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(54) **METHODS OF PRODUCING PROTEINS HAVING TRIPLE-HELIX STRUCTURE**

VERFAHREN ZUR HERSTELLUNG VON PROTEINEN MIT TRIPPELHELIXSTRUKTUR

PROCEDE POUR LA PRODUCTION D'UNE PROTEINE AYANT UNE STRUCTURE TRI-
 HELICOIDALE

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DescriptionTechnical Field

5 [0001] The present invention relates to methods of producing proteins having a triple-helix structure. More specifically, the present invention relates to methods of producing human collagen or partial peptides of human collagen. An objective of the present invention is to provide human collagen and partial peptides of human collagen that are safe for the living body and can be easily purified and obtained, and methods of producing them. More specifically, the present invention is to provide methods of producing human collagen and partial peptides thereof, by stably transducing Chinese hamster 10 ovary (CHO) cells with a mammalian expression vector into which human collagen cDNA has been inserted.

Background Art

15 [0002] Collagen is a protein that is distributed to almost all tissues of the body including the skin, bone and cartilage, and is well known to play important functions such as maintaining structures of tissues and organs by providing scaffolds for cells. Meanwhile, collagen is a bioabsorbable material that is decomposed by collagenases secreted from fibroblasts and collagenases present in phagocytes. Collagen is considered to be useful as a biomaterial because it is a biocompatible and bioabsorbable material as described above. Thus far, collagen has been used as a biomaterial for covering wounded skin and is reported to improve healing (Non-Patent Documents 1 and 2).

20 [0003] Forty percent of total collagen exists in the skin, and 70% or more of the dry weight of the skin and tendon is collagen; thus, collagen is important for developing artificial skin. It is applied as a useful material for cell and organ culture techniques, which gives great expectation in its applications in the booming field of regeneration medicine. It has been also pointed out that collagen (type II collagen) may be used to suppress articular rheumatism by oral intake (Non-Patent Documents 3 and 4). As a source material for such collagen, those derived from tissues of large non-human 25 animals such as pigs and cows have been mainly used.

[Non-Patent Document 1] Surg. Forum, 10, 303 (1960)

[Non-Patent Document 2] J. Surg. Res., 10, 485-491 (1970)

[Non-Patent Document 3] Lancet, 342, 799 (1993)

[Non-Patent Document 4] Science, 261, 1727-1730 (1993)

30 [Patent Document 1] Japanese Patent Application Kokai Publication No. (JP-A) H10-179169 (unexamined, published Japanese patent application)

Disclosure of the Invention

35 [Problems to be solved by the invention] .

[0004] As described above, collagen is useful as a biomaterial or medicine for regeneration therapy and live organ transplantation, but the collagen used so far is derived from tissues of large non-human animals such as pigs and cows. Although collagen is a protein with low immunogenicity by nature, it is reported that when collagen from a xenogenic 40 animal is transplanted, implanted or administered as a biomaterial, immune reactions are induced at a low frequency (J. Immunol., 136, 877-882 (1986), Biomaterials, 11, 176-180 (1990)). In addition, the use of cow-derived collagen has become impossible due to the problem of prion contamination in cows. Furthermore, there is no guarantee that problems similar to prion contamination will not occur in animals such as pigs which are currently used for collagen extraction. From the above-mentioned aspects, it is preferable to use human-derived collagen as a biomaterial to be directly applied 45 to the human body. However, extraction and purification of collagen from human tissues not only have ethical and technical problems, but is also qualitatively problematic in that the collagen obtained forms unspecific cross-linkages and is difficult to purify.

[0005] In order to obtain non-immunogenic collagen that is free from risk of pathogen contamination and easy to isolate and purify, collagen production using gene recombination techniques has been studied (Biochem. Soc., 28, 350-353 50 (2000)). However, it is very complicated to prepare an expression vector for introducing into host cells, a cDNA encoding a collagen molecule whose molecular weight is more than 100,000. In addition, conventional methods have low productivity and are far from practical application. Furthermore, it is known that collagen molecules have a triple-helix structure in which three peptides are associated.

This structure is formed as a result of several modifications to primary translation products of the gene (N. Engl. J. Med., 55 311, 376-386 (1984)); however, only specific cells are thought to have such modification ability.

[0006] Attempts have been made to produce recombinant human collagen by using mouse fibroblasts, hamster lung cells and the like as a host (Proc. Natl. Acad. Sci. USA., 84, 764-768 (1987), J. Biol. Chem., 264, 20683-20687 (1989)). Although the collagen produced in these examples have a normal molecular structure, they are mixed collagen molecules

of collagen gene products from both human and the host cell. In an example where human type II collagen was expressed (Biochem. J., 298, 31-37 (1994)), the amount produced was as small as 0.5 to 1 mg per liter of culture medium, and the type II collagen expressed by the introduced cDNA was found to be contaminated with a significant amount of host-derived type II collagen. Thus, it was necessary to separate endogenous type II collagen from type II collagen derived from the introduced gene.

[0007] In addition to the above-mentioned examples, there are examples of expressing human collagen using yeasts (Japanese Patent Kohyo Publication No. (JP-A) H7-501939 (unexamined, published Japanese national phase publication corresponding to a non-Japanese international publication)), insect cells (Japanese Patent Application Kokai Publication No. (JP-A) H8-23979 (unexamined, published Japanese patent application)), *Bacillus brevis* (JP-A H11-178574), and *Escherichia coli* (JP-A 2002-325584), but the post-expression modifications of collagen peptides may be different from those made in animal cells. As mentioned above, no method reported so far is satisfactory as a gene recombination method for producing human collagen in terms of quantity and quality. In addition, there has not been any investigation on methods for producing large quantities of proteins with a triple-helix structure such as collagen.

[0008] The present invention was achieved in view of the above circumstances. An objective of the present invention is to provide methods for producing proteins with a triple-helix structure. More specifically, the objective is to provide methods for producing human collagen molecules that are easy to isolate and purify, and have substantially the same structure as natural collagen molecules, by synthesizing large amounts of human collagen protein in host cells introduced with a collagen gene, incorporated in a high expression vector, where the large amounts of human collagen protein are derived from the introduced gene.

[Means for Solving the Problems]

[0009] The present inventors performed various studies to solve the above-mentioned problems. As a result, the inventors discovered that large amounts of human collagen hardly contaminated with host cell-derived collagen can be produced, by selecting from various mammalian cells a host cell that has low collagen expression and introducing a collagen gene construct into a vector capable of high exogenous gene expression, and thereby completed the present invention.

There has been no report of collagen production methods that preferentially produce human collagen in host cells by massively expressing an introduced collagen gene.

[0010] Specifically, the present inventors successfully developed methods for producing a large amount of human collagen that do not require a complex purification process, by inserting a human collagen gene into a vector capable of highly expressing a foreign gene and then introducing the resultant construct into a host mammalian cell with low expression of collagen (a triple-helix structural protein), and thereby completed the present invention.

[0011] Specifically, the present invention provides:

[1] a method of producing a protein having a triple-helix structure, wherein the method comprises:

- (a) introducing DNA encoding a protein having a triple-helix structure into a vector;
- (b) transforming a mammalian cell by transfer of the gene vector; and
- (c) culturing or breeding the transformant, and collecting the protein having a triple helix structure from the cell or culture supernatant thereof;

[2] the method of [1], wherein the protein having a triple-helix structure is human collagen or a partial peptide thereof;

[3] the method of [2], wherein the human collagen consists of at least one or more types of α chains;

[4] the method of [2], wherein the human collagen is human type I collagen;

[5] the method of [4], wherein the human type I collagen is a complex of $\alpha 1$ and $\alpha 2$ chains;

[6] the method of [2], wherein the human collagen is human type II collagen;

[7] the method of [2], wherein the human collagen is human type III collagen;

[8] the method of [1], wherein the DNA encoding a protein having a triple helix structure is at least a DNA selected from:

- (a) a DNA comprising any one of the nucleotide sequences of SEQ ID NOs:1, 4, 7, and 10; and
- (b) a DNA hybridizing under stringent conditions with a DNA comprising any one of the nucleotide sequences of SEQ ID NOs:1, 4, 7, and 10;

[9] the method of any one of [1] to [8], wherein the mammalian cell is a Chinese hamster ovary (CHO) cell;

[10] the method of any one of [1] to [8], wherein the mammalian cell is a human embryonic kidney (HEK293) cell;

[11] the method of any one of [1] to [10], wherein the vector to be introduced with the DNA encoding a protein having a triple helix structure is pNOW/CMV-AA;

[12] a human collagen produced according to the method of any one of [1] to [11];
 [13] a vector introduced with at least one DNA selected from:

5 (a) a DNA comprising any one of the nucleotide sequences of SEQ ID NOs: 1, 4, 7, and 10; and
 (b) a DNA hybridizing under stringent conditions with DNA comprising any one of the nucleotide sequences of SEQ ID NOs: 1, 4, 7, and 10;

[14] a mammalian cell carrying the vector of [13]; and

10 [15] a kit for producing a protein having a triple helix structure, wherein the kit comprises the vector of [13] or the mammalian cell of [14].

Brief Description of the Drawings

[0012]

15 Fig. 1 shows an expression construct of an α 1-chain of human type-I collagen. hColla1: human type I collagen α 1-chain cDNA, PCMV: cytomegalovirus promoter, BGHPA: poly (A) addition signal of bovine growth hormone gene, PSVd: simian virus 40 promoter devoid of enhancer, DHFR: mouse dihydrofolate reductase cDNA, SVpA: poly (A)-addition signal of simian virus 40, ColE1ori: replication origin of *Escherichia coli*, Neor: selection marker for mammalian cells (G418 resistance) and *Escherichia coli* (kanamycin resistance)

20 Fig. 2 shows an expression construct of an α 2-chain of human type-I collagen. hColla2: human type-I collagen α 2-chain gene cDNA, PCMV: cytomegalovirus promoter, BGHPA: poly (A) addition signal of bovine growth hormone gene, PSVd: simian virus 40 promoter devoid of enhancer, DHFR: mouse dihydrofolate reductase cDNA, SVpA: poly (A) addition signal of simian virus 40, ColE1ori: replication origin of *Escherichia coli*, Neor: selection marker for mammalian cells (G418 resistance) and *Escherichia coli* (kanamycin resistance)

25 Fig. 3 shows an expression construct of an α 1-chain of human type-II collagen. hColla1: human type-II collagen α 1-chain cDNA, PCMV: cytomegalovirus promoter, BGHPA: poly(A) addition signal of bovine growth hormone gene, PSVd: simian virus 40 promoter devoid of enhancer, DHFR: mouse dihydrofolate reductase cDNA, SVpA: poly (A)-addition signal of simian virus 40, ColE1ori: replication origin of *Escherichia coli*, Neor: selection marker for mammalian cells (G418 resistance) and *Escherichia coli* (kanamycin resistance)

30 Fig. 4 shows an expression construct of an α 1-chain of human type-III collagen. hColla1: human type-III collagen α 1-chain cDNA, PCMV: cytomegalovirus promoter, BGHPA: poly(A) addition signal of bovine growth hormone gene, PSVd: simian virus 40 promoter devoid of enhancer, DHFR: mouse dihydrofolate reductase cDNA, SVpA: poly(A) addition signal of simian virus 40, ColE 1 ori: replication origin of *Escherichia coli*, Neor: selection marker for mammalian cells (G418 resistance) and *Escherichia coli* (kanamycin resistance)

35 Fig. 5 is a photograph showing SDS-PAGE analysis of recombinant human type I collagen in culture supernatants. Lane 1: human type I collagen (100 μ g/mL), lane 2: recombinant type I collagen.

40 Fig. 6 is a photograph showing SDS-PAGE analysis of pepsin-digested products of recombinant human type I collagen in culture supernatants. Lane 1: recombinant human type I collagen (185 μ g/mL), lane 2: recombinant human type I collagen (20 times concentrated).

45 Fig. 7 is a set of photographs showing Western blot detection of purified recombinant human type I collagen and pepsin-digested products thereof.

A. Detection by an antibody against the α 1-chain of human type-I collagen, lane 1: human type I collagen (50 μ g/mL), lane 2: recombinant type I collagen, lane 3: pepsin digested products of recombinant type I collagen.

B. Detection by an antibody against the α 2-chain of human type-I collagen, lane 1: human type I collagen (10 μ g/mL), lane 2: recombinant type I collagen, lane 3: pepsin-digested products of recombinant type I collagen.

50 Fig. 8 is a photograph showing SDS-PAGE analysis of recombinant human type II collagen in culture supernatants. Lane 1: human type II collagen (100 μ g/mL), lane 2: recombinant type II collagen.

55 Fig. 9 is a photograph showing Western blot analysis of recombinant human type II collagen in culture supernatants. Lane 1: human type II collagen (10 μ g/mL), lane 2: recombinant type II collagen (10 times diluted)

Fig. 10 is a photograph showing SDS-PAGE analysis of the pepsin-digested products of recombinant human type II collagen in culture supernatants. Lane 1: human type II collagen (100 μ g/mL), lane 2: recombinant type II collagen (5 times concentrated).

Fig. 11 is a photograph showing Western blot analysis of the pepsin-digested products of recombinant human type II collagen in culture supernatants. Lane 1: human type II collagen (10 μ g/mL), lane 2: recombinant type II collagen.

Fig. 12 is a photograph showing SDS-PAGE analysis of recombinant human type III collagen in culture supernatants.

Lane 1: human type III collagen (100 µg/mL), lane 2: recombinant type III collagen.

Fig. 13 is a photograph showing Western blot analysis of recombinant human type III collagen in culture supernatants and pepsin-digested products thereof. Lane 1: human type III collagen (10 µg/mL), lane 2: recombinant type III collagen (10 times diluted), lane 3: pepsin-digested products of recombinant type III collagen.

5 Fig. 14 is a photograph showing SDS-PAGE analysis of purified recombinant human type III collagen in culture supernatants.

A. Type I collagen, lane 1: human type I collagen, lane 2: recombinant type I collagen.

B. Type III collagen, lane 1: human type III collagen, lane 2: recombinant type III collagen.

10 Best Mode for Carrying Out the Invention

[0013] Herein below, the best mode to conduct the present invention is shown and the present invention is explained in more detail.

15 [0014] The present invention relates to methods of producing proteins having a triple-helix structure, comprises the steps of:

(a) introducing into a vector a DNA encoding a protein having a triple-helix structure;

(b) transforming a mammalian cell by transfer of the gene vector;

20 (c) culturing or breeding the transformant, and collecting proteins with a triple-helix structure from the cells or culture supernatants thereof.

[0015] "Proteins having a triple-helix structure" in the present invention are not specifically limited as long as they has a triple-helix structure, but are preferably collagen or collectin, and more preferably collagen. Proteins having a triple-helix structure may be proteins whose triple-helix structure is constructed during the steps of culture and production, or after the steps of culture and production by manipulations such as purification. It is also possible to produce large quantities of proteins that can form a triple-helix structure in a single-chain structural state.

[0016] More than 20 different types of collagen and about 25 types of constituting α chains are known. Genes encoding them have been cloned and nucleotide sequences thereof have been elucidated ("Connective Tissue and Its Heritable Disorders", pp145-165, published by Wiley-Liss Inc. (1992)). These genes can be introduced into a vector used in the present invention that can highly express foreign genes by gene recombination techniques known to those skilled in the art (for example, "Molecular Cloning" second edition, published by Cold Spring Harbor Laboratory Press (1989)). The human collagen cDNA used in the present invention may be any one of these cloned cDNAs of collagen, and includes cDNAs of partial collagen peptides.

35 [0017] The collagen of the present invention does not have a specifically limited origin, but mammal-derived collagen is preferable, and human-derived collagen is more preferable.

[0018] Furthermore, the collagen of the present invention also includes collagen whose amino acid sequence is partially modified by substitution, deletion, or such, or has an addition of a non-collagen-derived amino acid sequence. In addition, there are known methods for obtaining transduced cells expressing protein molecules by introducing a vector into host mammalian cells. Similar methods can be applied to the present invention.

40 [0019] The following method can be used to examine whether collagen is synthesized as a recombinant protein by cells introduced with the above-mentioned high exogenous gene expression vector. Specifically, collagen peptides can be identified by immunochemical methods such as Western blotting by using commercially available antibodies that specifically bind to human collagen. Collagen usually does not migrate according to molecular weight in SDS-polyacrylamide gel electrophoresis (Nature, 227, 680-685 (1970)). Thus, the reactivity of a sample with an anti-collagen antibody can be examined after the sample is electrophoresed simultaneously with collagen as a marker and transferred to a nylon membrane or a nitrocellulose membrane according to the method by Matsudaira et al. (J. Biol. Chem., 261, 10035-10038 (1987)). Further, whether a molecule having a triple-helix structure is present in the recombinant collagen products generated by the expression vector can be examined as follows.

45 [0020] Typical fibrous collagen is a three-chain molecule formed from three subunits (α chains), and has an intramolecular triple-helix structure. Further, collagen having a triple-helix structure is known to be resistant to pepsin digestion. Thus, the presence of three-chain molecules in a protein sample can be confirmed by digesting culture supernatants of cells introduced with the above-mentioned high exogenous gene expression vector with pepsin in an acidic condition, and examining whether the sample has a pepsin-resistant structure.

50 [0021] Specifically, in the present invention, pepsin-treated protein samples were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions. As a result, the obtained recombinant collagen was shown to have pepsin resistance similar to that of natural collagen, and thus collagen peptides having a pepsin-resistant property were expected to be contained in culture supernatants of cells introduced with a high exogenous gene expression vector. The above-

mentioned results show that the expression vector of the present invention has ability to synthesize in host cells, collagen that has resistance to pepsin, which is a characteristic equivalent to collagen found in the living body.

[0022] Methods of producing and purifying the triple-helix structural proteins of the present invention are shown below, without being limited thereto.

5 [0023] Mammalian cells used as a host cell in the present invention are not particularly limited, but are preferably CHO cells or HEK293 cells.

[0024] Large-scale culture of CHO cells or HEK293 cells used in the present invention can be done by suspension culture. For example, 1×10^8 to 1×10^9 recombinant CHO cells introduced with a human collagen-expression vector containing a weakened neomycin phosphotransferase gene, mouse dihydrofolate reductase gene, and cDNA encoding 10 human collagen or a partial peptide thereof can be cultured in a shaker flask or a spinner flask using 100 ml to 1 L of culture medium. After culturing these cells for an appropriate period of time, proteins can be extracted from the collected culture supernatants in large quantities.

[0025] In the culture supernatants of recombinant CHO cells introduced with the human collagen-expression vector containing a weakened neomycin phosphotransferase gene, mouse dihydrofolate reductase gene, and cDNA encoding 15 human collagen or a partial peptide thereof, there exist not only three-chain collagen molecules with a triple-helix structure, but also collagen that has not formed into normal three-chain molecules. As mentioned above, collagen molecules that do not have a triple-helix structure are digested by pepsin. Thus, collagen molecules lacking a triple-helix structure can be removed by pepsin digestion. This treatment can also at the same time degrade and remove the non-collagen proteins 20 in culture supernatants. By using the above-mentioned characteristics, non-collagen proteins as well as collagen lacking a triple-helix structure can be digested and removed by direct pepsin treatment of total proteins present in the culture supernatants of recombinant CHO cells introduced with a human collagen expression vector containing a weakened neomycin phosphotransferase gene, mouse dihydrofolate reductase gene, and cDNA encoding human collagen or a partial peptide thereof.

[0026] In the present invention, the human collagen of interest is all human collagens that are currently known, including 25 type I to XXI collagens, and also includes partial peptides thereof. The type of collagen of the present invention is not particularly limited but includes, as representative examples, type I, type II, type III, type IV, type V, type VII, type IX, type XI, type XII, type XVII, and type XVIII, and preferably type I, type II, type III. Types I, IV, V, IX, and XI consist of two or three types of α chains, and types II, III, VII, XII, XVII, and XVIII consist of one type of α chain. They each have the 30 following molecular composition: type I: $[\alpha 1(I)]_2\alpha 2(I)$, type II: $[\alpha 1(II)]_3$, type III: $[\alpha 1(III)]_3$, type IV: $[\alpha 1(IV)]_2\alpha 2(IV)$, type V: $[\alpha 1(V)]_2\alpha 2(V)$ and $\alpha 1(V)\alpha 2(V)\alpha 3(V)$, type VII: $[\alpha 1(VII)]_3$, type IX: $\alpha 1(IX)\alpha 2(IX)\alpha 3(IX)$, type XI: $\alpha 1(XI)\alpha 2(XI)\alpha 3(XI)$, type XII: $[\alpha 1(XII)]_3$, type XVII: $[\alpha 1(XVII)]_3$, or type XVIII: $[\alpha 1(XVIII)]_3$; however, the molecular composition of the collagen of the present invention is not particularly limited. Further, the molecular composition of collagen of the present invention is not restricted to that of natural collagen, and may be artificially composed of three different types of α chains.

[0027] The nucleotide sequence of a DNA encoding the $\alpha 1$ chain of type I collagen of the present invention is indicated 35 in SEQ ID NO: 1, the nucleotide sequence of a DNA encoding the $\alpha 2$ chain of type I collagen is indicated in SEQ ID NO: 4, the nucleotide sequence of a DNA encoding the $\alpha 1$ chain of type II collagen is indicated in SEQ ID NO: 7, and the nucleotide sequence of a DNA encoding the $\alpha 1$ chain of type III collagen is indicated in SEQ ID NO: 10.

[0028] DNAs encoding the collagen of the present invention include oligonucleotides that have any one of the nucleotide 40 sequences of SEQ ID NOs: 1, 4, 7, and 10, and preferably include oligonucleotides that selectively hybridize to oligonucleotides having any one of the nucleotide sequences of SEQ ID NOs: 1, 4, 7, and 10. "Selectively hybridizing" refers to nucleic acid molecules that hybridize with, form double strands with, or bind substantially to a molecule having a predetermined sequence (i.e. a second oligonucleotide) present in a DNA or RNA sample under hybridization conditions of appropriate stringency. The stringent conditions are, for example, usually conditions of 42°C, 2x SSC, and 0.1% SDS, preferably conditions of 50°C, 2x SSC, and 0.1% SDS, and more preferably conditions of 65°C, 0.1x SSC, and 0.1% SDS, but are not particularly limited to these conditions. Conditions affecting hybridization stringency may include plural factors such as temperature and salt concentration, and those skilled in the art can appropriately select these factors to achieve the most appropriate stringency.

[0029] Collagen produced by the present invention may be procollagen molecules in which a propeptide links to the N- and C-termini, or may be in a form in which the propeptide is removed.

50 [0030] In the present invention, "partial peptides of collagen" refers to polypeptides that are encoded by 20% or more (for example, 20, 30, 40, 50, 60, 70, 80, or 90%) of the polynucleotides of a collagen-encoding cDNA. The peptides also include those in which the collagen amino acid sequences are partially modified or those that have an added non-collagen amino acid sequence.

[0031] In the present invention, "mammalian cells with low collagen expression" refers to cells producing 50 ng/mL of collagen or less when cultured at a density of 1×10^6 cells/mL; and preferred examples are CHO cells and HEK293 cells. In the present invention, "high expression" refers to expression of 10 μ g/mL of collagen or more, preferably expression of 50 μ g/mL or more of collagen.

[0032] In the present invention, "high exogenous gene expression vector" refers to, for example, vectors comprising

a weak drug-selectable marker gene in mammalian cells, such that the exogenous gene carried by the vector is selectively inserted into an actively transcribed region of chromosome in mammalian cells. Such vectors preferably include the pNOW/CMV-AA vector. The pNOW/CMV-AA vector is known in JP-A H10-179169. In the present invention, the culture method may be either suspension or adhesion culture.

- 5 [0033] All prior art literatures cited in the present specification are incorporated herein by reference.
 [0034] Hereinbelow, the present invention will be described more specifically using Examples; however, it is not to be construed as being limited thereto.

10 [Example 1] Preparation of the pNOW/CMV-AA vector

[0035] The pNOW/CMV-AA vector used was prepared by a known method (JP-A H10-179169)

[Example 2] Preparation of collagen expression vectors (1): isolation of human type-I α 1-chain cDNA

15 [0036] The human type-I α 1-chain collagen gene has already been cloned, and the nucleotide sequence thereof has been reported (EMBL Gene Database Accession No: NM 000088). The sequence is shown in SEQ ID NO: 1. Human type-I α 1 cDNA was amplified from human testis-derived cDNA by the polymerase chain reaction (PCR) method ("PCR Technology", published by Stockton Press (1989)). Specifically, the full-length sequence of SEQ ID NO: 1 was amplified by PCR using human testis-derived cDNA (Becton, Dickinson and Company) as a template and the oligonucleotides of SEQ ID NO: 2 (GC_{GG}CCGCCACC_{AT}GTCAGCTTGTGGACCTCCG) and SEQ ID NO: 3 (TTCTAGATTACAGGAAG-CAGACAGGCCAA) as primers. More specifically, the reaction was carried out using a commercially available PCR amplification kit (TaKaRa LA Taq with GC Buffer: Takara Bio Inc.). The reaction mixture was heated at 94°C for 5 minutes, and then subjected to 35 cycles of the following three steps: denaturation (94°C, 20 seconds), annealing of primers (60°C, 30 seconds), and amplification (72°C, 3 minutes 30 seconds), followed by an additional treatment at 72°C for 7 minutes to end the reaction. Hereinafter, all the PCR reactions in the Examples were carried out in the same reaction cycle. The PCR product obtained was separated by agarose gel electrophoresis, and ligated into a cloning vector for PCR products (pT7Blue kits: Novagen Inc.) using a ligation kit (DNA ligation kit ver.2: Takara Bio Inc.). After the ligated DNA was introduced into *Escherichia coli* strain XL-I Blue, plasmid DNA was obtained by culturing ampicillin-resistant colonies appeared on LB agar medium (Difco Inc.). A DNA fragment encoding human type I α 1-chain collagen was excised from the plasmid DNA, and ligated with a *Not* I and *Xba* I-digested product of the pNOW/CMV-AA vector prepared in Example 1, using DNA Ligation Kit ver.2. After the ligated DNA was introduced into *Escherichia coli* strain XL-I Blue, plasmid DNA (pNOW-hColla1, Fig. 1) was obtained by culturing one ampicillin-resistant colony that appeared on LB agar medium.

35 [Example 3] Preparation of collagen expression vectors (2): isolation of human type-I α 2-chain cDNA

[0037] The human type-I α 2-chain collagen gene has already been cloned, and its nucleotide sequence has been reported (EMBL Gene Database Accession No: NM 000089). The sequence is shown in SEQ ID NO: 4. The human type-I α 2 cDNA was amplified from human liver-derived cDNA by PCR. Specifically, the full-length sequence of SEQ ID NO: 4 was amplified by PCR using human liver-derived cDNA (Wako Pure Chemical Industries, Ltd) as a template and the oligonucleotides of SEQ ID NO: 5 (GC_{GG}CCGCCACC_{AT}GCTCAGCTTGTGGATACGCCGA) and SEQ ID NO: 6 (ACTAGTTATTGAAACAGACTGGGCCAA) as primers. The resultant PCR product was separated by agarose gel electrophoresis, and was ligated into a cloning vector for PCR products (pT7Blue kits: Novagen Inc.) by using a ligation kit (DNA ligation kit ver.2: Takara Bio Inc.). After the ligated DNA was introduced into the *Escherichia coli* strain XL-I Blue, plasmid DNA was obtained by culturing four ampicillin-resistant colonies that appeared on LB agar medium (Difco Inc.). A DNA fragment encoding human type-I α 2-chain collagen was excised from the plasmid DNA, and ligated into pNOW/CMV-AA vector cleaved with *Not* I and *Xba* I using DNA Ligation Kit ver.2. After the ligated DNA was introduced into *Escherichia coli* strain XL-I Blue, plasmid DNA (pNOW-hColla2, Fig. 2) was obtained by culturing one ampicillin-resistant colony that appeared on LB agar medium.

50 [Example 4] Preparation of collagen-expression vector (3): isolation of human type-II α 1-chain cDNA

[0038] The human type-II α 1-chain collagen gene has already been cloned, and its nucleotide sequence has been reported (EMBL Gene Database Accession No: NM 001844.1). The sequence is shown in SEQ ID NO: 7. Human type-II α 1 cDNA was amplified from human testis-derived cDNA by PCR. Specifically, the full-length sequence of SEQ ID NO: 7 was amplified by PCR using human testis-derived cDNA (Becton, Dickinson and Company) as a template and the oligonucleotides of SEQ ID NO: 8 (GGCCCCGCGGTGAGCCATGATTGCCCTCG) and SEQ ID NO: 9 (TCTAGAT-TACAAGAACGACCGGCCCTAT) as primers. The PCR product obtained was separated by agarose gel electro-

5 phoresis, and ligated to a cloning vector for PCR products (pT7Blue kits: Novagen Inc.) using a ligation kit (DNA ligation kit ver.2: Takara Bio Inc.). After the ligated DNA was introduced into *Escherichia coli* strain XL-I Blue, plasmid DNA was obtained by culturing four ampicillin-resistant colonies that appeared on LB agar medium (Difco Inc.). A DNA fragment encoding human type-II α 1-chain collagen was excised from the plasmid DNA, and ligated with pNOW/CMV-AA vector cleaved with *Not* I and *Xba* I using DNA Ligation Kit ver.2. After the ligated DNA was introduced into *Escherichia coli* strain XL-I Blue, plasmid DNA (pNOW-hColla1, Fig. 3) was obtained by culturing one ampicillin-resistant colony that appeared on LB agar medium.

10 [Example 5] Preparation of collagen expression vectors (4): isolation of human type-III α 1-chain cDNA

15 [0039] The human type-III α 1-chain collagen gene has already been cloned, and its nucleotide sequence has been reported (EMBL Gene Database Accession No: X14420). The sequence is shown in SEQ ID NO: 10. Human type-III α 1 cDNA was amplified from human liver-derived cDNA by PCR. Specifically, the full-length sequence of SEQ ID NO: 10 was amplified by PCR using human liver-derived cDNA (Wako Pure Chemical Industries, Ltd) as a template and the oligonucleotides of SEQ ID NO: 11 (GCGGCCGCCACCATGATGAGCTTGCAAAAGGGGA) and SEQ ID NO: 12 (TCTAGATTATAAAAGCAAACAGGGCAAC) as primers. The PCR product obtained was separated by agarose gel electrophoresis, and ligated into a cloning vector for PCR products (pT7Blue kits : Novagen Inc.) using a ligation kit (DNA ligation kit ver.2: Takara Bio Inc.). After the ligated DNA was introduced into *Escherichia coli* strain XL-I Blue, plasmid DNA was obtained by culturing four ampicillin-resistant colonies that appeared on LB agar medium. A DNA fragment encoding human type-III α 1-chain collagen was excised from the plasmid DNA, and ligated into pNOW/CMV-AA vector cleaved with *Not* I and *Xba* I using DNA Ligation Kit ver.2. After the ligated DNA was introduced into *Escherichia coli* strain XL-I Blue, plasmid DNA (pNOW-hColla1, Fig. 4) was obtained by culturing one ampicillin-resistant colony that appeared on LB agar medium.

20 25 [Example 6] Production of human type I collagen: transfer of the human type-I collagen gene using expression vectors pNOW-hColla1 and pNOW-hColla2, and establishment of primary G418-resistant clones.

30 [0040] One microgram each of pNOW-hColla1 and pNOW-hColla2 obtained in Examples 2 and 3 was transferred into 1.5 million DHFR-deficient CHO cells (CHO DG44 cells; provided by Dr. Gail Urlaub) in a 25 cm² culture flask by the 35 lipofectin method (Effectene Transfection Reagent, QIAGEN Inc.). The transfer method was carried out according to the manufacturer's instructions. After 48 hours, the cells were removed by trypsin treatment and the number of cells was counted. Then, 5x10⁵ cells were diluted with 100 mL of Iscove's Modified Dulbecco's Medium containing 0.8 mg/mL G418 and 10% dialyzed fetal bovine serum, and then were seeded into ten 96-well microtiter plates (960 wells), followed by culturing at 37°C for three weeks under the presence of 5% carbon dioxide gas. Live cells in 197 wells were transferred to 40 24-well plates with 1 mL of Iscove's Modified Dulbecco's Medium containing 0.8 mg/mL G418 and 10% dialyzed fetal bovine serum, and were cultured until confluent. After discarding culture supernatants, 1 mL of PBS (Invitrogen Inc.) was added to each well, and culture supernatants were discarded again. 0.5 mL of ProCH04 (Takara Bio Inc.), a CD medium for CHO cells, was added to each well and cultured at 37°C for 96 hours under the presence of 5% carbon dioxide gas. Subsequently, the amount of human type I collagen produced in the culture supernatants was examined.

45 [Example 7] Quantitative assay of the human type I collagen produced in pNOW-hColla1- and pNOW-hColla2-transduced cell clones

50 [0041] The amount produced was assayed by SDS-polyacrylamide gel electrophoresis. 12.5 μ L of the culture supernatant was mixed with an equal volume of Tris-SDS β -ME sample treatment solution (Daiichi Pure Chemicals Co., Ltd.), and heat-treated at 95°C for 5 minutes. This mixture was loaded onto an SDS-polyacrylamide gel (PAGEL, ATTO Inc.) and fractionated by electrophoresis. After the electrophoresis, human type I collagen in the polyacrylamide gel was detected and quantified by treating the gel with Coomassie Brilliant Blue Staining Solution (Amersham Biosciences). As a comparative control, 12.5 μ g/mL to 100 μ g/mL of human type I collagen (Cosmo Bio Co., Ltd.) treated in the same way was used.

55 [Example 8] Production of human type I collagen

[0042] Among the G418-resistant cell lines, a cell clone that produced the largest amount of human type I collagen was stabilized by passaging and culturing. The level of human type I collagen produced was 85 μ g/mL culture medium (four days).

[Example 9] SDS-PAGE analysis of recombinant human type I collagen in culture supernatants

[0043] The cell clone massively producing human type I collagen obtained by gene amplification was adjusted to 1 x 10⁶ cells/mL in a 25 cm² culture flask using the cell culture solution IS CHO-CD (IS Japan Co., Ltd.). After culturing at 37°C for 96 hours under the presence of 5% carbon dioxide gas, the culture fluid was collected. The cells were removed by centrifugation to obtain a culture supernatant. 12.5 µL of the culture supernatant was mixed with an equal volume Tris-SDSβ-ME sample treatment solution (Daiichi Pure Chemicals Co., Ltd.), and heat-treated at 95°C for 5 minutes. This mixture was loaded onto an SDS-polyacrylamide gel (PAGEL, ATTO Inc.) and fractionated by electrophoresis. The SDS-polyacrylamide gel electrophoresis described below was carried out in the same way. After the electrophoresis was finished, human type I collagen in the polyacrylamide gel was detected by treating the gel with Coomassie Brilliant Blue Staining Solution (Amersham Biosciences). 100 µg/mL of human type I collagen treated in the same way was used as a comparative control. Fig. 5 shows the result of SDS-PAGE analysis of the culture supernatant obtained from the human type I collagen-producing cell clone. 150- and 170-kDa polypeptides which may be recombinant human type I collagen α1 chains, and 130- and 150-kDa polypeptides which may be recombinant human type I collagen α2 chains were detected in the culture supernatant.

[Example 10] Pepsin digestion and SDS-PAGE analysis of recombinant human type I collagen in the culture supernatant

[0044] Pepsin digestion of the culture supernatant obtained from the human type I collagen-producing cell clone was carried out by adding 99.7% acetic acid to the supernatant at a final concentration of 0.5 M and then pepsin (Sigma Inc.) at a final concentration of 24 units/ml, followed by incubation at 20°C for two hours. The pepsin digestion described below was carried out in the same way. The sample obtained from pepsin digestion was analyzed by SDS-polyacrylamide gel electrophoresis. 185 µg/mL of commercially available recombinant human type I collagen (Beckton, Dickinson and Company) was used as a comparative control. Fig. 6 shows the analytical result of the pepsin-digested products by SDS-polyacrylamide gel electrophoresis. As observed with the commercially available human type I atelocollagen, when treated with pepsin, the recombinant human type I collagen in the culture supernatant was detected as 130- and 120-kDa polypeptides, which may be α1 chain and α2 chain, respectively. These facts showed that recombinant human type I collagen that has a pepsin resistance substantially equivalent to that of the natural type was contained in the culture supernatant obtained from the human type I collagen-producing cell clone.

[Example 11] Western blot analysis of the recombinant human type I collagen in the culture supernatant

[0045] The polyacrylamide gel after SDS-polyacrylamide gel electrophoresis was immersed in a transfer buffer, and then human type I collagen in the polyacrylamide gel was transferred to a PVDF membrane by a conventional method. After blocking with Block Ace, the membrane was reacted with 2 µg/mL of an antibody against human type I collagen α1 chain and then with an anti-goat IgG antibody labeled with horseradish peroxidase (HRP). Reacted antibodies were detected by a method that uses the TMB peroxidase reagent for detecting HRP activity (Funakoshi Co.). 50 µg/mL of recombinant human type I collagen (Beckton, Dickinson and Company) was used as a comparative control. Human type I collagen α2 chain was detected using an antibody against human type I collagen α2 chain instead of an anti-human type I collagen α1 chain antibody. 10 µg/mL of human type I collagen was used as a comparative control. Fig. 7 shows the result of the Western blotting analysis. A 170 kDa polypeptide that may be a recombinant human type I collagen α1 chain which can be bound by an anti-human type I collagen α1 chain antibody, and 130- and 150-kDa polypeptides that may be recombinant human type I collagen α2 chains which can be bound by an anti-human type I collagen α2 chain antibody, were detected in the culture supernatant.

[Example 12] Purification of human type I collagen in the culture supernatant

[0046] 100 mL of the culture supernatant containing human type I collagen was purified as follows.

[0047] The 100 ml culture supernatant filtrated through a 0.45 µm membrane filter (Millipore Co.) was concentrated to 30 mL by centrifugation at 3,000 rpm at 4°C using a centrifugal concentration filter (VIVASPIN20 (MWCO 10,000): Sartorius).

[0048] Salting out was carried out by gradually adding 30 mL of 90% ammonium sulfate solution to the above concentrated culture supernatant while stirring at 4°C. After all the ammonium sulfate solution was added, the mixture was further stirred for an hour. Then, the mixture was allowed to stand on ice for one hour, and then centrifuged at 18,000 rpm, 4°C for 30 minutes in a high-speed refrigerated centrifuge. Collagen in the solution was insolubilized by salting out and floated on the surface of the solution, and then collected and solubilized completely in 5 mL of D-PBS (Sigma Co.). This solution was filtrated through a 0.45 µm membrane filter (Millipore Co.), and then purified by gel filtration using Superose 6 (Amersham Biosciences) equilibrated with D-PBS, and the first peak was isolated. The collected peak

fraction was concentrated about 20 times using VIVASPIN6 (MWCO 100,000). An appropriate amount of D-PBS was added to the concentrated collagen solution for further concentration, and low molecular fragments were removed. This D-PBS addition was repeated at least three times or more.

5 [0049] A purified collagen solution obtained from the original 100 mL culture supernatant was concentrated to approximately 300 μ L and electrophoresed by SDS-PAGE to confirm its purity.

[Example 13] Test of human type II collagen production: transfer of the human type II collagen gene using expression vector pNOW-hColla1 and establishment of primary G418-resistant clones.

10 [0050] One microgram of pNOW-hColla1 was transferred into 1.5 million CHO-DG44 cells in a 25 cm^2 culture flask using the lipofectin method. The transfer method was carried out according to the manufacturer's instructions. After 48 hours, the cells were removed by trypsin treatment and the number of cells was counted. 5×10^5 cells were diluted with 100 mL of Iscove's Modified Dulbecco's Medium containing 0.8 mg/mL G418 and 10% dialyzed fetal bovine serum, and then seeded into ten 96-well microtiter plates (960 wells), followed by culturing at 37°C for three weeks under the presence of 5% carbon dioxide gas. Live cells in 126 wells were transferred to 24 well plates with 1 mL of Iscove's Modified Dulbecco's Medium containing 0.8 mg/mL G418 and 10% dialyzed fetal bovine serum, and were cultured until confluent. After culture supernatants were discarded, 1 mL PBS (Invitrogen Inc.) was added to each well, and culture supernatants were discarded again. 0.5 mL of ProCH04 (Takara Bio Inc.), a serum-free CD medium for CHO cells, was added to each well and cultured at 37°C for 96 hours under the presence of 5% carbon dioxide gas. Next, the amount of human type II collagen produced in the culture supernatants was examined.

[Example 14] Quantitative assay of the human type II collagen produced by pNOW-hColla1-transduced cell clones

25 [0051] The amount produced was assayed by SDS-polyacrylamide gel electrophoresis. 7.5 μ L of the culture supernatant was mixed with an equal volume of Tris-SDS β -ME sample treatment solution (Daiichi Pure Chemicals Co., Ltd.), and heat-treated at 95°C for 5 minutes. This mixture was loaded onto an SDS-polyacrylamide gel (PAGEL, ATTO Inc.) and fractionated by electrophoresis. After the electrophoresis was finished, human type II collagen in the polyacrylamide gel was detected and quantified by treating the gel with Coomassie Brilliant Blue Staining Solution (Amersham Biosciences). 12.5 μ g/mL to 100 μ g/mL of human type II collagen (Cosmo Bio Co., Ltd.) treated in the same way was used as a comparative control.

30 [Example 15] Gene amplification in G418-resistant cell lines

35 [0052] Among G418-resistant cell lines, a cell clone that produced the largest amount of human type II collagen was stabilized by passaging and culturing, and then gene amplification was carried out using MTX. Amplification was first carried out in a medium containing 5 nM MTX for one week, a medium containing 25 nM MTX for one week, a medium containing 50 nM MTX for one week, a medium containing 250 nM MTX for three weeks, and a medium containing 1 μ M MTX for three weeks. As a result, the production level of human type II collagen increased to 70 μ g/mL culture medium (four days) when MTX reached 25 nM. Generally, multiple MTX concentrations between 10 nM and 10 μ M are used for gene amplification, and 10 μ M is often used as a final concentration. However, exposure to high concentration is problematic when establishing stable recombinant cell lines because of cellular toxicity. Thus, it is also an important criterion that high productivity is achieved at low MTX concentrations, and thus concentrations up to 1 μ M were used in the present experiment.

40 Further, although the period of MTX exposure, including selection, is usually six to twelve months, the present experiment was done in about nine weeks. Despite these experimental conditions, the amount of human type II collagen produced was found to be effectively increased. Gene amplification in the G418-resistant cell lines described below was carried out in the same way.

45 [Example 16] Analysis of recombinant human type II collagen in the culture supernatant by SDS-polyacrylamide gel electrophoresis

50 [0053] The cell clone massively producing human type II collagen obtained by gene amplification was adjusted to 1×10^6 cells/mL in a 25 cm^2 culture flask using the IS CHO-CD culture medium (IS Japan Co., Ltd.). After culturing at 37°C for 96 hours under the presence of 5% carbon dioxide gas, the culture fluid was collected and the cells were removed by centrifugation to obtain a culture supernatant. 7.5 μ L of the culture supernatant was mixed with an equal volume Tris-SDS β -ME sample treatment solution (Daiichi Pure Chemicals Co., Ltd.), and heat-treated at 95°C for 5 minutes. This mixture was loaded onto an SDS-polyacrylamide gel (PAGEL, ATTO Inc.) and fractionated by electrophoresis. The SDS-polyacrylamide gel electrophoresis described below was carried out in the same way. After the

electrophoresis was finished, human type II collagen in the polyacrylamide gel was detected by treating the gel with Coomassie Brilliant Blue Staining Solution (Amersham Biosciences). 100 $\mu\text{g}/\text{mL}$ of human type II collagen (Cosmo Bio Co., Ltd.) treated in the same way was used as a comparative control. Fig. 8 shows the SDS-PAGE analysis result of the culture supernatant obtained from the human type II collagen-producing cell clone. 170- and 200-kDa polypeptides that may be recombinant human type II collagen were detected in the culture supernatant.

5 [Example 17] Western blot analysis of recombinant human type II collagen in the culture supernatant

10 [0054] The polyacrylamide gel after SDS-polyacrylamide gel electrophoresis was immersed in a transfer buffer, and then human type II collagen in the polyacrylamide gel was transferred to a PVDF membrane by a conventional method. After blocking with Block Ace, the membrane was reacted with 1 $\mu\text{g}/\text{mL}$ of an antibody against the human type II collagen chain (Cosmo Bio Co., Ltd.), and then with an anti-rabbit IgG antibody labeled with horseradish peroxidase (HRP). Reacted antibodies were detected by a method of detecting HRP activity using the TMB peroxidase reagent (Funakoshi Co.). 10 $\mu\text{g}/\text{mL}$ of human type II collagen (Cosmo Bio Co., Ltd.) was used as a comparative control. 170-kDa polypeptide 15 which may be recombinant human type II collagen that can be bound by an antibody against the human type II collagen chain was detected in the culture supernatant (Fig. 9).

20 [Example 18] Pepsin digestion, SDS-PAGE analysis, and Western blot analysis of recombinant human type II collagen in the culture supernatant

25 [0055] A sample obtained from pepsin digestion was analyzed by SDS-polyacrylamide gel electrophoresis. 100 $\mu\text{g}/\text{mL}$ of human type II collagen (Cosmo Bio Co., Ltd.) was used as a comparative control. Fig. 10 shows the result of analyzing the pepsin-digested products by SDS-polyacrylamide gel electrophoresis. As observed with commercially available human type II atelocollagen, when treated with pepsin, the recombinant human type II collagen in the culture supernatant was detected as a polypeptide of 130kDa. These facts showed that recombinant human type II collagen that has a pepsin resistance substantially equivalent to that of the natural type collagen was contained in the culture supernatant obtained from the human type II collagen-producing cell clone. The same results were obtained by Western blot analysis (Fig. 11).

30 [Example 19] Test of human type III collagen production: transfer of human type III collagen gene using expression vector pNOW-hCollIia1 and establishment of primary G418-resistant clones.

35 [0056] One microgram of pNOW-hCollIia1 was transferred into 1.5 million CHO DG44 cells in a 25 cm^2 culture flask by the lipofectin method. The transfer method was carried out according to the manufacturer's instructions. After 48 hours, the cells were removed by trypsin treatment, and the number of cells was counted. Then, 3×10^3 cells were diluted with 100 mL of Iscove's Modified Dulbecco's Medium containing 0.8 mg/mL G418 and 10% dialyzed fetal bovine serum, and seeded in ten 96-well microtiter plates (960 wells), followed by culturing at 37°C under the presence of 5% carbon dioxide gas for three weeks. As a result, live cells were found only in 117 wells (G418 resistant). The live cells were transferred to 24 well plates with 1 mL of Iscove's Modified Dulbecco's Medium containing 0.8 mg/mL G418 and 10% dialyzed fetal bovine serum, and cultured until confluent. After culture supernatants were discarded, 1 mL of PBS (Invitrogen Inc.) was added to each well, and culture supernatants were discarded again. 0.5 mL of CHO-S-SFM II (Invitrogen Inc.), a serum-free medium for CHO cells, was added to each well and cultured at 37°C for 72 hours under the presence of 5% carbon dioxide gas. Subsequently, the amount of human type III collagen produced in the culture supernatants was examined.

45 [Example 20] Quantitative assay of the human type III collagen produced in pNOW-hCollIia1-transduced cell clones

50 [0057] The amount produced was assayed by a dot blotting method. A nylon membrane was dotted with 1 μL of 72-hour culture supernatant, 1 μL each of commercially available human type III collagen (Beckton, Dickinson and Company) 2x diluted (0.125 to 8 $\mu\text{g}/\text{mL}$) in a serum-free medium for CHO cells, CHO-S-SFM II, and 1 μL of CHO-S-SFM II alone; and was then air dried for one hour. After blocking with Block Ace, the membrane was reacted with 1 $\mu\text{g}/\text{mL}$ of an anti-human type III collagen antibody (Cosmo Bio Co., Ltd.) and then with an HRP-labeled anti-rabbit IgG antibody. Reacted antibodies were detected by a method of detecting HRP activity with the SuperSignal West Pico reagent using Lumino Capture.

55 [Example 21] Gene amplification in G418-resistant cell lines

[0058] Among G418-resistant cell lines, a cell clone that produced the largest amount of human type III collagen was

stabilized by passaging and culturing, and then gene amplification was carried out with MTX. Gene amplification was carried out first in a medium containing 15 nM MTX for two weeks, a medium containing 60 nM MTX for two weeks, a medium containing 250 nM MTX for two weeks, and a medium containing 1 μ M MTX for four weeks. As a result, the production level of human type III collagen was increased to 225 μ g/mL culture medium (three days).

5

[Example 22] SDS-PAGE analysis of recombinant human type III collagen in the culture supernatant

[0059] The cell clone massively producing human type III collagen obtained by gene amplification was adjusted to 1 $\times 10^6$ cells/mL in a 25 cm² culture flask by using the IS CHO-CD culture medium (IS Japan Co., Ltd.). After culturing at 37°C for 96 hours under the presence of 5% carbon dioxide gas, the culture fluid was collected and the cells were removed by centrifugation to obtain a culture supernatant. 6.0 μ L of the culture supernatant was mixed with an equal volume Tris-SDS β -ME sample treatment solution (Daiichi Pure Chemicals Co., Ltd.), and heat-treated at 95°C for 5 minutes. This mixture was loaded onto an SDS-polyacrylamide gel (PAGEL, ATTO Inc.) and fractionated by electrophoresis. The SDS-polyacrylamide gel electrophoresis described below was carried out in the same way. After the electrophoresis was finished, human type III collagen in the polyacrylamide gel was detected by treating the gel with Coomassie Brilliant Blue Staining Solution (Amersham Biosciences). 100 μ g/mL of human type III collagen (Beckton, Dickinson and Company) treated in the same way was used as a comparative control. Fig. 12 shows the result of SDS-PAGE analysis of the culture supernatant obtained from the human type III collagen-producing cell clone. 140- and 170-kDa polypeptides that may be recombinant human type III collagen were detected in the culture supernatant.

20

[Example 23] Western blot analysis of recombinant human type III collagen in the culture supernatant

[0060] The polyacrylamide gel after SDS-polyacrylamide gel electrophoresis was immersed in a transfer buffer, and then human type III collagen in the polyacrylamide gel was transferred to a PVDF membrane by a conventional method. After blocking with Block Ace, the membrane was reacted with 1 μ g/mL of an antibody against the human type III collagen chain (Cosmo Bio Co., Ltd.), and then with an anti-rabbit IgG antibody labeled with horseradish peroxidase (HRP). Reacted antibodies were detected by a method of detecting HRP activity using the TMB peroxidase reagent (Funakoshi Co.). 100 μ g/mL of human type III collagen (Beckton, Dickinson and Company) was used as a comparative control. 140- and 170-kDa polypeptides that may be recombinant human type III collagen which can be bound by an antibody against the human type III collagen chain were detected in the culture supernatant (Fig. 13).

[0061] As observed with commercially available human type III atelocollagen (Beckton, Dickinson and Company), when treated with pepsin, the recombinant human type III collagen in the supernatant was detected as a polypeptide at 130kDa. These facts showed that recombinant human type III collagen that has a pepsin resistance substantially equivalent to that of the natural type was contained in the culture supernatant obtained from the human type III collagen-producing cell clone.

[Example 24] Purification of human type I and type III collagens in the culture supernatants

[0062] Purification was carried out using 100 mL of the culture supernatant containing human type I or type III collagen in Example 12. A purified collagen solution obtained from the original 100 mL culture supernatant was concentrated to approximately 300 μ L and electrophoresed by SDS-PAGE to confirm its purity. (Fig. 14).

Industrial Applicability

[0063] The present invention can provide expression vectors and human collagen-producing cells that enable production of recombinant human collagen that has high quality and is closer to the natural type. The invention can also provide cells that produce triple-helix structure human collagen.

[0064] The production methods of the present invention can be applied not only to collagen but also to proteins that have a triple-helix structure, such as collectin.

[0065] Furthermore, the collagen production method of the present invention may be used to produce large quantities of triple-helix structural collagen with a novel molecular composition, which cannot be produced (or has not been discovered) in nature, by simultaneously expressing different types of α chains. Triple-helix structure collagen with a novel molecular composition may have properties that are different from those of known collagen, and is therefore expected to be applied as a new material.

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SEQUENCE LISTING

[0066]

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OSAKA PREFECTURAL GOVERNMENT

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30 Claims

1. A method of producing a protein having a triple-helix structure, wherein the method comprises:

35 (a) introducing DNA encoding a protein having a triple-helix structure into a vector;
 (b) transforming a Chinese hamster ovary (CHO) cell by transfer of the gene vector; and
 (c) culturing or breeding the transformant, and collecting the protein having a triple helix structure from the cell or culture supernatant thereof,

40 wherein the protein having a triple-helix structure is human type I collagen or a partial peptide thereof.

2. The method of claim 1, wherein the human type I collagen is a complex of $\alpha 1$ and $\alpha 2$ chains.

3. The method of claim 1, wherein the DNA encoding a protein having a triple helix structure is at least a DNA selected 45 from:

(a) a DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 4; and
 (b) a DNA hybridizing under stringent conditions with a DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 4.

50 4. The method of any one of claims 1 to 3, wherein the vector to be introduced with the DNA encoding a protein having a triple helix structure is pNOW/CMV-AA.

55 Patentansprüche

1. Verfahren zur Herstellung eines Proteins mit einer Triple-Helix-Struktur, wobei das Verfahren umfasst:

- 5 (a) Einbringen einer DNA, welche ein Protein mit einer Triple-Helix-Struktur kodiert, in einen Vektor;
(b) Transformieren einer CHO (Chinese hamster ovary)-Zelle mittels Transfer des Genvektors; und
(c) Kultivieren oder Züchten der Transformante, und Sammeln des Proteins mit einer Triple-Helix-Struktur aus der Zelle oder dem Kulturüberstand davon,

wobei das Protein mit einer Triple-Helix-Struktur menschliches Typ I-Kollagen oder ein Teilpeptid davon ist.

2. Verfahren nach Anspruch 1, wobei das menschliche Typ I-Kollagen ein Komplex aus $\alpha 1$ - und $\alpha 2$ -Ketten ist.

10 3. Verfahren nach Anspruch 1, wobei die DNA, welche ein Protein mit einer Triple-Helix-Struktur kodiert, mindestens eine DNA ist, welche ausgewählt ist aus:

- 15 (a) einer DNA umfassend die Nukleotidsequenz von SEQ ID Nr: 1 oder 4; und
(b) einer DNA, welche unter stringenten Bedingungen mit einer DNA umfassend die Nukleotidsequenz von SEQ ID Nr: 1 oder 4 hybridisiert.

20 4. Verfahren nach einem der Ansprüche 1 bis 3, wobei der Vektor, in welchen die DNA, welche für ein Protein mit einer Triple-Helix-Struktur kodiert, einzubringen ist, pNOW/CMV-AA ist.

Revendications

1. Procédé de production d'une protéine possédant une structure à triple hélice, le procédé comprenant :

- 25 (a) l'introduction d'ADN codant une protéine possédant une structure à triple hélice dans un vecteur ;
(b) la transformation d'une cellule ovarienne de hamster chinois (OHC) par transfert du vecteur génique ; et
(c) culture ou élevage du transformant et collecte de la protéine possédant une structure à triple hélice à partir de la cellule ou du surnageant de culture de celle-ci,

30 dans lequel la protéine possédant une structure à triple hélice est du collagène de type 1 humain ou un peptide partiel de celui-ci.

2. Procédé selon la revendication 1, dans lequel le collagène de type 1 humain est un complexe de chaînes $\alpha 1$ et $\alpha 2$.

35 3. Procédé selon la revendication 1, dans lequel l'ADN codant une protéine possédant une structure à triple hélice est au moins un ADN choisi à partir :

- 40 (a) d'un ADN comprenant la séquence de nucléotides de SEQ ID NO : 1 ou 4 ; et
(b) un ADN hybridant dans des conditions strictes avec un ADN comprenant la séquence de nucléotides de SEQ ID NO : 1 ou 4.

45 4. Procédé selon l'une des revendications 1 à 3, dans lequel le vecteur à introduire avec l'ADN codant une protéine possédant une structure à triple hélice est pNOW/CMV-AA.

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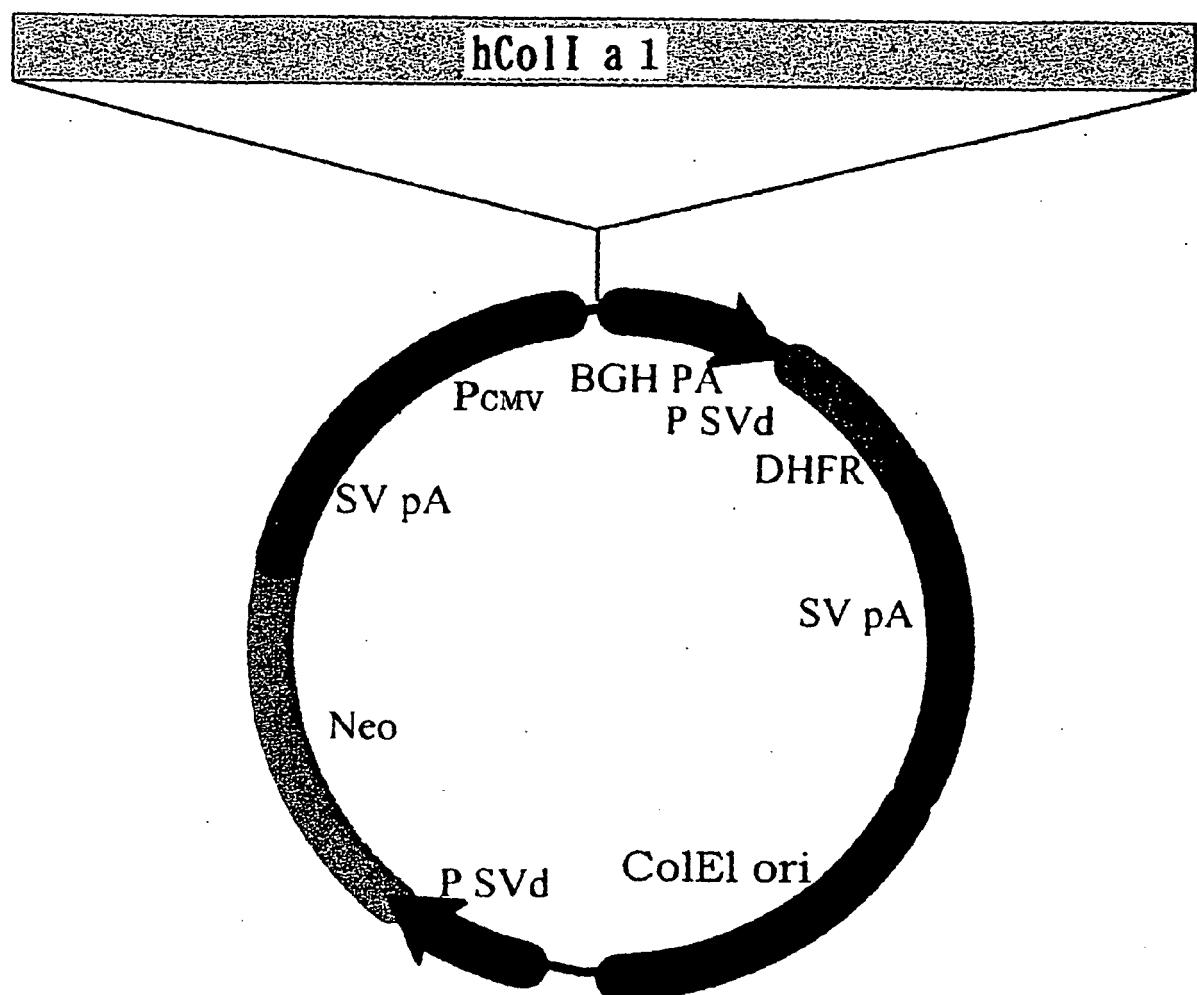


FIG. 1

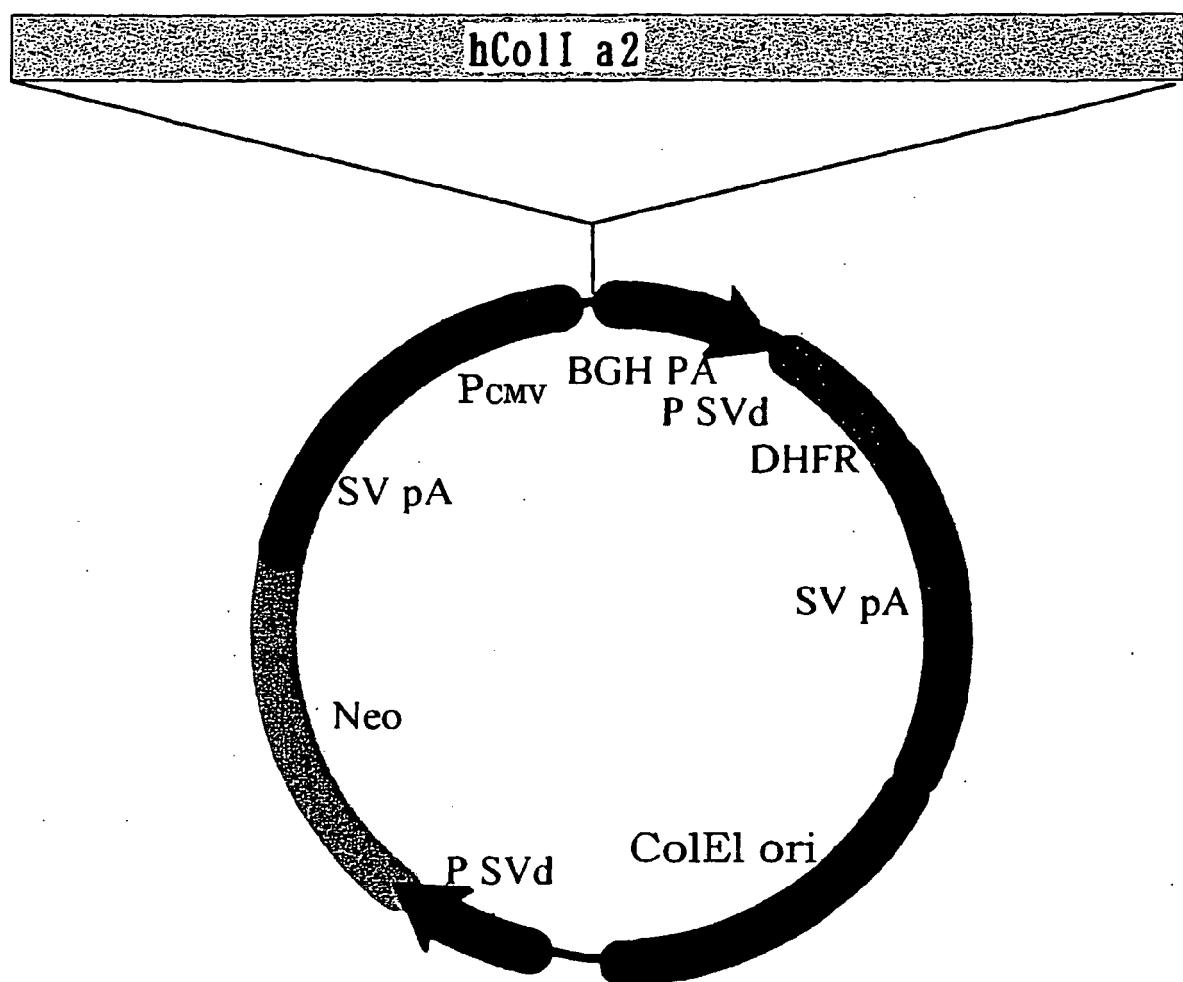


FIG. 2

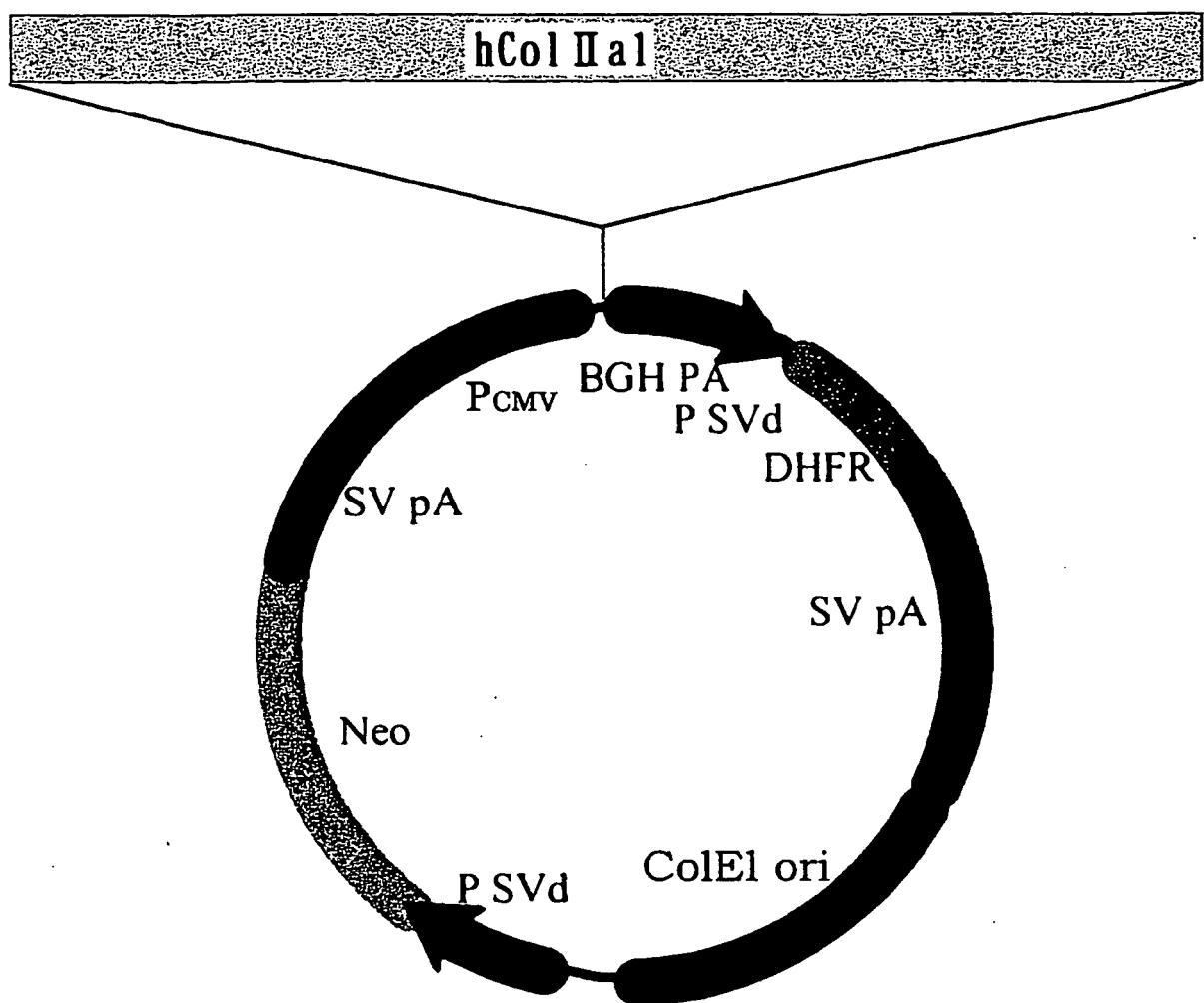


FIG. 3

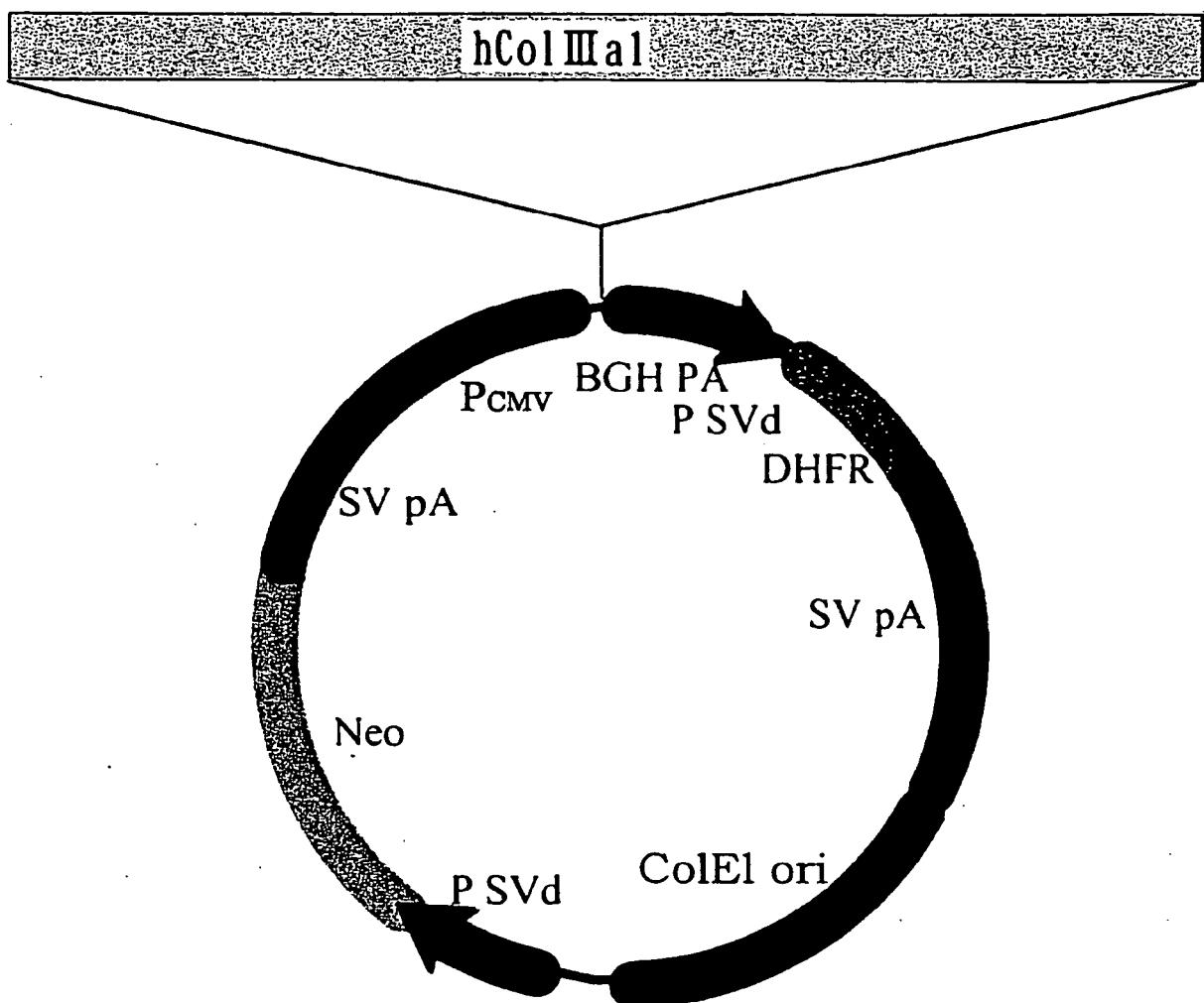


FIG. 4

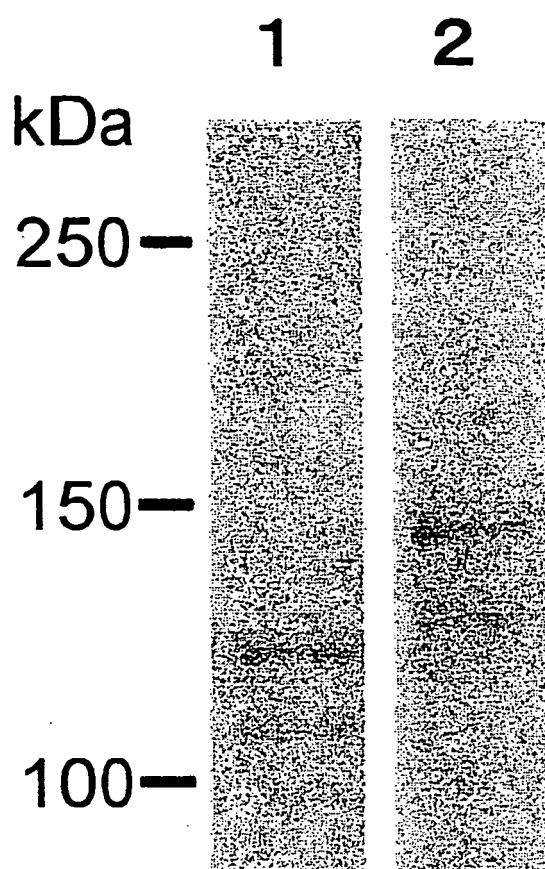


FIG. 5

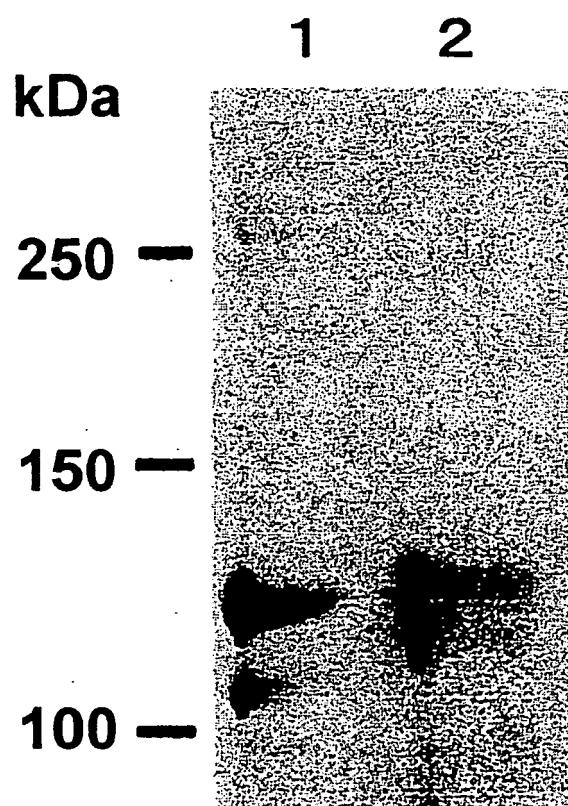


FIG. 6

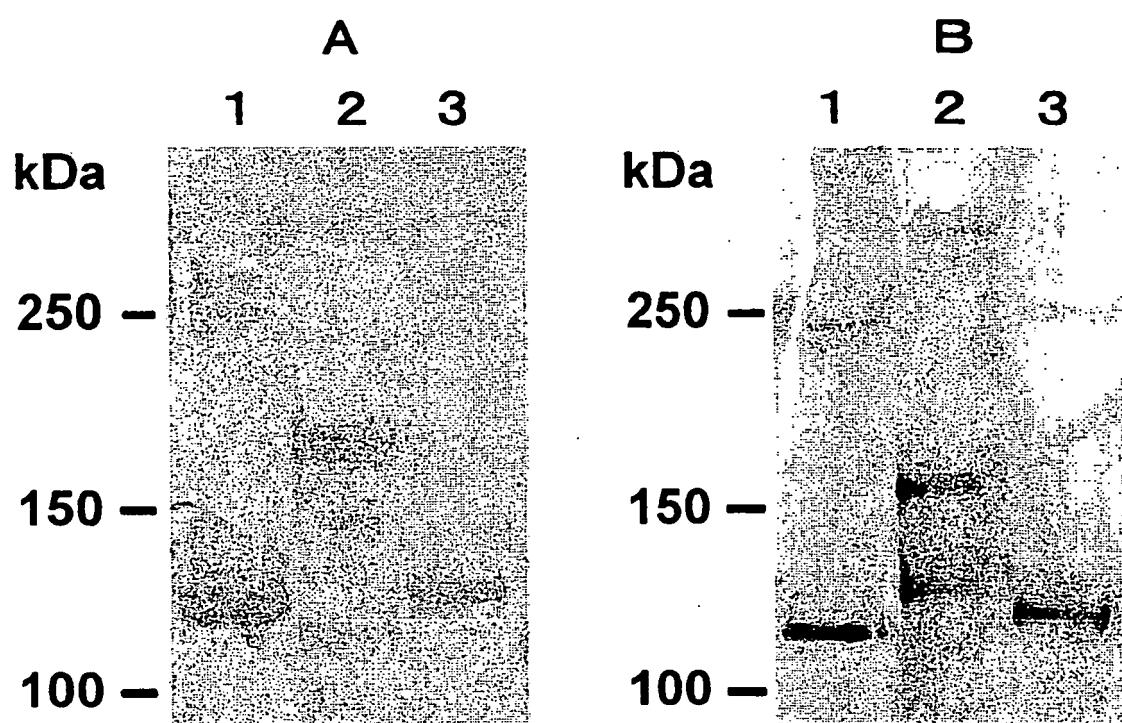


FIG. 7

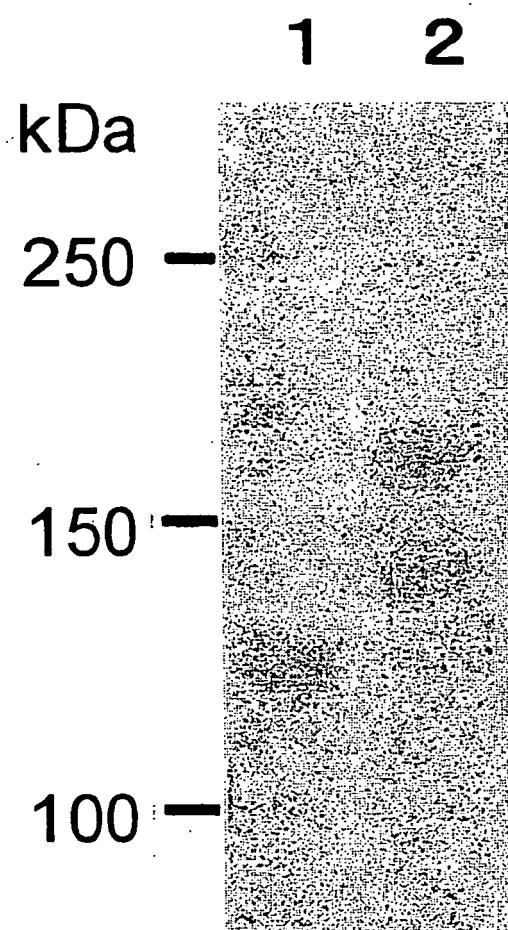


FIG. 8

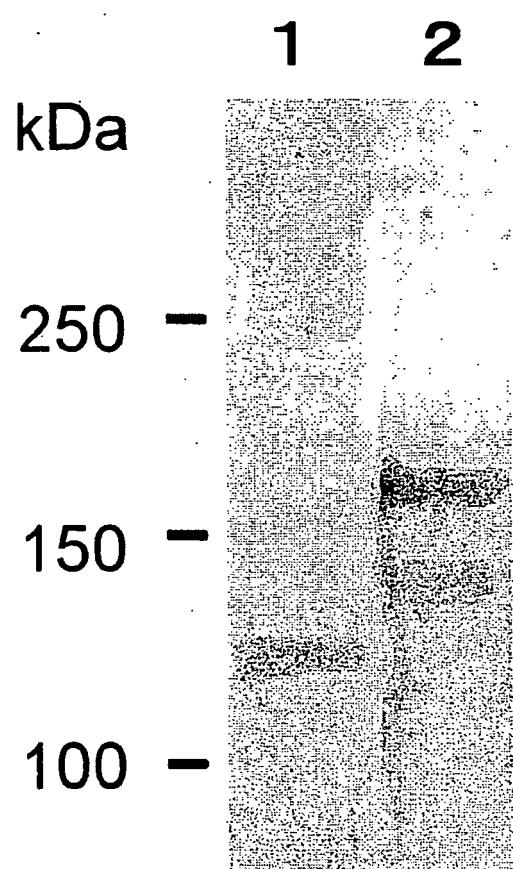


FIG. 9

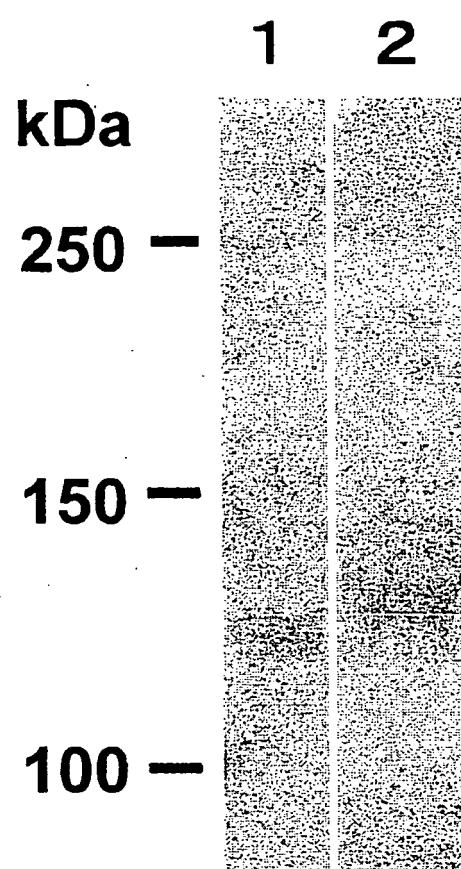


FIG. 10

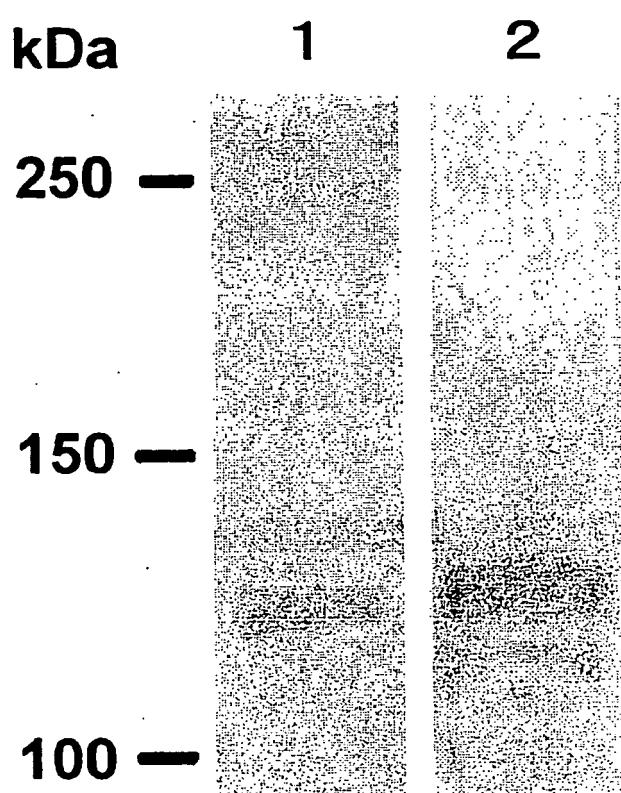


FIG. 11

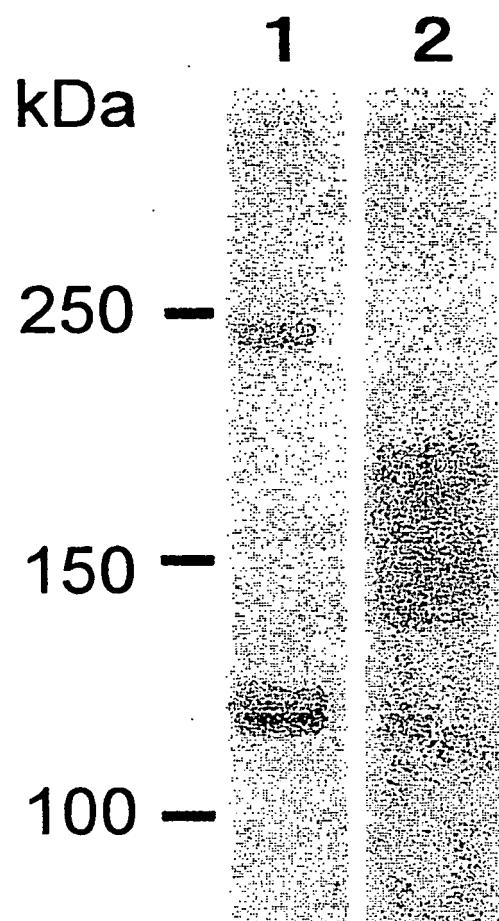


FIG. 12

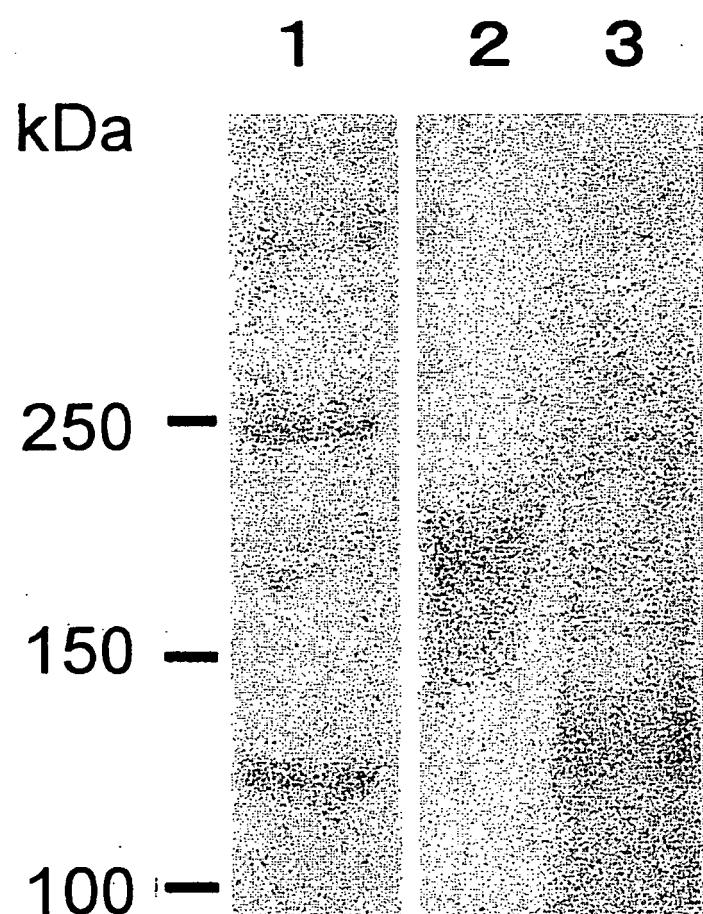


FIG. 13

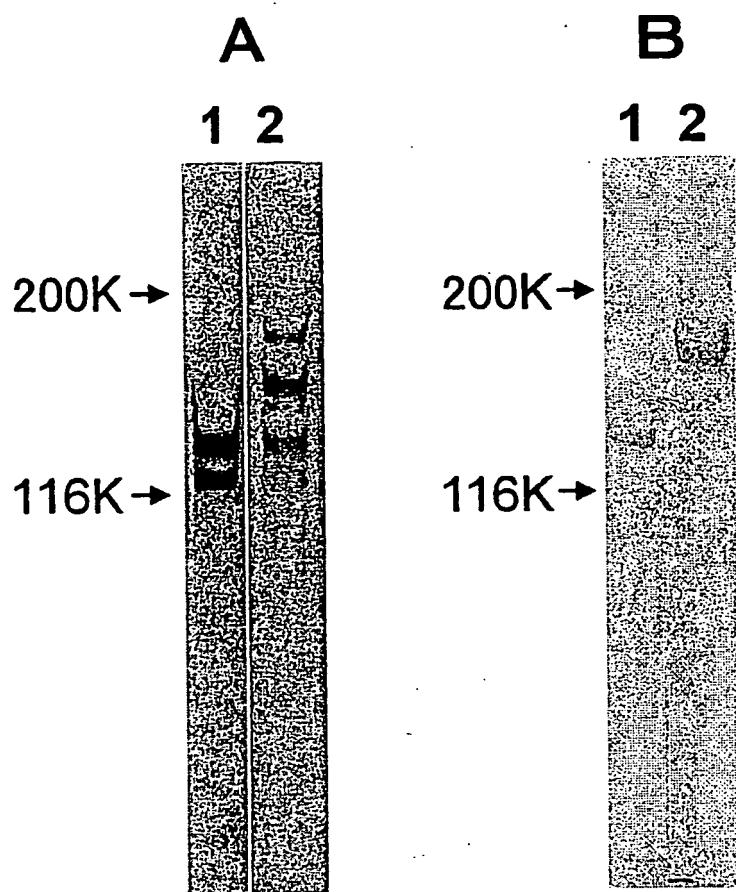


FIG. 14

REFERENCES CITED IN THE DESCRIPTION

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