40 45

Gly Ala Arg Phe Pro Gly Val Gly Val Leu Pro Gly Val Pro Thr Gly
50 55 60

Ala Gly Val Lys Pro Lys Ala Pro Gly Val Gly Gly Ala Phe Ala Gly
65 70 75 80

Ile Pro Gly Val Gly Pro Phe Gly Gly Pro Gln Pro Gly Val Pro Leu
85 90 95

Gly Tyr Pro Ile Lys Ala Pro Lys Leu Pro Gly Gly Tyr Gly Leu Pro
100 105 110

Tyr Thr Thr Gly Lys Leu Pro Tyr Gly Tyr Gly Pro Gly Gly Val Ala
115 120 125

Gly Ala Ala Gly Lys Ala Gly Tyr Pro Thr Gly Thr Gly Val Gly Pro
130 135 140

Gln Ala Ala Ala Ala Ala Ala Ala Lys Ala Ala Ala Lys Phe Gly Ala
145 150 155 160

Gly Ala Ala Gly Val Leu Pro Gly Val Gly Gly Ala Gly Val Pro Gly
165 170 175

Val Pro Gly Ala Ile Pro Gly Ile Gly Gly Ile Ala Gly Val Gly Thr
180 185 190

Pro Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Lys Ala Ala Lys Tyr

195

200

205

Gly Ala Ala Ala Gly Leu Val Pro Gly Gly Pro Gly Phe Gly Pro Gly
 210 215 220

Val Val Gly Val Pro Gly Ala Gly Val Pro Gly Val Gly Val Pro Gly
 225 230 235 240

Ala Gly Ile Pro Val Val Pro Gly Ala Gly Ile Pro Gly Ala Ala Val
 245 250 255

Pro Gly Val Val Ser Pro Glu Ala Ala Ala Lys Ala Ala Ala Lys Ala
 260 265 270

Ala Lys Tyr Gly Ala Arg Pro Gly Val Gly
 275 280

<210> 9

<211> 468

<212> PRT

<213> Artificial Sequence

<220>

<223> type I keratin 26

<400> 9

Met Ser Phe Arg Leu Ser Gly Val Ser Arg Arg Leu Cys Ser Gln Ala
 1 5 10 15

Gly Thr Gly Arg Leu Thr Gly Gly Arg Thr Gly Phe Arg Ala Gly Asn
 20 25 30

Val Cys Ser Gly Leu Gly Ala Gly Ser Ser Phe Ser Gly Pro Leu Gly
 35 40 45

Ser Val Ser Ser Lys Gly Ser Phe Ser His Gly Gly Gly Gly Leu Gly
 50 55 60

Ser Gly Val Cys Thr Gly Phe Leu Glu Asn Glu His Gly Leu Leu Pro
 65 70 75 80

Gly Asn Glu Lys Val Thr Leu Gln Asn Leu Asn Asp Arg Leu Ala Ser
85 90 95

Tyr Leu Asp His Val Cys Thr Leu Glu Glu Ala Asn Ala Asp Leu Glu
100 105 110

Gln Lys Ile Lys Gly Trp Tyr Glu Lys Tyr Gly Pro Gly Ser Gly Arg
115 120 125

Gln Leu Ala His Asp Tyr Ser Lys Tyr Phe Ser Val Thr Glu Asp Leu
130 135 140

Lys Arg Gln Ile Ile Ser Val Thr Thr Cys Asn Ala Ser Ile Val Leu
145 150 155 160

Gln Asn Glu Asn Ala Arg Leu Thr Ala Asp Asp Phe Arg Leu Lys Cys
165 170 175

Glu Asn Glu Leu Ala Leu His Gln Ser Val Glu Ala Asp Ile Asn Gly
180 185 190

Leu His Arg Val Met Asp Glu Leu Thr Leu Cys Thr Ser Asp Leu Glu
195 200 205

Met Gln Cys Glu Ala Leu Ser Glu Glu Leu Thr Tyr Leu Lys Lys Asn
210 215 220

His Gln Glu Glu Met Lys Val Met Gln Gly Ala Ala Arg Gly Asn Val
225 230 235 240

Asn Val Glu Ile Asn Ala Ala Pro Gly Val Asp Leu Thr Val Leu Leu
245 250 255

Asn Asn Met Arg Ala Glu Tyr Glu Asp Leu Ala Glu Gln Asn His Glu
260 265 270

Asp Ala Glu Ala Trp Phe Ser Glu Lys Ser Thr Ser Leu His Gln Gln
275 280 285

Ile Ser Asp Asp Ala Gly Ala Ala Met Ala Ala Arg Asn Glu Leu Met

290

295

300

Glu Leu Lys Arg Asn Leu Gln Thr Leu Glu Ile Glu Leu Gln Ser Leu
 305 310 315 320

Leu Ala Met Lys His Ser Tyr Glu Cys Ser Leu Ala Glu Thr Glu Ser
 325 330 335

Asn Tyr Cys His Gln Leu Gln Gln Ile Gln Glu Gln Ile Gly Ala Met
 340 345 350

Glu Asp Gln Leu Gln Gln Ile Arg Met Glu Thr Glu Gly Gln Lys Leu
 355 360 365

Glu His Glu Arg Leu Leu Asp Val Lys Ile Phe Leu Glu Lys Glu Ile
 370 375 380

Glu Met Tyr Cys Lys Leu Ile Asp Gly Glu Gly Arg Lys Ser Lys Ser
 385 390 395 400

Thr Cys Tyr Lys Ser Glu Gly Arg Gly Pro Lys Asn Ser Glu Asn Gln
 405 410 415

Val Lys Asp Ser Lys Glu Glu Ala Val Val Lys Thr Val Val Gly Glu
 420 425 430

Leu Asp Gln Leu Gly Ser Val Leu Ser Leu Arg Val His Ser Val Glu
 435 440 445

Glu Lys Ser Ser Lys Ile Ser Asn Ile Thr Met Glu Gln Arg Leu Pro
 450 455 460

Ser Lys Val Pro
 465

<210> 10

<211> 36

<212> PRT

<213> Artificial Sequence

<220>

<223> HisTag

<400> 10

Met Glu Thr His Ile Ser His Ile Ser His Ile Ser His Ile Ser His
1 5 10 15

Ile Ser His Ile Ser Ser Glu Arg Ser Glu Arg Gly Leu Tyr Ser Glu
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Arg Ser Glu Arg
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Asn Gly Pro Gly Ser Gly Gln Gln Gly Pro Gly Gln Ser Gly Gln Tyr
35 40 45

Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln Gly Pro Gly Ser Ser Ala
50 55 60

Ala Ala Ala Ala Gly Pro Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro
65 70 75 80

Ser Ala Ser Ala Ala Ala Ala Ala Gly Pro Gly Ser Gly Gln Gln Gly
85 90 95

Pro Gly Ala Ser Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro Gly Gln
100 105 110

Gln Gly Pro Gly Ser Ser Ala Ala Ala Ala Ala Gly Gln Tyr Gly Ser
115 120 125

Gly Pro Gly Gln Gln Gly Pro Tyr Gly Ser Ala Ala Ala Ala Gly
130 135 140

Pro Gly Ser Gly Gln Tyr Gly Gln Gly Pro Tyr Gly Pro Gly Ala Ser
145 150 155 160

Gly Pro Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro Ser Ala Ser Ala
165 170 175

Ala Ala Ala Ala Gly Ser Gly Gln Gln Gly Pro Gly Gln Tyr Gly Pro
180 185 190

Tyr Ala Ser Ala Ala Ala Ala Ala Gly Gln Tyr Gly Ser Gly Pro Gly
195 200 205

Gln Gln Gly Pro Tyr Gly Pro Gly Gln Ser Gly Ser Gly Gln Gln Gly
210 215 220

Pro Gly Gln Gln Gly Pro Tyr Ala Ser Ala Ala Ala Ala Gly Pro
225 230 235 240

Gly Gln Gln Gly Pro Tyr Gly Pro Gly Ser Ser Ala Ala Ala Ala Ala
245 250 255

Gly Gln Tyr Gly Tyr Gly Pro Gly Gln Gln Gly Pro Tyr Gly Pro Gly
260 265 270

Ala Ser Gly Gln Asn Gly Pro Gly Ser Gly Gln Tyr Gly Pro Gly Gln
275 280 285

Gln Gly Pro Gly Gln Ser Ala Ala Ala Ala Ala Gly Pro Gly Gln Gln
290 295 300

Gly Pro Tyr Gly Pro Gly Ala Ser Ala Ala Ala Ala Gly Gln Tyr
305 310 315 320

Gly Pro Gly Gln Gln Gly Pro Gly Gln Tyr Gly Pro Gly Ser Ser Gly

325

330

335

Pro Gly Gln Gln Gly Pro Tyr Gly Pro Gly Ser Ser Ala Ala Ala Ala
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Ala Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro Tyr Gly Pro Gly Gln
 355 360 365

Ser Ala Ala Ala Ala Ala Gly Gln Tyr Gln Gln Gly Pro Gly Gln Gln
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Gly Pro Tyr Gly Pro Gly Ala Ser Gly Pro Gly Gln Gln Gly Pro Tyr
 385 390 395 400

Gly Pro Gly Ala Ser Ala Ala Ala Ala Gly Pro Gly Gln Tyr Gly
 405 410 415

Pro Gly Gln Gln Gly Pro Ser Ala Ser Ala Ala Ala Ala Ala Gly Gln
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Tyr Gly Ser Gly Pro Gly Gln Tyr Gly Pro Tyr Gly Pro Gly Gln Ser
 435 440 445

Gly Pro Gly Ser Gly Gln Gln Gly Gln Gly Pro Tyr Gly Pro Gly Ala
 450 455 460

Ser Ala Ala Ala Ala Ala Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro
 465 470 475 480

Tyr Gly Pro Gly Gln Ser Ala Ala Ala Ala Ala Gly Pro Gly Ser Gly
 485 490 495

Gln Tyr Gly Pro Gly Ala Ser Gly Gln Asn Gly Pro Gly Ser Gly Gln
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Tyr Gly Pro Gly Gln Gln Gly Pro Gly Gln Ser Ala Ala Ala Ala Ala
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Gly Gln Tyr Gln Gln Gly Pro Gly Gln Gln Gly Pro Tyr Gly Pro Gly
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Ala Ser Ala Ala Ala Ala Ala Gly Gln Tyr Gly Ser Gly Pro Gly Gln
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Gln Gly Pro Tyr Gly Pro Gly Gln Ser Gly Ser Gly Gln Gln Gly Pro
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Gly Gln Gln Gly Pro Tyr Ala Ser Ala Ala Ala Ala Ala Gly Pro Gly
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Ser Gly Gln Gln Gly Pro Gly Ala Ser Gly Gln Gln Gly Pro Tyr Gly
595 600 605

Pro Gly Ala Ser Ala Ala Ala Ala Ala Gly Gln Asn Gly Pro Gly Ser
610 615 620

Gly Gln Gln Gly Pro Gly Gln Ser Gly Gln Tyr Gly Pro Gly Gln Gln
625 630 635 640

Gly Pro Gly Gln Gln Gly Pro Gly Ser Ser Ala Ala Ala Ala Ala Gly
645 650 655

Pro Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro Ser Ala Ser Ala Ala
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Ala Ala Ala Gly Pro Gly Ser Gly Gln Gln Gly Pro Gly Ala Ser Gly
675 680 685

Gln Tyr Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln Gly Pro Gly Ser
690 695 700

Ser Ala Ala Ala Ala Ala Gly Gln Tyr Gly Ser Gly Pro Gly Gln Gln
705 710 715 720

Gly Pro Tyr Gly Ser Ala Ala Ala Ala Ala Gly Pro Gly Ser Gly Gln
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Tyr Gly Gln Gly Pro Tyr Gly Pro Gly Ala Ser Gly Pro Gly Gln Tyr
740 745 750

Gly Pro Gly Gln Gln Gly Pro Ser Ala Ser Ala Ala Ala Ala Gly
755 760 765

Ser Gly Gln Gln Gly Pro Gly Gln Tyr Gly Pro Tyr Ala Ser Ala Ala
770 775 780

Ala Ala Ala Gly Gln Tyr Gly Ser Gly Pro Gly Gln Gln Gly Pro Tyr
785 790 795 800

Gly Pro Gly Gln Ser Gly Ser Gly Gln Gln Gly Pro Gly Gln Gln Gly
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Pro Tyr Ala Ser Ala Ala Ala Ala Ala Gly Pro Gly Gln Gln Gly Pro
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Tyr Gly Pro Gly Ser Ser Ala Ala Ala Ala Gly Gln Tyr Gly Tyr
835 840 845

Gly Pro Gly Gln Gln Gly Pro Tyr Gly Pro Gly Ala Ser Gly Gln Asn
850 855 860

Gly Pro Gly Ser Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro Gly Gln
865 870 875 880

Ser Ala Ala Ala Ala Ala Gly Pro Gly Gln Gln Gly Pro Tyr Gly Pro
885 890 895

Gly Ala Ser Ala Ala Ala Ala Gly Gln Tyr Gly Pro Gly Gln Gln
900 905 910

Gly Pro Gly Gln Tyr Gly Pro Gly Ser Ser Gly Pro Gly Gln Gln Gly
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Pro Tyr Gly Pro Gly Ser Ser Ala Ala Ala Ala Ala Gly Gln Tyr Gly
930 935 940

Pro Gly Gln Gln Gly Pro Tyr Gly Pro Gly Gln Ser Ala Ala Ala Ala
945 950 955 960

Ala Gly Gln Tyr Gln Gln Gly Pro Gly Gln Gln Gly Pro Tyr Gly Pro
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Gly Ala Ser Gly Pro Gly Gln Gln Gly Pro Tyr Gly Pro Gly Ala Ser
980 985 990

Ala Ala Ala Ala Ala Gly Pro Gly Gln Tyr Gly Pro Gly Gln Gln Gly
995 1000 1005

Pro Ser Ala Ser Ala Ala Ala Ala Ala Gly Gln Tyr Gly Ser Gly
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Pro Gly Gln Tyr Gly Pro Tyr Gly Pro Gly Gln Ser Gly Pro Gly
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Gln Tyr Gly Pro Gly Gln Gln Gly Pro Gly Gln Ser Ala Ala Ala
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Gly Gln Gln Gly Pro Gly Gln Gln Gly Pro Tyr Ala Ser Ala Ala

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1415						1420					1425			
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1430						1435					1440			
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1460						1465					1470			
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1475						1480					1485			
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1490						1495					1500			
Tyr	Gly	Pro	Gly	Ser	Ser	Gly	Pro	Gly	Gln	Gln	Gly	Pro	Tyr	Gly
1505						1510					1515			
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1640						1645					1650			
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Ala Ser Gly Gln Gln Gly Pro Tyr Gly Pro Gly Ala Ser Ala Ala
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Ala Ala Ala Gly Gln Asn Gly Pro Gly Ser Gly Gln Gln Gly Pro
1790 1795 1800

Gly Gln Ser Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro Gly Gln
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Gln Gly Pro Gly Ser Ser Ala Ala Ala Ala Ala Gly Pro Gly Gln
1820 1825 1830

Tyr Gly Pro Gly Gln Gln Gly Pro Ser Ala Ser Ala Ala Ala Ala
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Ala Gly Pro Gly Ser Gly Gln Gln Gly Pro Gly Ala Ser Gly Gln
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Tyr Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln Gly Pro Gly Ser
1865 1870 1875

Ser Ala Ala Ala Ala Ala Gly Gln Tyr Gly Ser Gly Pro Gly Gln
1880 1885 1890

Gln Gly Pro Tyr Gly Ser Ala Ala Ala Ala Ala Gly Pro Gly Ser
1895 1900 1905

Gly Gln Tyr Gly Gln Gly Pro Tyr Gly Pro Gly Ala Ser Gly Pro
1910 1915 1920

Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro Ser Ala Ser Ala Ala
1925 1930 1935

Ala Ala Ala Gly Ser Gly Gln Gln Gly Pro Gly Gln Tyr Gly Pro
1940 1945 1950

Tyr Ala Ser Ala Ala Ala Ala Ala Gly Gln Tyr Gly Ser Gly Pro

1955				1960				1965						
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1985						1990					1995			
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2000						2005					2010			
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2015						2020					2025			
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Gln	Tyr	Gly	Pro	Gly	Gln	Gln	Gly	Pro	Gly	Gln	Ser	Ala	Ala	Ala
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2060						2065					2070			
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2075						2080					2085			
Gly	Gln	Tyr	Gly	Pro	Gly	Ser	Ser	Gly	Pro	Gly	Gln	Gln	Gly	Pro
2090						2095					2100			
Tyr	Gly	Pro	Gly	Ser	Ser	Ala	Ala	Ala	Ala	Ala	Gly	Gln	Tyr	Gly
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2210						2215					2220			
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2225						2230					2235			
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2270						2275					2280			
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2285						2290					2295			
Gln	Gly	Pro	Tyr	Gly	Pro	Gly	Ala	Ser	Ala	Ala	Ala	Ala	Ala	Gly
2300						2305					2310			
Gln	Tyr	Gly	Ser	Gly	Pro	Gly	Gln	Gln	Gly	Pro	Tyr	Gly	Pro	Gly
2315						2320					2325			
Gln	Ser	Gly	Ser	Gly	Gln	Gln	Gly	Pro	Gly	Gln	Gln	Gly	Pro	Tyr
2330						2335					2340			
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His His
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 35 40 45

Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln Gly Pro Gly Ser Ser Ala
 50 55 60

Ala Ala Ala Ala Gly Pro Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro
 65 70 75 80

Ser Ala Ser Ala Ala Ala Ala Ala Gly Pro Gly Ser Gly Gln Gln Gly
 85 90 95

Pro Gly Ala Ser Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro Gly Gln
 100 105 110

Gln Gly Pro Gly Ser Ser Ala Ala Ala Ala Ala Gly Gln Tyr Gly Ser
 115 120 125

Gly Pro Gly Gln Gln Gly Pro Tyr Gly Ser Ala Ala Ala Ala Ala Gly
 130 135 140

Pro Gly Ser Gly Gln Tyr Gly Gln Gly Pro Tyr Gly Pro Gly Ala Ser
 145 150 155 160

Gly Pro Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro Ser Ala Ser Ala
 165 170 175

Ala Ala Ala Ala Gly Ser Gly Gln Gln Gly Pro Gly Gln Tyr Gly Pro
 180 185 190

Tyr Ala Ser Ala Ala Ala Ala Ala Gly Gln Tyr Gly Ser Gly Pro Gly
 195 200 205

Gln Gln Gly Pro Tyr Gly Pro Gly Gln Ser Gly Ser Gly Gln Gln Gly
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Pro Gly Gln Gln Gly Pro Tyr Ala Ser Ala Ala Ala Ala Gly Pro
 225 230 235 240

Gly Gln Gln Gly Pro Tyr Gly Pro Gly Ser Ser Ala Ala Ala Ala Ala
 245 250 255

Gly Gln Tyr Gly Tyr Gly Pro Gly Gln Gln Gly Pro Tyr Gly Pro Gly
 260 265 270

Ala Ser Gly Gln Asn Gly Pro Gly Ser Gly Gln Tyr Gly Pro Gly Gln
 275 280 285

Gln Gly Pro Gly Gln Ser Ala Ala Ala Ala Ala Gly Pro Gly Gln Gln
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Ala Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro Tyr Gly Pro Gly Gln
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370 375 380

Gly Pro Tyr Gly Pro Gly Ala Ser Gly Pro Gly Gln Gln Gly Pro Tyr
385 390 395 400

Gly Pro Gly Ala Ser Ala Ala Ala Ala Ala Gly Pro Gly Gln Tyr Gly
405 410 415

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Tyr Gly Ser Gly Pro Gly Gln Tyr Gly Pro Tyr Gly Pro Gly Gln Ser
435 440 445

Gly Pro Gly Ser Gly Gln Gln Gly Gln Gly Pro Tyr Gly Pro Gly Ala
450 455 460

Ser Ala Ala Ala Ala Ala Gly Gln Tyr Gly Pro Gly Gln Gln Gly Pro
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Gln Tyr Gly Pro Gly Ala Ser Gly Gln Asn Gly Pro Gly Ser Gly Gln
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Tyr Gly Pro Gly Gln Gln Gly Pro Gly Gln Ser Ala Ala Ala Ala Ala
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Pro Gly Ala Ser Ala Ala Ala Ala Ala Gly Gln Asn Gly Pro Gly Ser
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Gln Tyr Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln Gly Pro Gly Ser
690 695 700

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705 710 715 720

Gly Pro Tyr Gly Ser Ala Ala Ala Ala Ala Gly Pro Gly Ser Gly Gln
725 730 735

Tyr Gly Gln Gly Pro Tyr Gly Pro Gly Ala Ser Gly Pro Gly Gln Tyr
740 745 750

Gly Pro Gly Gln Gln Gly Pro Ser Ala Ser Ala Ala Ala Ala Gly
755 760 765

Ser Gly Gln Gln Gly Pro Gly Gln Tyr Gly Pro Tyr Ala Ser Ala Ala

770

775

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