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CA 2684883 C 2016/06/21

(11)(21) **2 684 883**

(12) **BREVET CANADIEN**
CANADIAN PATENT

(13) **C**

(86) **Date de dépôt PCT/PCT Filing Date:** 2008/04/24
(87) **Date publication PCT/PCT Publication Date:** 2008/11/06
(45) **Date de délivrance/Issue Date:** 2016/06/21
(85) **Entrée phase nationale/National Entry:** 2009/10/21
(86) **N° demande PCT/PCT Application No.:** JP 2008/058323
(87) **N° publication PCT/PCT Publication No.:** 2008/133350
(30) **Priorité/Priority:** 2007/04/24 (US60/913,713)

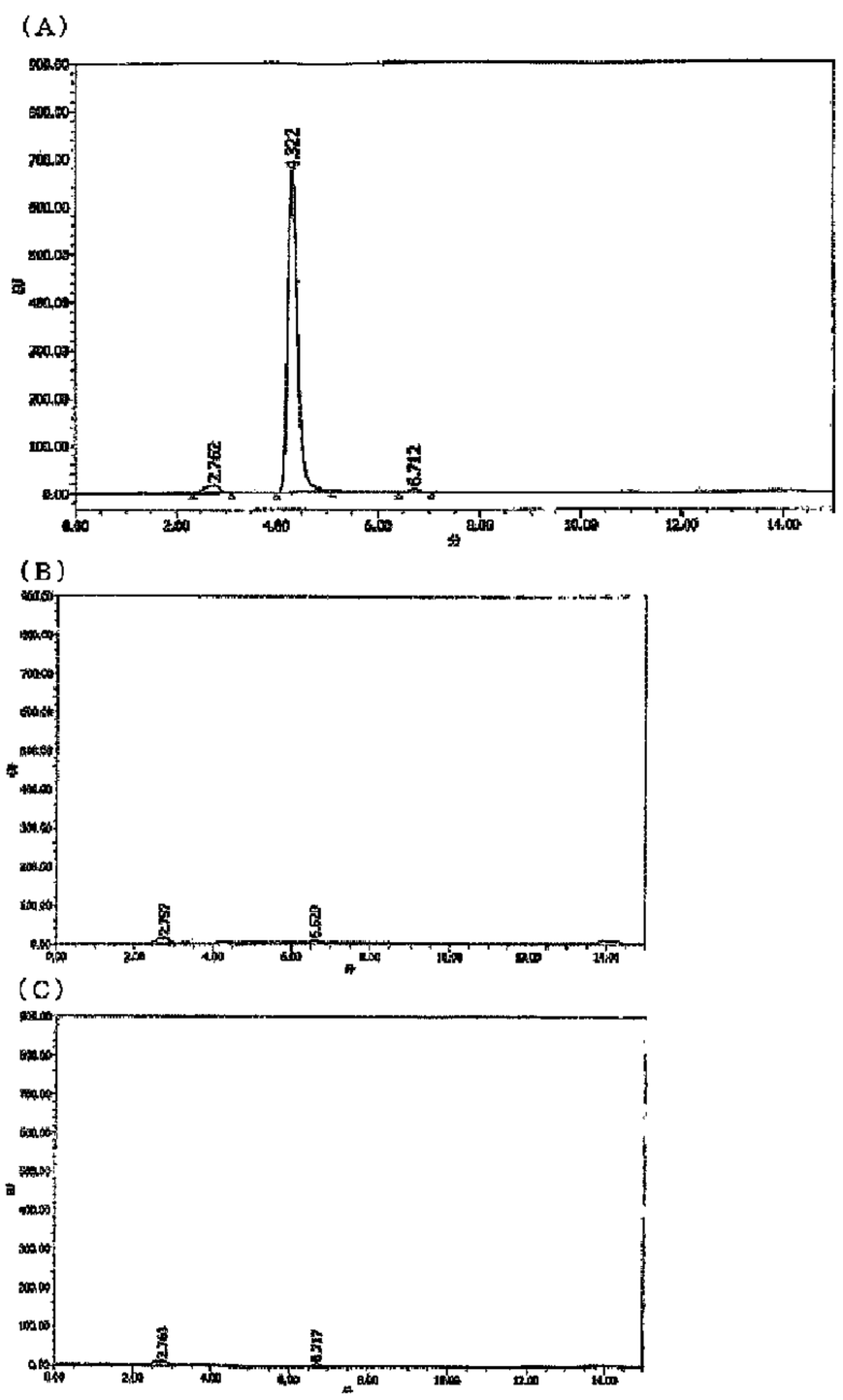
(51) **Cl.Int./Int.Cl.** ***C12N 9/10*** (2006.01),
C12N 9/90 (2006.01)

(72) **Inventeurs/Inventors:**
SUZUKI, KIYOSHI, JP;
MIYAMOTO, KENTARO, JP;
KASEYAMA, HIROMI, JP

(73) **Propriétaire/Owner:**
SEIKAGAKU CORPORATION, JP

(74) **Agent:** SMART & BIGGAR

(54) **Titre : BACTERIE PRODUISANT DE LA CHONDROITINE ET PROCEDE DE PRODUCTION DE CHONDROITINE**
(54) **Title: CHONDROITIN-PRODUCING BACTERIUM AND METHOD OF PRODUCING CHONDROITIN**



(57) **Abrégé/Abstract:**
Chondroitin is produced by: culturing a UDP-glucuronic acid-producing bacterium introduced with a kfoA gene derived from Escherichia coli K4 strain and a kfoC gene derived from Escherichia coli K4 strain and having chondroitin-producing ability; and collecting chondroitin from the bacterium.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 November 2008 (06.11.2008)

PCT

(10) International Publication Number
WO 2008/133350 A1

(51) International Patent Classification:
C12N 9/10 (2006.01) *C12N 9/90* (2006.01)

(21) International Application Number:
PCT/JP2008/058323

(22) International Filing Date: 24 April 2008 (24.04.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/913,713 24 April 2007 (24.04.2007) US

(71) Applicant *(for all designated States except US)*: **SEIKAGAKU CORPORATION** [JP/JP]; 6-1, Marunouchi 1-chome, Chiyoda-ku, Tokyo, 1000005 (JP).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SUZUKI, Kiyoshi** [JP/JP]; c/o SEIKAGAKU CORPORATION Central Research Laboratories, 1253, Tateno 3-chome, Higashiyamato-shi, Tokyo, 2070021 (JP). **MIYAMOTO, Kentaro** [JP/JP]; c/o SEIKAGAKU CORPORATION Central Research Laboratories, 1253, Tateno 3-chome, Higashiyamato-shi, Tokyo, 2070021 (JP). **KASEYAMA, Hiromi** [JP/JP]; c/o SEIKAGAKU CORPORATION Central Research Laboratories, 1253, Tateno 3-chome, Higashiyamato-shi, Tokyo, 2070021 (JP).

(74) Agents: **KAWAGUCHI, Yoshiyuki** et al.; Acropolis 21 Building 6th floor, 4-10, Higashi Nihonbashi 3-chome, Chuo-ku, Tokyo, 1030004 (JP).

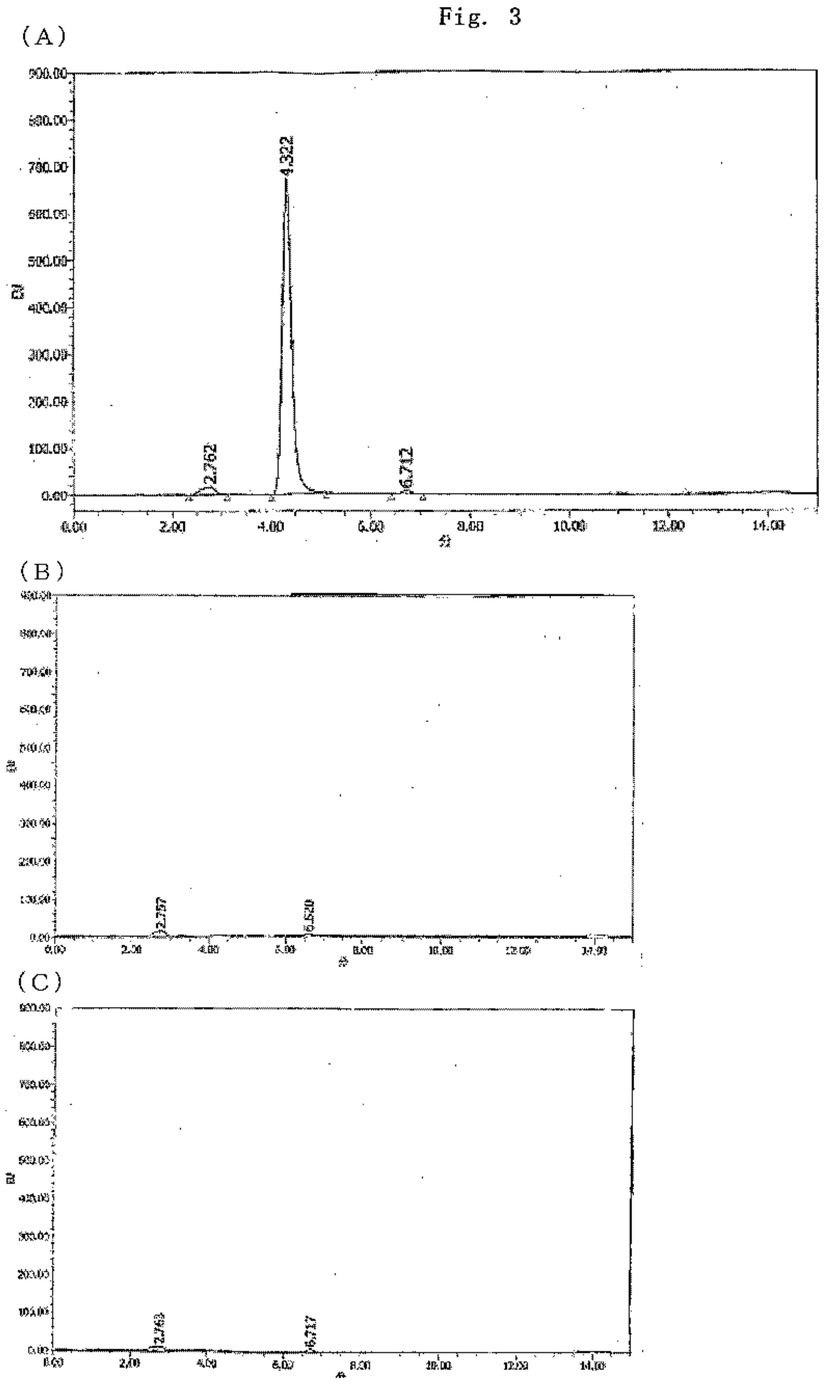
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH,

[Continued on next page]

(54) Title: CHONDROITIN-PRODUCING BACTERIUM AND METHOD OF PRODUCING CHONDROITIN

Fig. 3



(57) Abstract: Chondroitin is produced by: culturing a UDP-glucuronic acid-producing bacterium introduced with a kfoA gene derived from Escherichia coli K4 strain and a kfoC gene derived from Escherichia coli K4 strain and having chondroitin-producing ability; and collecting chondroitin from the bacterium.

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WO 2008/133350 A1

GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

DESCRIPTION

CHONDROITIN-PRODUCING BACTERIUM AND METHOD OF PRODUCING CHONDROITIN

[0001]

Technical Field

The present invention relates to a chondroitin-producing bacterium and a method of producing chondroitin.

[0002]

Background Art

Chondroitin is a polysaccharide comprising a repeated structure of disaccharides of a glucuronic acid (GlcUA) residue and a N-acetyl-D-galactosamine (GalNAc) residue (-GlcUAβ(1-3)-GalNAcβ(1-4)-; in the present specification, also referred to as a chondroitin carbohydrate backbone). Chondroitin sulfate is a polysaccharide which consists by sulfation of chondroitin.

Conventionally, chondroitin and chondroitin sulfate are extracted and purified from cartilages, organs, and the like of animals. However, in recent years, a technique for artificially synthesizing a carbohydrate backbone common to chondroitin and chondroitin sulfate has been studied because of shortage of such materials as cartilages and organs.

[0003]

Chondroitin synthases, which produce chondroitin by alternately transferring GlcUA and GalNAc from their donor substrates to acceptor oligosaccharides, have been reported, and a method of producing chondroitin using the enzyme has been proposed.

J. Biol. Chem. 275(31), 24124-24129 (2000) discloses a chondroitin synthase derived from *Pasteurella multocida*.

In addition, WO 2003/102193 and WO 2003/102194 disclose chondroitin synthases derived from human.

Moreover, US 2003-0109693 (JP 2003-199583 A) discloses a novel

chondroitin synthase (KfoC) produced by *Escherichia coli* K4 strain.

However, in the case of producing chondroitin by the enzymatic methods, it is necessary to prepare expensive materials such as oligosaccharides as acceptors and sugar nucleotides for donors of GlcUA and GalNAc, and thus a method of producing chondroitin using more inexpensive materials has been desired.

[0004]

Escherichia coli K4 strain is known to produce a polysaccharide having a chondroitin backbone structure as a capsule. However, its structure consists of a repeating unit comprising trisaccharides of a GalNAc residue, a GlcUA residue and a fructose residue that is bound to a C3-hydroxyl group of a GlcUA residue. In addition, over 100 chemically different capsular polysaccharides have been detected in *Escherichia coli*. For example, *Escherichia coli* K5 strain produces a capsule polysaccharide K5 which has a carbohydrate backbone of heparin/heparan sulfate (J. Biol. Chem., 272(5), p2682-2687, 1997). However, the existence of *Escherichia coli* which produces chondroitin itself has not been known. Therefore, a method of producing chondroitin using an *Escherichia* bacterium is unknown.

Although US Published Patent Application 2007-0281342 discloses a method of producing chondroitin by using a recombinant gram-positive *Bacillus* bacterium introduced with a chondroitin synthase gene derived from *Pasteurella multocida*, further development in fermentative production of chondroitin has been desired.

[0005]

Disclosure of the Invention

An object of the present invention is to provide a novel microorganism capable of producing chondroitin and a novel method of producing chondroitin using the microorganism.

[0006]

The inventors of the present invention have made extensive studies, and as a result, have discovered that a UDP-glucuronic

acid-producing bacterium such as *Escherichia coli* K5 strain which is introduced with kfoA and kfoC genes derived from *Escherichia coli* K4 strain produces chondroitin at high efficiency, thus completed the present invention.

[0007]

An object of the present invention is to provide a UDP-glucuronic acid-producing bacterium, which is introduced with a kfoA gene derived from *Escherichia coli* K4 strain and a kfoC gene derived from *Escherichia coli* K4 strain and has chondroitin-producing ability.

Here, the kfoA gene derived from *Escherichia coli* K4 strain is preferably a gene encoding a protein selected from the group consisting of the following (A) and (B):

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

(B) a protein comprising an amino acid sequence of SEQ ID NO: 2 including substitution, deletion, insertion, or addition of one or several amino acids and having UDP-glucose-4-epimerase activity.

The kfoA gene derived from *Escherichia coli* K4 strain is preferably a DNA selected from the group consisting of the following (a) and (b):

(a) a DNA comprising the nucleotide sequence of SEQ ID NO: 1; and

(b) a DNA that hybridizes with a DNA comprising the nucleotide sequence complementary to SEQ ID NO: 1 under stringent conditions and encodes a protein comprising UDP-glucose-4-epimerase activity.

In addition, the kfoC gene derived from *Escherichia coli* K4 strain is preferably a gene encoding a protein selected from the group consisting of the following (C) and (D):

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

(D) a protein comprising an amino acid sequence of SEQ ID NO: 4 including substitution, deletion, insertion, or addition of one

or several amino acids and having chondroitin synthase activity.

In addition, the kfoC gene derived from *Escherichia coli* K4 strain is preferably a DNA selected from the group consisting of the following (c) and (d):

(c) a DNA comprising the nucleotide sequence of SEQ ID NO: 3; and

(d) a DNA that hybridizes with a DNA comprising the nucleotide sequence complementary to SEQ ID NO: 3 under stringent conditions and encodes a protein having chondroitin synthase activity.

In addition, the kfoC gene derived from *Escherichia coli* K4 strain is preferably a gene encoding a protein selected from the group consisting of the following (E) and (F):

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

(F) a protein comprising an amino acid sequence of SEQ ID NO: 6 including substitution, deletion, insertion, or addition of one or several amino acids and having chondroitin synthase activity.

In addition, the kfoC gene derived from *Escherichia coli* K4 strain is preferably a DNA selected from the group consisting of the following (e) and (f):

(e) a DNA comprising the nucleotide sequence of SEQ ID NO: 5; and

(f) a DNA that hybridizes with a DNA comprising the nucleotide sequence complementary to SEQ ID NO: 5 under stringent conditions and encodes a protein having chondroitin synthase activity.

The UDP-glucronic acid-producing bacterium is preferably *Escherichia coli* K5 strain.

[0008]

Another object of the present invention is to provide a method of producing chondroitin comprising at least the following steps (1) and (2):

(1) culturing the bacterium as described above; and

(2) collecting chondroitin from the culture.

[0009]

Another object of the present invention is to provide a method of producing chondroitin sulfate comprising: producing chondroitin by the method as described above; and then sulfating the chondroitin to yield chondroitin sulfate.

[0010]

Another object of the present invention is to provide a vector comprising a kfoA gene derived from *Escherichia coli* K4 strain and a kfoC gene derived from *Escherichia coli* K4 strain.

[0010A]

The present invention as claimed relates to:

- a UDP-glucuronic acid-producing recombinant bacterium, which is introduced with a kfoA gene which encodes a protein selected from the group consisting of (A) and (B) and a kfoC gene which encodes a protein selected from the group consisting of (C), (D), (E) and (F), and said bacterium produces chondroitin as a capsular polysaccharide: (A) a protein comprising the amino acid sequence of SEQ ID NO: 2; (B) a protein comprising an amino acid sequence of SEQ ID NO: 2 including substitution, deletion, insertion, or addition of 1-20 amino acids and having UDP-glucose-4-epimerase activity; (C) a protein comprising the amino acid sequence of SEQ ID NO: 4; (D) a protein comprising an amino acid sequence of SEQ ID NO: 4 including substitution, deletion, insertion, or addition of 1-20 amino acids and having chondroitin synthase

activity; (E) a protein comprising the amino acid sequence of SEQ ID NO: 6; and (F) a protein comprising an amino acid sequence of SEQ ID NO: 6 including substitution, deletion, insertion, or addition of 1-20 amino acids and having chondroitin synthase activity; and

- a UDP-glucuronic acid-producing recombinant bacterium, which is introduced with the kfoA gene which is a DNA selected from the group consisting of (a) and (b), and a kfoC gene which is a DNA selected from the group consisting of (c), (d), (e) and (f), and said bacterium produces chondroitin as a capsular polysaccharide: (a) a DNA comprising the nucleotide sequence of SEQ ID NO: 1; (b) a DNA that hybridizes with a DNA comprising the nucleotide sequence complementary to SEQ ID NO: 1 under stringent conditions and encodes a protein having UDP-glucose-4-epimerase activity; (c) a DNA comprising the nucleotide sequence of SEQ ID NO: 3; (d) a DNA that hybridizes with a DNA comprising the nucleotide sequence complementary to SEQ ID NO: 3 under stringent conditions and encodes a protein having chondroitin synthase activity; (e) a DNA comprising the nucleotide sequence of SEQ ID NO: 5; and (f) a DNA that hybridizes with a DNA comprising the nucleotide sequence complementary to SEQ ID NO: 5 under stringent conditions and encodes a protein having chondroitin synthase activity; wherein the stringent conditions are conditions for hybridization at 42°C in a solution containing 50% formamide, 4 × SSC, 50 mM HEPES (pH 7.0), 10 × Denhardt's solution, 100 µg/ml salmon sperm DNA and washing with 2 × SSC, 0.1% SDS solution at room temperature and then 0.1 × SSC, 0.1% SDS solution at 60°C.

[0011]

Brief Description of the Drawings

Fig. 1 is a drawing showing a structure of a vector for expressing kfoA and kfoC genes.

Fig. 2 is a drawing (photograph) showing expression of KfoA and KfoC proteins. Lane 1 shows a strain introduced with no plasmid (control), Lane 2 shows a strain introduced with pTrcHis-kfoCA, and M shows a molecular weight marker.

Fig. 3 shows the results of disaccharide composition analyses using fluorescent HPLC system for the fraction L treated with chondroitinase ABC (cABC) (A), the fraction L treated with no cABC (B), and the fraction L treated with heat-inactivated cABC (C).

Fig. 4 shows results of fluorescence disaccharide analyses for the fraction E treated with cABC (A), the fraction E treated with no cABC (B), and the fraction E treated with heat-inactivated cABC (C).

Fig. 5 shows results of fluorescence disaccharide analyses for the fraction S treated with cABC (A), the fraction S treated with no cABC (B), and the fraction S treated with heat-inactivated cABC (C).

Fig. 6 shows results of fluorescence disaccharide analyses for the chondroitin standard treated with cABC (A) and a mixture of the

chondroitin standard treated with cABC and the fraction L treated with cABC (B).

Fig. 7 is a drawing (photograph) showing expression of KfoA, KfoC and Kfo⊿C proteins using (A) an anti-KfoC antibodies (EK-C) as 1st antibody, (B) an anti-KfoA antibodies (EK-A) as 1st antibody and (C) normal rabbit serum. Lane 1 and 5 show molecular marker, MagicMarkXP standard (Invitrogen), Lane 2 shows a strain introduced with pTrcHis-kfoCA, Lane 3 shows a strain introduced with pTrcHis-kfo⊿CA and Lane 4 shows a strain introduced with pTrcHis-kfoA.

[0012]

Description of the Preferred Embodiments

Hereinafter, the present invention will be described in detail.

<1> Bacterium of the present invention

The bacterium of the present invention is a UDP-glucuronic acid-producing bacterium introduced with kfoA and kfoC genes derived from *Escherichia coli* K4 strain and having chondroitin-producing ability.

The "UDP-glucuronic acid-producing bacterium" provides UDP-glucuronic acid, which is used for production of polysaccharide composed of glucuronic acid. The "UDP-glucuronic acid-producing bacterium" is not limited as long as it produces UDP-glucuronic acid, and examples thereof include a bacterium belonging to the genus *Gluconacetobacter* such as *Gluconacetobacter hansenii* and *Gluconacetobacter xylinus*, a bacterium belonging to the genus *Rhizobium* such as *Rhizobium meffloti*, a bacterium belonging to the genus *Acetobacter* such as *Acetobacter xylinum*, a bacterium belonging to the genus *Erwinia* such as *Erwinia amylovora*, a bacterium belonging to the genus *Thiobacillus* such as *Thiobacillus ferrooxidans*, a bacterium belonging to the genus *Xylella* such as *Xylella fastidiosa*, a bacterium belonging to the genus *Sinorhizobium* such as *Sinorhizobium meliloti*, a bacterium belonging to the genus *Rhodococcus* such as *Rhodococcus rhodochrous*, a bacterium belonging to the genus *Klebsiella*

such as *Klebsiella aerogenes*, a bacterium belonging to the genus *Enterobacter* such as *Enterobacter aerogenes*, and a bacterium belonging to the genus *Escherichia* such as *Escherichia coli*.

The "UDP-glucuronic acid-producing bacterium" is preferably a bacterium belonging to the genus *Escherichia* and capable of producing UDP-glucuronic acid, and more preferably a bacterium belonging to *Escherichia coli* and capable of producing UDP-glucuronic acid.

A specific example thereof includes *Escherichia coli* K5 strain. The K5 strain has been deposited at the American Type Culture Collection (ATCC: P.O. Box 1549, Manassas, VA 20108, United States of America) under the accession number of ATCC23506 and is available from the catalogue or homepage of ATCC.

The strain to be introduced with the kfoA and kfoC genes may be a strain which is a derivative of the *Escherichia coli* K5 strain, and such derivative strains may be obtained by introducing a gene mutation into the *Escherichia coli* K5 strain or introducing a gene into the *Escherichia coli* K5 strain by genetic recombination. That is, one aspect of the bacterium of the present invention includes: a strain obtained by introducing kfoA and kfoC genes derived from *Escherichia coli* K4 strain into *Escherichia coli* K5 strain; a strain obtained by introducing kfoA and kfoC genes derived from *Escherichia coli* K4 strain into *Escherichia coli* K5 strain and further introducing a gene mutation; and a strain obtained by introducing kfoA and kfoC genes derived from *Escherichia coli* K4 strain into *Escherichia coli* K5 strain and further introducing another gene.

[0013]

Examples of the kfoA gene derived from *Escherichia coli* K4 strain include a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2 and a DNA comprising the nucleotide sequence of SEQ ID NO: 1.

In general, substitution, deletion, insertion, or addition in one or several amino acids that constitute a protein has no effect

on the activity of the protein, in many cases, and therefore, the kfoA gene derived from *Escherichia coli* K4 strain may be a gene encoding a protein comprising an amino acid sequence of SEQ ID NO: 2 including substitution, deletion, insertion, or addition of one or several amino acids and having UDP-glucose-4-epimerase activity.

The phrase "one or several amino acids" as used herein refers to the number of amino acids that may cause a substitution, deletion, insertion, or addition without impairing UDP-glucose 4-epimerase activity. Specifically, the number is, for example, an integer of 1 to 20, preferably an integer of 1 to 10, more preferably an integer of 1 to 5.

Meanwhile, the kfoA gene derived from *Escherichia coli* K4 strain may be a gene encoding a protein comprising an amino acid sequence that is not less than 90% identical, preferably not less than 95% identical, more preferably not less than 98% identical to the entire sequence of SEQ ID NO: 2, and having UDP-glucose 4-epimerase activity.

[0014]

The kfoA gene derived from *Escherichia coli* K4 strain may be obtained by PCR using a chromosomal DNA of *Escherichia coli* K4 strain as a template.

Escherichia coli K4 strain has been deposited at the American Type Culture Collection (ATCC) under the accession number of ATCC23502 and is available from the catalogue or homepage of ATCC.

The gene encoding a protein comprising an amino acid sequence of SEQ ID NO: 2 including substitution, deletion, insertion, or addition of one or several amino acids or an amino acid sequence that is not less than 90% identical to the entire sequence of SEQ ID NO: 2 and having UDP-glucose-4-epimerase activity may be obtained by modifying the nucleotide sequence of SEQ ID NO: 1 so that substitution, deletion, insertion, or addition of one or several amino acids is introduced into the amino acid sequence of SEQ ID NO: 2 by site-specific mutagenesis (Kramer, W. and Frits, H. J., Meth. in Enzymol., 154,

350(1987); Kunkel, T.A. et al., Meth. in Enzymol., 154, 367(1987)) or the like.

The gene may also be obtained by: screening a DNA encoding a protein having UDP-glucose 4-epimerase activity by hybridization with a DNA comprising the nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1 or a partial sequence thereof under stringent conditions.

The term "stringent conditions" refers to conditions under which so-called specific hybrid is formed and non-specific hybrid is not formed (see Sambrook, J. et al., Molecular Cloning A Laboratory Manual, second Edition, Cold Spring Harbor Laboratory Press (1989), etc.). Specific examples of the "stringent conditions" include conditions for hybridization at 42°C in a solution containing 50% formamide, 4 × SSC, 50 mM HEPES (pH 7.0), 10 × Denhardt's solution, 100 µg/ml salmon sperm DNA and washing with 2 × SSC, 0.1% SDS solution at room temperature and then 0.1 × SSC, 0.1% SDS solution at 60°C.

Whether the gene obtained as described above encodes a protein having UDP-glucose 4-epimerase activity can be judged by introducing the resultant gene into an appropriate host to express the protein and then measuring the UDP-glucose 4-epimerase activity in accordance with the method described in J. Biol. Chem., 277(24), p21567-21575, 2002.

[0015]

Examples of the kfoC gene derived from *Escherichia coli* K4 strain include a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 4 and a DNA comprising the nucleotide sequence of SEQ ID NO: 3. Examples of the kfoC gene derived from *Escherichia coli* K4 strain also include a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 6 and a DNA comprising the nucleotide sequence of SEQ ID NO: 5.

The kfoC gene derived from *Escherichia coli* K4 strain may be a gene encoding a protein comprising an amino acid sequence of SEQ

ID NO: 4 or 6 including substitution, deletion, insertion, or addition of one or several amino acids and having chondroitin synthase activity.

The kfoC gene derived from *Escherichia coli* K4 strain may be a gene encoding a protein comprising an amino acid sequence that is not less than 90% identical, preferably not less than 95% identical, more preferably not less than 98% identical to the entire sequence of SEQ ID NO: 4 or 6 and having chondroitin synthase activity.

The term "chondroitin synthase activity" as used herein refers to an activity to alternately transfer GlcUA from a GlcUA donor or GalNAc from a GalNAc donor to the non-reduced end of a sugar chain.

[0016]

The kfoC gene derived from *Escherichia coli* K4 strain may be obtained by PCR using a chromosomal DNA of *Escherichia coli* K4 strain as a template. Furthermore, the kfoC gene encoding the amino acid sequence of SEQ ID NO: 6 such as a DNA comprising the nucleotide sequence of SEQ ID NO: 5 may be obtained according to the method described in WO2007/145197.

The gene encoding a protein comprising an amino acid sequence of SEQ ID NO: 4 or 6 including substitution, deletion, insertion, or addition of one or several amino acids or an amino acid sequence that is not less than 90% identical to the entire sequence of SEQ ID NO: 4 or 6 and having chondroitin synthase activity may be obtained by modifying the nucleotide sequence of SEQ ID NO: 3 or 5 so that substitution, deletion, insertion, or addition of one or several amino acids is introduced into the amino acid sequence of SEQ ID NO: 4 or 6 by site-specific mutagenesis or the like.

The gene may also be obtained by: screening a DNA encoding a protein having chondroitin synthase activity by hybridization with a DNA comprising the nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 3 or 5 or a partial sequence thereof under stringent conditions.

The definitions of the terms "one or several" and "stringent

conditions" are the same as described above.

Whether the gene obtained as described above encodes a protein having chondroitin synthase activity can be judged by introducing the resultant gene into an appropriate host to express the protein and then measuring the chondroitin synthase activity in accordance with the method described in US 2003-0109693 (JP 2003-199583 A).

[0017]

The bacterium of the present invention can be obtained by introducing a kfoA gene derived from *Escherichia coli* K4 strain and a kfoC gene derived from *Escherichia coli* K4 strain into a UDP-glucuronic acid-producing bacterium as described above.

The term "introduction" used in the present invention includes introduction of both of the genes into a UDP-glucuronic acid-producing bacterium using a vector such as a plasmid or a bacteriophage and introduction of both of the genes on a chromosome of the UDP-glucuronic acid-producing bacterium by homologous recombination or the like.

The vector such as a plasmid or a bacteriophage to be used herein is not particularly limited as long as they can be used for gene introduction into *Escherichia coli*, and examples thereof include pTrcHis (Invitrogen Corporation), pET vector (Novagen), and pGEX vector (Amersham Pharmacia).

In introduction of a kfoA gene derived from *Escherichia coli* K4 strain and a kfoC gene derived from *Escherichia coli* K4 strain, native promoters of the genes may be used, but it is preferable to use a promoter that is potent in *Escherichia coli*, such as lac promoter, trp promoter, trc promoter, PR promoter, or lacUV promoter.

The kfoA gene and kfoC gene derived from *Escherichia coli* K4 strain may be introduced one by one or simultaneously introduced using a single vector. It is more preferable to use a vector such as a plasmid or a bacteriophage carrying both of the kfoA and kfoC genes because introduction and expression of the genes can be easily performed.

The kfoA and kfoC genes derived form *Escherichia coli* K4 strain may be introduced so that they are expressed as fusion proteins with other peptides.

Examples of the other peptides include polyhistidine tag and GST (glutathione-S-transferase) tag. Expression of the gene products as fusion proteins is preferable because detection (confirmation of expression) of the gene products can be easily performed.

Introduction of a gene may be performed by a known transformation method. Examples of the method include the electroporation method, the DEAE-dextran method, and the calcium phosphate method.

Introduction of a gene can be confirmed by: a method of detecting a gene, such as RT-PCR or Northern blotting; a method of peculiarly detecting expression of a recombinant protein, such as Western blotting; and an activity measurement method as described above.

If the kfoA gene derived from *Escherichia coli* K4 strain and the kfoC gene derived from *Escherichia coli* K4 strain introduced work in a UDP-glucuronic acid-producing bacterium, the bacterium acquires the ability to produce chondroitin as a capsular polysaccharide.

[0018]

<2> Method of producing chondroitin

The method of producing chondroitin of the present invention comprises at least the following steps (1) and (2).

(1) culturing a bacterium of the present invention.

(2) collecting chondroitin from the culture.

[0019]

The culture may be performed by a general method for culturing bacteria belonging to the genus *Escherichia*.

The culture medium is not particularly limited as long as it can be used for culture of bacteria belonging to the genus *Escherichia*, and preferable examples thereof include LB medium (Luria-Bertani medium) (containing 10.0 g of Bacto-tryptone, 5.0 g of Bacto-yeast

extract, and 5.0 g of NaCl per liter) and CYG medium (2.0% casamino acid, 0.5% yeast extract, and 0.2% glucose, adjust pH to 7.0 before autoclaving). In the case where a gene is introduced using a vector containing an antibiotics-resistant gene, the medium preferably contains an antibiotics corresponding to the gene.

Culture conditions are not particularly limited as long as bacteria belonging to the genus *Escherichia* can grow, and in order to produce chondroitin at high efficiency, culture is preferably performed at 20 to 40°C for 8 to 72 hours.

In order to produce chondroitin at high efficiency, a bacterium may be precultured in a plate or liquid medium.

[0020]

The method of collecting chondroitin from culture is not particularly limited as long as chondroitin can be collected, and examples thereof include the method as described in the Examples shown below. Specifically, harvested recombinant cells are suspended into PBS (phosphate-buffered saline) or the like; treating the cells with lysozyme, DNaseI, and proteinase K; and removing proteins. Chondroitin can be detected by, for example, treating the fraction with chondroitinase and performing a disaccharide composition analysis.

[0021]

<3> Method of producing chondroitin sulfate

Chondroitin sulfate can be produced by further sulfating chondroitin prepared from the recombinant bacteria disclosed in this invention.

The sulfation may be performed by a known method of sulfating glycosaminoglycan, which is not particularly limited, and examples thereof include the method described in JP 61-47701 A.

In addition, the sulfation may be performed using an enzyme for transferring a sulfate group to chondroitin (sulfotransferase). Under existence of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as

a sulfate donor, sulfotransferases can produce chondroitin sulfate by reacting to chondroitin produced by the recombinant bacteria disclosed in this invention. Examples of a known sulfotransferase for transferring a sulfate group to chondroitin include chondroitin 6-O-sulfate group transferase (J. Biol. Chem., 275(28), 21075-21080 (2000)) and galactosaminoglycan 4-sulfate group transferase (JP 2000-4877 A).

[0022]

EXAMPLES

Hereinafter, the present invention will be described in detail by referring to the examples.

[0023]

Example 1: Preparation of KfoC/KfoA co-expression vector

<Amplification of kfoA gene>

PCR was performed using a chromosomal DNA of *E. coli* K4 strain as a template according to the method described in J. Biol. Chem., Vol. 277, Issue 24, 21567-21575, and the resultant PCR product was inserted into pTrcHis (histidine fusion protein expression vector, Invitrogen), to thereby yield pTrcHis-kfoA.

Primers used are as follows:

K4A-SP: 5'-CGGGATCCCGATGAGTATTCTTAATCAAGC-3' (SEQ ID NO: 7) and

K4A-AS: 5'-GGAATTCCGGCCAGTCTACATGTTTATCAC-3' (SEQ ID NO: 8)

[0024]

<Amplification of kfoC gene>

PCR was performed using a chromosomal DNA of *E. coli* K4 strain as a template according to the method described in JP 2003-199583 A, and the resultant PCR product was inserted into pTrcHis, to thereby yield pTrcHis-kfoC.

Primers used are as follows:

K4C-SP: 5'-CGGGATCCCGATGAGTATTCTTAATCAAGC-3' (SEQ ID NO: 9) and

K4C-AS: 5'-GGAATTCCGGCCAGTCTACATGTTTATCAC-3' (SEQ ID NO: 10).

[0025]

<Preparation of pTrcHis-kfoCA>

pTrcHis-kfoCA was prepared from the above-mentioned pTrcHis-kfoA and pTrcHis-kfoC as follows.

First, the NotI-SalI site was introduced into the C-terminal sequence of pTrcHis-kfoC by PCR. PCR was performed using the primers shown below to amplify the full length of pTrcHis-kfoC from the C-terminal side of kfoC, and the resultant PCR product was self-ligated, to thereby yield a plasmid pTrcHis-kfoC-NotI-SalI.

Primers used are as follows:

NotSal-pTrcHisC-rev: 5′-GCGGCCGCAAAACAGCCAAGCTTCGAATTC-3′ (SEQ ID NO: 11) and

NotSal-pTrcHisC-for: 5′-ACGCGTCGACGGCGGATGAGAGAAGATTTTCA-3′ (SEQ ID NO: 12)

[0026]

Then, Primers (NotI-RBS-KfoA-N: 5′-GCGGCCGCAAAATTAAAGAGGTATATATTAATGTATCGA-3′ (SEQ ID NO: 13) and SalI-KfoA-C: 5′-GTCGACCTCTCATCCGCCAAAACA-3′(SEQ ID NO: 14)) were designed at the upstream of the ribosome binding site (RBS) and the C-terminal region of kfoA of pTrcHis-kfoA, respectively and used for PCR using pTrcHis-kfoA as a template. Then, the resultant PCR product was inserted into pCR4-TOPO (Invitrogen), to thereby yield pCR4-TOPO-kfoA.

An insert containing kfoA was excised from pCR4-TOPO-kfoA with NotI-SalI and introduced into the NotI-SalI site of pTrcHis-kfoC-NotI-SalI, and the resultant plasmid was named pTrcHis-kfoCA (Fig. 1).

[0027]

Example 2

Co-expression of the recombinant KfoC and KfoA

The expression vector, pTrcHis-kfoCA, was introduced into *Escherichia coli* K5 strain by electroporation (cell 100 μL, 200 Ω, 25 μF, 2.5 kV, cuvette 0.1 cm), to thereby yield *E. coli* K5/pTrcHis-kfoCA

strain. Colonies were transferred into LB medium supplemented with 100 ppm ampicillin, and then the seed culture was incubated at 37°C overnight. One mL of the seed culture was transferred to 20 mL of CYG medium supplemented with 100 ppm ampicillin. The recombinant bacteria were cultured at 37°C for 3 hours (OD600 = 1.8, 1.7-2.0), and then to the culture was added IPTG at a final concentration of 1.0 mM. The resulting culture was cultivated at 37°C for further 5 hours to induce expression of the recombinant proteins.

Bacterial cells were harvested from 900 µL of the culture by centrifugation. The obtained cells were suspended in 90 µL of Laemmli buffer (×1). After heating in a boiling water bath for 10 min, 10 µL of the supernatant was subjected to SDS-PAGE in a 5/20% gradient gel and transferred to a PVDF membrane, followed by Western analysis to detect expression of recombinant proteins. Anti-Penta-His-HRP (QIAGEN) diluted to 1/2,000 was used for detection of recombinant histidine fusion proteins. As a result, expressions of the recombinant proteins KfoA and KfoC were confirmed (Fig. 2).

[0028]

Example 3

Preparation of polysaccharide and its detection by disaccharide analysis

<Preparation of bacterial cells>

E. coli K5/pTrcHis-kfoCA strain was cultured in LB medium supplemented with 100 ppm ampicillin as a seed culture. The seed culture (750µL) was transferred into CYG medium supplemented with 100 ppm ampicillin (15 mL), and then it was cultured at 37°C for 3 hours. To the culture was added IPTG (final concentration: 1 mM), and cultivation was further performed for 5 hours.

The bacterial cells were harvested by centrifugation.

<Preparation of culture supernatant>

The seed culture (1 mL) was transferred into CYG medium supplemented with 100 ppm ampicillin (20 mL), and then it was cultured

at 37°C for 3 hours. To the culture was added IPTG (final concentration: 0.1 mM), and cultivation was further performed for 5 hours. The culture supernatant was harvested by centrifugation.

[0029]

<Preparation of polysaccharide fraction>

Polysaccharides were prepared from the recombinant cells and culture supernatant by the following three methods.

Preparation 1) The obtained bacterial described above were resuspended in PBS and treated with lysozyme and DNaseI at 37°C for 1 hour, followed by treatment with proteinase K at 37°C for 1 hour. Then, the enzymes were subjected to heat inactivation, and proteins were removed by precipitation with 70% ammonium sulfate. The resulting supernatant fraction was dialyzed against 10 mM Tris-HCl (pH 8.0) to yield Sample L.

[0030]

Preparation 2) The obtained bacterial described above were treated with the agents for purifying plasmid: P1 (containing lysozyme), P2, and P3 (QIAGEN) sequentially, according to the manufacturer's instructions. After harvesting by centrifugation, the supernatant was subjected to ethanol precipitation. The precipitates were harvested by centrifugation and resuspended in 100 µL of sterilized water. The suspension was treated with DNaseI at 37°C for 1 hour to digest DNAs, followed by treatment with proteinase K at 37°C for 1 hour. Then, the enzymes were subjected to heat inactivation, and proteins were removed by precipitation with 70% ammonium sulfate. The resulting supernatant fraction was dialyzed against 10 mM Tris-HCl (pH 8.0) to yield Sample E.

Preparation 3) The culture supernatant (20 mL) was evaporated to dryness. The dried materials were dissolved into 2 ml of distilled water. The solution was dialyzed against 10 mM Tris-HCl (pH 8.0) to yield Sample S.

[0031]

<Detection of chondroitin by disaccharide analysis>

Samples L, E and S (100 μL each) were treated with cABC (chondroitinase ABC; manufactured by Seikagaku Corporation) in 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaOAc at 37°C overnight.

As controls, Samples L, E and S treated with no cABC and Samples L, E and S treated with heat-inactivated cABC were prepared. Ultrafiltration was performed to remove macromolecules with sizes equal to or more than 10,000, and the samples were analyzed by disaccharide composition analysis using a fluorescent HPLC system (J Biol Chem. 2000 Jul 21;275(29):2269-2275.) (Figs. 3, 4 and 5). Senshu Pak Decosil C22 (4.6 I.D. ×150mm) was used as a separation column in the HPLC system, and excitation and emission wavelength of the fluorescence detector were set to 346nm and 410nm, respectively.

As a result, only in the cases of the samples treated with cABC, specific peaks were detected. The disaccharide composition profile of each sample was closely corresponding to the profile of the sample obtained by treating the chondroitin standard with cABC as shown in Fig. 6 (A). Meanwhile, in the case where the chondroitin standard treated with cABC was mixed with Sample L treated with cABC, only one peak was detected, and thus Sample L was confirmed to be containing chondroitin (Fig. 6 (B)). In the case of Sample E and S, the same results were obtained (data not shown).

[0032]

Example 4

Preparation of Kfo⊿C/KfoA co-expression vector

<Amplification of kfo⊿C gene>

According to the method described in WO2007/145197, a DNA encoding the N-terminal-truncated KfoC comprising the nucleotide sequence of SEQ ID NO: 5 (kfo⊿C gene) was cloned. The kfo⊿C gene was inserted into pTrcHis to obtain pTrcHis-kfo⊿C.

<Preparation of pTrcHis-kfo⊿CA>

The plasmid pTrcHis-kfoCA prepared in the Example 1 was cleaved

using restriction enzymes SphI and SmaI.
DNA fragment (2.9 kDa) was obtained by digestion of the plasmid pTrcHis-kfo⊿C using the same restriction enzymes. According to the conventional method, the DNA fragment was inserted into the SphI-SmaI site of the cleaved plasmid, and then the resulting plasmid was named pTrcHis-kfo⊿CA.

[0033]

Example 5

Preparation of anti-KfoA and anti-KfoC antibodies

<Preparation of anti-KfoA antibodies>

Oligopeptide comprising the peptide sequence of SEQ ID No: 15 (CIVSR RDGDI AESWS SPEKA NK, Purity : > 70 %) was synthesized, and 4 mg of the oligopeptide was conjugated with KLH (keyhole limpet hemocyanin) by conventional method. After preparing the antigen solution by mixing with complete adjuvant, immunization was carried out 5 times in the interval for 2 weeks. The whole blood was collected, after antibody titre is confirmed. From each rabbit blood, anti-KfoA serum was obtained 48 mL (Sample A) and 60 mL (Sample B), respectively. After purification using Protein A column, IgG fraction EK-A was obtained 32 ml (IgG concentration: 5.30 mg/mL) from Sample A.

[0034]

<Preparation of anti-KfoC antibodies>

Oligopeptide comprising the peptide sequence of SEQ ID No: 16 (CQEPP GKENE TDRAA GK, Purity : > 70 %) was synthesized, and 4 mg of the oligopeptide was conjugated with KLH (keyhole limpet hemocyanin) by conventional method. After preparing the antigen solution by mixing with complete adjuvant, immunization was carried out 5 times in the interval for 2 weeks. The whole blood was collected, after antibody titre is confirmed. From each rabbit blood, anti-KfoA serum was obtained 65 mL (Sample A) and 39 mL (Sample B), respectively. After purification using Protein A column, IgG fraction EK-A was obtained 39 ml (IgG concentration: 4.41 mg/mL) from Sample A.

[0035]

Example 6

In the same manner as in Example 2, the plasmid pTrcHis-kfo⊿CA was introduced into the *E. coli* K5 strain to obtain *E. coli* K5/pTrcHis-kfo⊿CA strain, and the recombinant strain was cultured to evaluate the expression of the recombinant proteins. The seed culture grown in LB medium supplemented with 100 ppm ampicillin (0.5 mL) was transferred into CYG medium supplemented with 100 ppm ampicillin (10 mL), and then the culture was carried out at 37 °C for 1.5 hours. After adding IPTG (final concentration: 1 mM), the cultivation was continued for further 4 hours. The bacterial cells were harvested from 900 μL of the culture and suspended into 90 μL of Laemmli buffer (×1). After heating in a boiling water bath for 10 min, 10 μL of the supernatant was subjected to SDS-PAGE in a 7.5 % gel and transferred to a PVDF membrane, followed by Western analysis to detect expression of recombinant proteins. Anti-KfoA antibodies (EK-A) and anti-KfoC antibodies (EK-C) diluted to 1/1,000 was used as 1st antibody for detection of recombinant proteins KfoA, KfoC and Kfo⊿C. Anti-Rabbit Immunoglobulins HRP (DAKO,#P0448) was used as 2nd antibody. In addition, the same examinations using the culture of *E. coli* K5/pTrcHis-kfoCA and *E. coli* K5/pTrcHis-kfoA were carried out to compare the expression level of recombinant proteins. As a result, there was no the great difference on expression level of KfoA and KfoC(Kfo⊿C) between *E. coli* K5/pTrcHis-kfoCA and *E. coli* K5/pTrcHis-kfoA (Fig. 7).

[0036]

Example 7

Preparation of polysaccharide and its detection by disaccharide analysis

<Preparation of bacterial cell>

In the same manner as in Example 3, bacterial cells and supernatant from the culture of *E. coli* K5/pTrcHis-kfo⊿CA, *E. coli*

K5/pTrcHis-kfoCA and *E. coli* K5/pTrcHis-kfoA strains were prepared after autoclaving at 121 °C for 5 min.

<Preparation of polysaccharide fraction>

The supernatant (1 mL) was dialyzed against running tap water overnight, and then dialyzed solution was lyophilized. The lyophilized materials were dissolved into 100μL of 50mM Tris-HCl(pH8.0)to use as a polysaccharide fraction.

[0037]

<Detection of chondroitin by disaccharide analysis>

In the same manner as in Example 3, the obtained fraction was analyzed by the disaccharide composition analysis. Chondroitin standard solution (200μg/mL and 100μg/mL) was analyzed by the fluorescent HPLC to make the calibration curve of chondroitin. The relationship between chondroitin concentration and peak area of unsaturated double sugar, ⊿ Di-0S, was shown in Equation-1.

[Chondroitin ; μg/mL of supernatant] =

(0.000591×[Peak area]+1.219)/[concentration rate]

(r^2=0.9984) (Equation−1)

[0038]

The peak agreed with unsaturated disaccharide (⊿ Di-0S) was detected in the samples prepared from the *E. coli* K5/pTrcHis-kfo ⊿CA and *E. coli* K5/pTrcHis-kfoCA (Data not shown). Table 1 shows the result of chondroitin concentration calculated using the Equation-1 in the recombinant cultures.

[0039]

Table 1 Comparison of chondroitin content in the culture of the recombinant strains.

E. coli K5	Concentration	Peak area	Chondroitin [μg/mL]
K5/pTrcHis-kfoA	10	10,187.4	0.7
K5/pTrcHis-kfoCA	10	505,032.3	30.0
K5/pTrcHis-kfo⊿CA	10	888,677.6	52.6

[0040]

By comparing chondroitin productivity between *E. coli* K5/pTrcHis-kfo⊿CA and *E. coli* K5/pTrcHis-kfoCA, the recombinant strain *E. coli* K5/pTrcHis-kfo⊿CA is more suitable for producing chondroitin by fermentation. This result suggested that chondroitin production could be enhanced by using the kfo⊿C gene which encodes N-terminal-truncated KfoC.

[0041]

Industrial Applicability

By using the bacterium of the present invention, chondroitin and chondroitin sulfate can be produced at high efficiency and at low cost.

SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 72689-189 Seq 26-OCT-09 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

The sequences in the sequence listing in electronic form are reproduced in the following table.

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Gly Lys Asn Ile Thr Val Gln Leu Leu Gln Gln Lys Val Pro Tyr Phe
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tat aga aaa aaa gaa aaa ata gaa tcc gcg aca tta aaa aga gta cca     1296
Tyr Arg Lys Lys Glu Lys Ile Glu Ser Ala Thr Leu Lys Arg Val Pro
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Leu Val Ser Ile Tyr Ile Pro Ala Tyr Asn Cys Ser Lys Tyr Ile Val
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cgt tgt gtt gaa agc gcc ctt aat cag aca ata act gac tta gaa gta     1392
Arg Cys Val Glu Ser Ala Leu Asn Gln Thr Ile Thr Asp Leu Glu Val
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tgc ata tgc gat gat ggt tcc aca gat gat aca ttg cgg att ctt cag     1440
Cys Ile Cys Asp Asp Gly Ser Thr Asp Asp Thr Leu Arg Ile Leu Gln
465                 470                 475                 480

gag cat tat gca aac cat cct cga gtt cgt ttt att tca caa aaa aac     1488
Glu His Tyr Ala Asn His Pro Arg Val Arg Phe Ile Ser Gln Lys Asn
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aaa gga att ggt tca gca tct aat aca gca gtt aga ttg tgt cgg gga     1536
Lys Gly Ile Gly Ser Ala Ser Asn Thr Ala Val Arg Leu Cys Arg Gly
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ttc tat ata ggt cag tta gac tct gat gac ttt ctt gaa cca gat gct     1584
Phe Tyr Ile Gly Gln Leu Asp Ser Asp Asp Phe Leu Glu Pro Asp Ala
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gtt gaa cta tgt cta gat gaa ttt aga aaa gat cta tca ttg gca tgt     1632
Val Glu Leu Cys Leu Asp Glu Phe Arg Lys Asp Leu Ser Leu Ala Cys
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gtt tat aca act aac cgt aat ata gat cgt gaa ggt aat ttg ata tca     1680
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aat ggc tat aat tgg ccc att tat tcg cga gaa aaa ctt act agt gca     1728
Asn Gly Tyr Asn Trp Pro Ile Tyr Ser Arg Glu Lys Leu Thr Ser Ala
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atg ata tgt cat cat ttc agg atg ttc aca gca aga gca tgg aac cta     1776
Met Ile Cys His His Phe Arg Met Phe Thr Ala Arg Ala Trp Asn Leu
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Thr Glu Gly Phe Asn Glu Ser Ile Ser Asn Ala Val Asp Tyr Asp Met
        595                 600                 605

tat tta aaa ctt agt gaa gtt gga ccg ttc aag cat ata aac aaa att     1872
Tyr Leu Lys Leu Ser Glu Val Gly Pro Phe Lys His Ile Asn Lys Ile
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tgt tat aat cgc gta ttg cat ggt gaa aat acg tct ata aaa aag ttg     1920
Cys Tyr Asn Arg Val Leu His Gly Glu Asn Thr Ser Ile Lys Lys Leu
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agg cta ggc ata aaa aaa tat aaa tat tca cca tta act aat ttg aat     2016
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Arg Ala Lys Ile Leu Ala Ile Thr Leu Ala Cys Leu Cys Asn Gln Lys
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225 230 235 240
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355 360 365
Leu Tyr Arg Glu Gly Cys Tyr Phe Arg Ser Val Glu Gly Ala Met Ala
370 375 380

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                405                 410                 415
Tyr Arg Lys Lys Glu Lys Ile Glu Ser Ala Thr Leu Lys Arg Val Pro
            420                 425                 430
Leu Val Ser Ile Tyr Ile Pro Ala Tyr Asn Cys Ser Lys Tyr Ile Val
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Arg Cys Val Glu Ser Ala Leu Asn Gln Thr Ile Thr Asp Leu Glu Val
    450                 455                 460
Cys Ile Cys Asp Asp Gly Ser Thr Asp Asp Thr Leu Arg Ile Leu Gln
465                 470                 475                 480
Glu His Tyr Ala Asn His Pro Arg Val Arg Phe Ile Ser Gln Lys Asn
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Lys Gly Ile Gly Ser Ala Ser Asn Thr Ala Val Arg Leu Cys Arg Gly
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Phe Tyr Ile Gly Gln Leu Asp Ser Asp Asp Phe Leu Glu Pro Asp Ala
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Val Glu Leu Cys Leu Asp Glu Phe Arg Lys Asp Leu Ser Leu Ala Cys
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Met Ile Cys His His Phe Arg Met Phe Thr Ala Arg Ala Trp Asn Leu
            580                 585                 590
Thr Glu Gly Phe Asn Glu Ser Ile Ser Asn Ala Val Asp Tyr Asp Met
        595                 600                 605
Tyr Leu Lys Leu Ser Glu Val Gly Pro Phe Lys His Ile Asn Lys Ile
    610                 615                 620
Cys Tyr Asn Arg Val Leu His Gly Glu Asn Thr Ser Ile Lys Lys Leu
625                 630                 635                 640
Asp Ile Gln Lys Glu Asn His Phe Lys Val Val Asn Glu Ser Leu Ser
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Arg Leu Gly Ile Lys Lys Tyr Lys Tyr Ser Pro Leu Thr Asn Leu Asn
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gcc aaa gca att agt ctg aac gag gtt gaa aaa aat gaa ata ata agc      96
Ala Lys Ala Ile Ser Leu Asn Glu Val Glu Lys Asn Glu Ile Ile Ser
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gaa gtc gaa ccc att cct tta gat tgg cct agt gat tta act tta ccg      192
Glu Val Glu Pro Ile Pro Leu Asp Trp Pro Ser Asp Leu Thr Leu Pro
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ccg tta cct gag agc aca aac gat tat gtt tgg gcg ggg aaa aga aaa      240
Pro Leu Pro Glu Ser Thr Asn Asp Tyr Val Trp Ala Gly Lys Arg Lys
65                  70                  75                  80

gag ctt gat gat tat cca aga aaa cag tta atc att gac ggg ctt agt      288
Glu Leu Asp Asp Tyr Pro Arg Lys Gln Leu Ile Ile Asp Gly Leu Ser
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att gta att cct aca tat aat cga gca aaa ata ctt gca att aca ctt      336
Ile Val Ile Pro Thr Tyr Asn Arg Ala Lys Ile Leu Ala Ile Thr Leu
            100                 105                 110

gct tgt ctt tgt aac caa aag acc ata tac gac tat gaa gtt att gtt      384
Ala Cys Leu Cys Asn Gln Lys Thr Ile Tyr Asp Tyr Glu Val Ile Val
        115                 120                 125

gcc gat gat gga agt aaa gaa aat att gaa gaa ata gta aga gaa ttt      432
Ala Asp Asp Gly Ser Lys Glu Asn Ile Glu Glu Ile Val Arg Glu Phe
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gaa agt tta tta aat ata aaa tat gta cgt cag aag gat tat gga tat      480
Glu Ser Leu Leu Asn Ile Lys Tyr Val Arg Gln Lys Asp Tyr Gly Tyr
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caa ctg tgt gct gtt aga aat ctt ggg ctt agg gct gca aag tat aat      528
Gln Leu Cys Ala Val Arg Asn Leu Gly Leu Arg Ala Ala Lys Tyr Asn
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tat gtt gca att ctg gat tgt gat atg gct ccg aac cca cta tgg gtt      576
Tyr Val Ala Ile Leu Asp Cys Asp Met Ala Pro Asn Pro Leu Trp Val
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cag tca tat atg gaa cta tta gcg gtg gac gat aat gtt gct cta att      624
Gln Ser Tyr Met Glu Leu Leu Ala Val Asp Asp Asn Val Ala Leu Ile
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ggc cct aga aaa tat ata gat aca agc aag cat aca tat tta gat ttc      672
Gly Pro Arg Lys Tyr Ile Asp Thr Ser Lys His Thr Tyr Leu Asp Phe
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ctt tcc caa aaa tca cta ata aat gaa att cct gaa atc att act aat      720
Leu Ser Gln Lys Ser Leu Ile Asn Glu Ile Pro Glu Ile Ile Thr Asn
225                 230                 235                 240

aat cag gtt gca ggc aag gtt gag caa aac aaa tca gtt gac tgg cga      768
Asn Gln Val Ala Gly Lys Val Glu Gln Asn Lys Ser Val Asp Trp Arg
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ata gaa cat ttc aaa aat acc gat aat cta aga tta tgc aac aca cca      816
Ile Glu His Phe Lys Asn Thr Asp Asn Leu Arg Leu Cys Asn Thr Pro
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ttt cga ttt ttt agc gga ggt aat gtc gct ttt gcg aaa aaa tgg ctt      864
Phe Arg Phe Phe Ser Gly Gly Asn Val Ala Phe Ala Lys Lys Trp Leu
        275                 280                 285

ttc cgt gca gga tgg ttt gat gaa gag ttt acg cat tgg ggg ggg gag      912
Phe Arg Ala Gly Trp Phe Asp Glu Glu Phe Thr His Trp Gly Gly Glu
    290                 295                 300

gat aat gag ttt gga tat cgt ctc tac aga gaa gga tgt tac ttt cgg      960
Asp Asn Glu Phe Gly Tyr Arg Leu Tyr Arg Glu Gly Cys Tyr Phe Arg
305                 310                 315                 320

tct gtt gaa gga gca atg gca tat cat caa gaa cca ccc ggg aaa gaa     1008
Ser Val Glu Gly Ala Met Ala Tyr His Gln Glu Pro Pro Gly Lys Glu
                325                 330                 335

aac gag acg gat cgt gcg gca ggg aaa aat att act gtt caa ttg tta     1056
Asn Glu Thr Asp Arg Ala Ala Gly Lys Asn Ile Thr Val Gln Leu Leu
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cag caa aaa gtt cct tat ttc tat aga aaa aaa gaa aaa ata gaa tcc     1104
Gln Gln Lys Val Pro Tyr Phe Tyr Arg Lys Lys Glu Lys Ile Glu Ser
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gcg aca tta aaa aga gta cca cta gta tct ata tat att ccc gcc tat     1152
Ala Thr Leu Lys Arg Val Pro Leu Val Ser Ile Tyr Ile Pro Ala Tyr
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Asn Cys Ser Lys Tyr Ile Val Arg Cys Val Glu Ser Ala Leu Asn Gln
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Thr Ile Thr Asp Leu Glu Val Cys Ile Cys Asp Asp Gly Ser Thr Asp
                405                 410                 415

gat aca ttg cgg att ctt cag gag cat tat gca aac cat cct cga gtt     1296
Asp Thr Leu Arg Ile Leu Gln Glu His Tyr Ala Asn His Pro Arg Val
            420                 425                 430

cgt ttt att tca caa aaa aac aaa gga att ggt tca gca tct aat aca     1344
Arg Phe Ile Ser Gln Lys Asn Lys Gly Ile Gly Ser Ala Ser Asn Thr
        435                 440                 445

gca gtt aga ttg tgt cgg gga ttc tat ata ggt cag tta gac tct gat     1392
Ala Val Arg Leu Cys Arg Gly Phe Tyr Ile Gly Gln Leu Asp Ser Asp
    450                 455                 460

gac ttt ctt gaa cca gat gct gtt gaa cta tgt cta gat gaa ttt aga     1440
Asp Phe Leu Glu Pro Asp Ala Val Glu Leu Cys Leu Asp Glu Phe Arg
465                 470                 475                 480

aaa gat cta tca ttg gca tgt gtt tat aca act aac cgt aat ata gat     1488
Lys Asp Leu Ser Leu Ala Cys Val Tyr Thr Thr Asn Arg Asn Ile Asp
                485                 490                 495

cgt gaa ggt aat ttg ata tca aat ggc tat aat tgg ccc att tat tcg     1536
Arg Glu Gly Asn Leu Ile Ser Asn Gly Tyr Asn Trp Pro Ile Tyr Ser
            500                 505                 510
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    530                 535                 540

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                565                 570                 575

aat acg tct ata aaa aag ttg gat att caa aag gaa aat cat ttt aaa     1776
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            580                 585                 590

gtt gtt aac gaa tca tta agt agg cta ggc ata aaa aaa tat aaa tat     1824
Val Val Asn Glu Ser Leu Ser Arg Leu Gly Ile Lys Lys Tyr Lys Tyr
        595                 600                 605

tca cca tta act aat ttg aat gaa tgt aga aaa tat acc tgg gaa aaa     1872
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    50                  55                  60
Pro Leu Pro Glu Ser Thr Asn Asp Tyr Val Trp Ala Gly Lys Arg Lys
65                  70                  75                  80
Glu Leu Asp Asp Tyr Pro Arg Lys Gln Leu Ile Ile Asp Gly Leu Ser
                85                  90                  95
Ile Val Ile Pro Thr Tyr Asn Arg Ala Lys Ile Leu Ala Ile Thr Leu
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Lys

CLAIMS:

1. A UDP-glucuronic acid-producing recombinant bacterium, which is introduced with a kfoA gene which encodes a protein selected from the group consisting of (A) and (B) and a kfoC gene which encodes a protein selected from the group consisting of (C), (D), (E) and (F), and said bacterium produces chondroitin as a capsular polysaccharide:

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

(B) a protein comprising an amino acid sequence of SEQ ID NO: 2 including substitution, deletion, insertion, or addition of 1-20 amino acids and having UDP-glucose-4-epimerase activity;

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

(D) a protein comprising an amino acid sequence of SEQ ID NO: 4 including substitution, deletion, insertion, or addition of 1-20 amino acids and having chondroitin synthase activity;

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

(F) a protein comprising an amino acid sequence of SEQ ID NO: 6 including substitution, deletion, insertion, or addition of 1-20 amino acids and having chondroitin synthase activity.

2. A UDP-glucuronic acid-producing recombinant bacterium, which is introduced with the kfoA gene which is a DNA selected from the group consisting of (a) and (b), and a kfoC gene which is a DNA selected from the group consisting of (c), (d), (e) and (f), and said bacterium produces chondroitin as a capsular polysaccharide:

(a) a DNA comprising the nucleotide sequence of SEQ ID NO: 1;

(b) a DNA that hybridizes with a DNA comprising the nucleotide sequence complementary to SEQ ID NO: 1 under stringent conditions and encodes a protein having UDP-glucose-4-epimerase activity;

(c) a DNA comprising the nucleotide sequence of SEQ ID NO: 3;

(d) a DNA that hybridizes with a DNA comprising the nucleotide sequence complementary to SEQ ID NO: 3 under stringent conditions and encodes a protein having chondroitin synthase activity;

(e) a DNA comprising the nucleotide sequence of SEQ ID NO: 5; and

(f) a DNA that hybridizes with a DNA comprising the nucleotide sequence complementary to SEQ ID NO: 5 under stringent conditions and encodes a protein having chondroitin synthase activity;

wherein the stringent conditions are conditions for hybridization at 42°C in a solution containing 50% formamide, 4 × SSC, 50 mM HEPES (pH 7.0), 10 × Denhardt's solution,

100 µg/ml salmon sperm DNA and washing with 2 × SSC, 0.1% SDS solution at room temperature and then 0.1 × SSC, 0.1% SDS solution at 60°C.

3. The bacterium according to Claim 1 or 2, wherein said bacterium is *Escherichia coli* K5 strain.

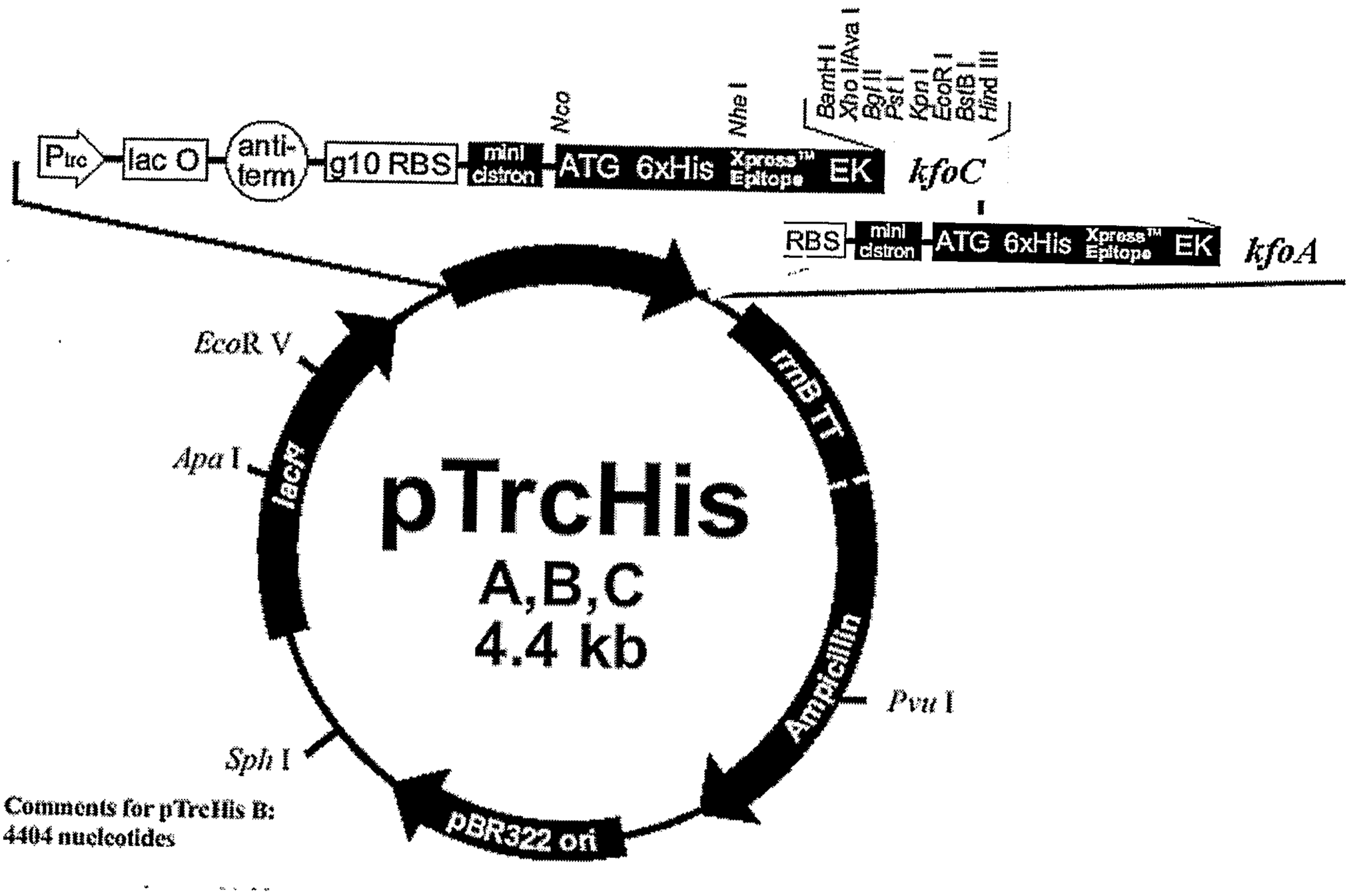
4. A method of producing chondroitin comprising at least the following steps (1) and (2):

(1) culturing the bacterium according to any one of Claims 1 to 3; and

(2) collecting chondroitin from the culture.

5. A method of producing chondroitin sulfate comprising: producing chondroitin by the method according to Claim 4; and sulfating the chondroitin to yield chondroitin sulfate.

Fig. 1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Kpn I
EcoR I
BstB I
Hind III
ATG 6xHis
Xpress™ Epitope
EK
kfoC
RBS
mini cistron
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Xpress™ Epitope
EK
kfoA
pTrcHis
A,B,C
4.4 kb
EcoR V
Apa I
lacI
rrnB TT
Ampicillin
Pvu I
Sph I
pBR322 ori
Comments for pTrcHis B:
4404 nucleotides

Fig. 2

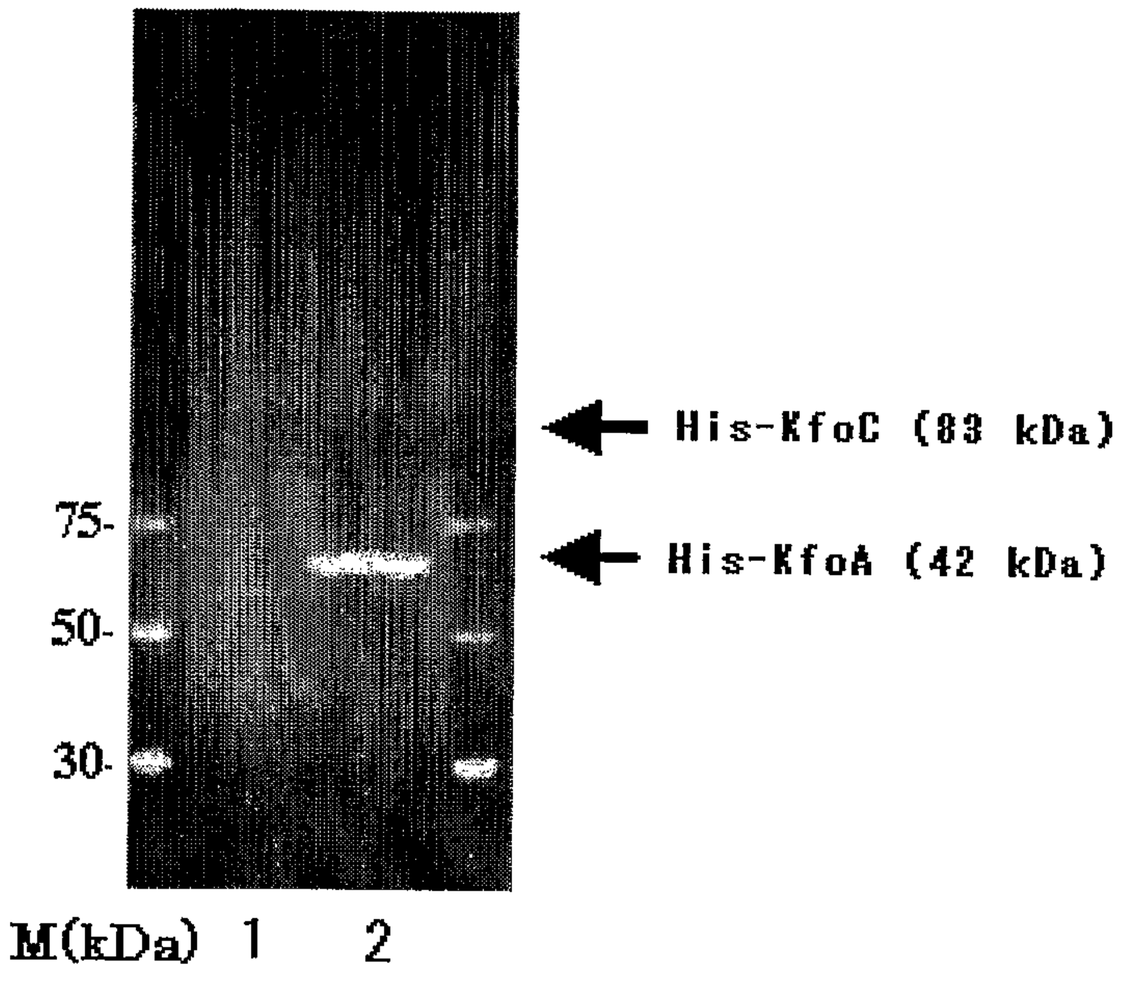
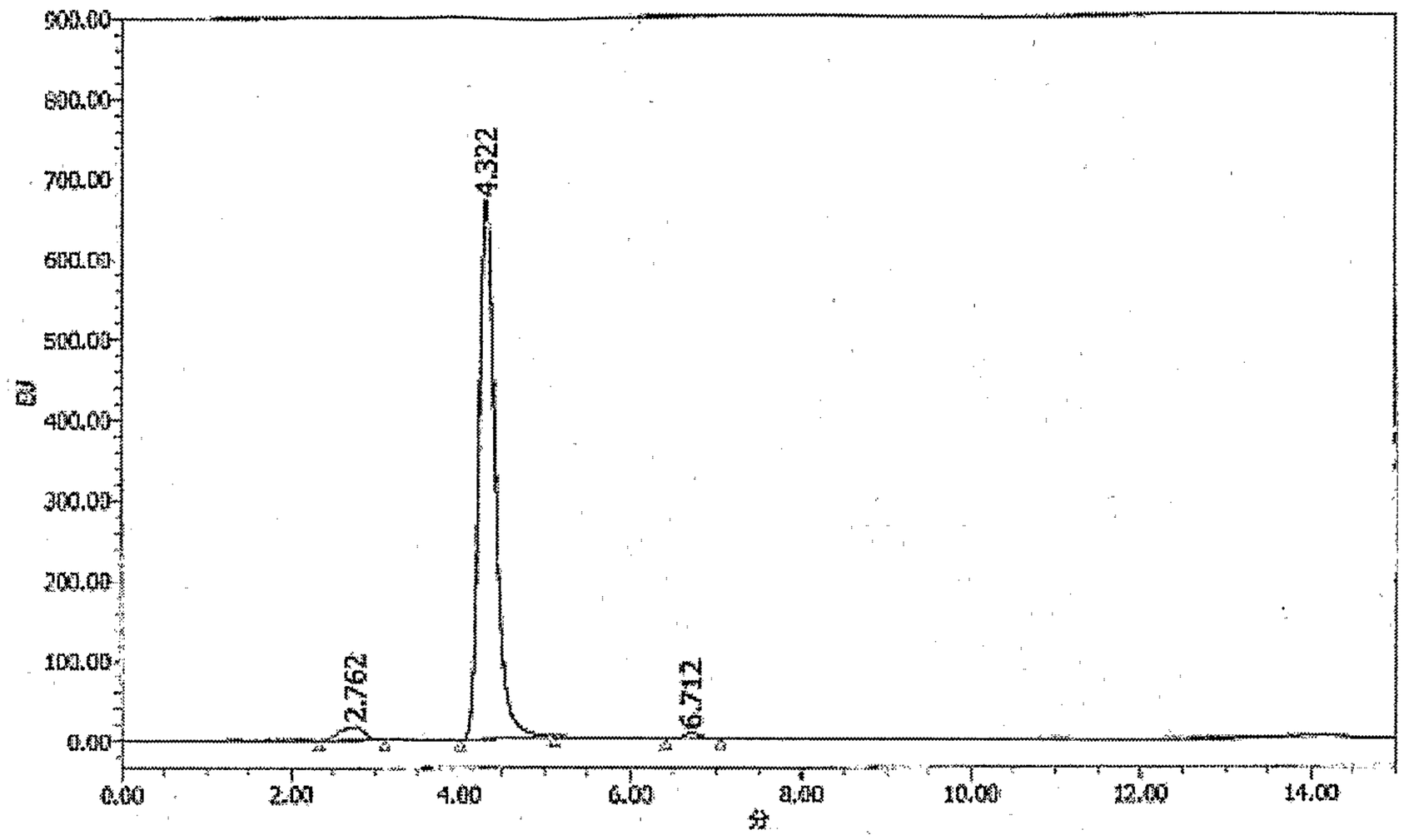
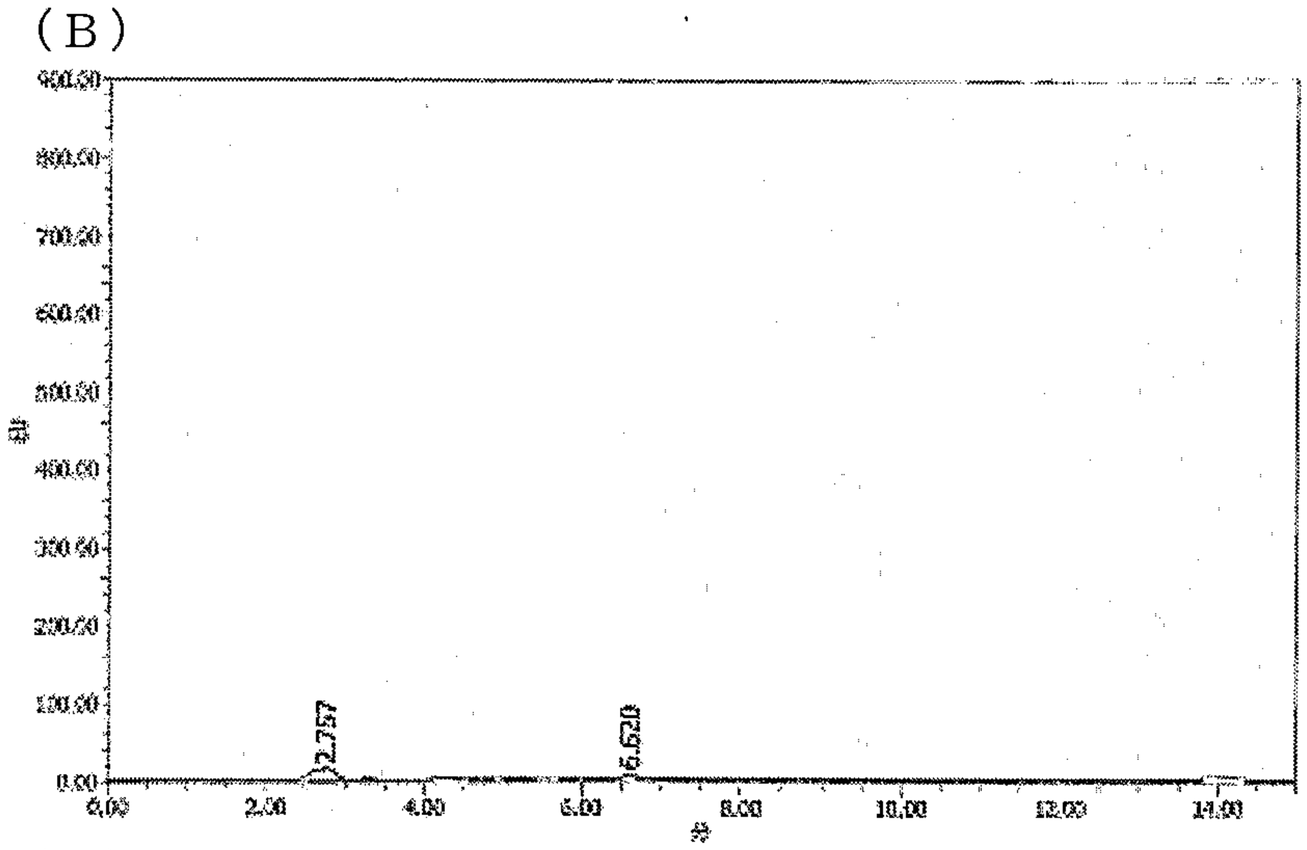


Fig. 3

(A)


900.00
800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
mV
4.322
2.762
6.712
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
分

(B)


mV
2.757
6.620
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
分

(C)

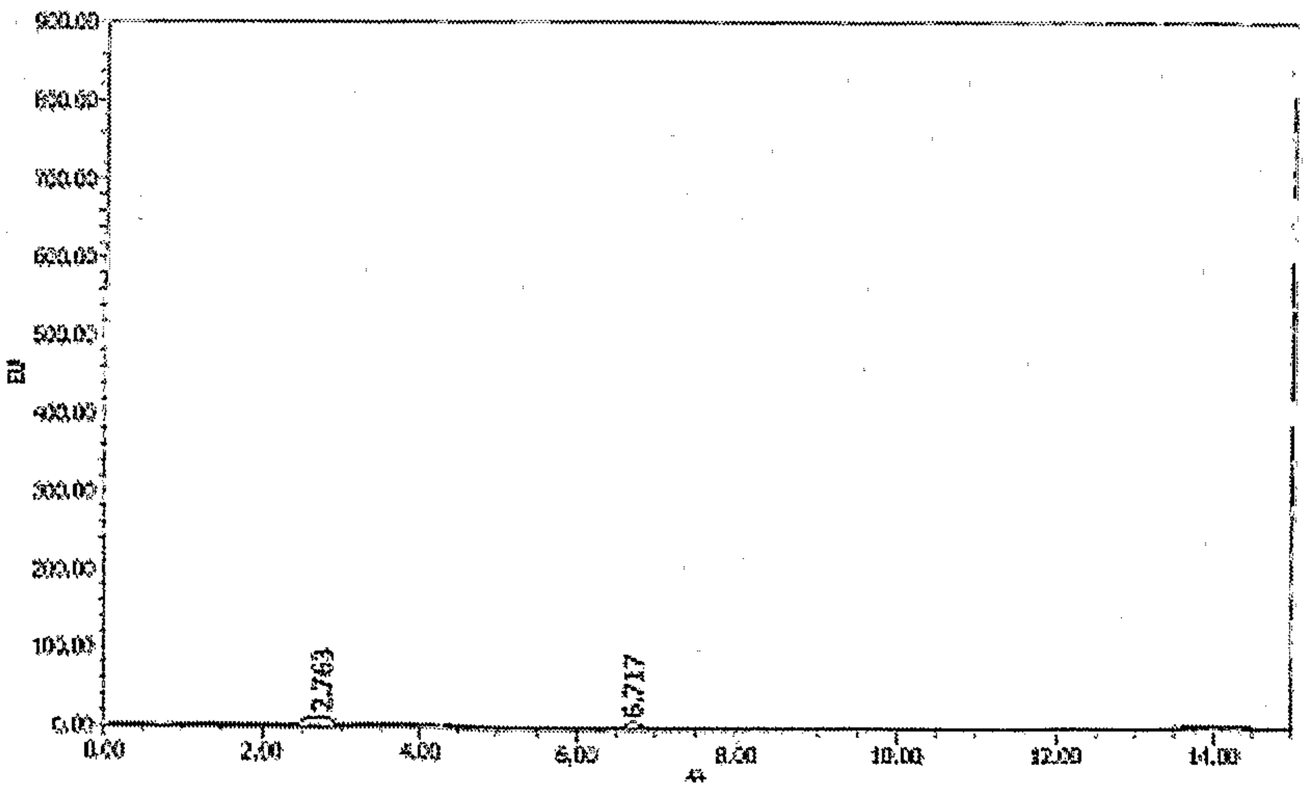
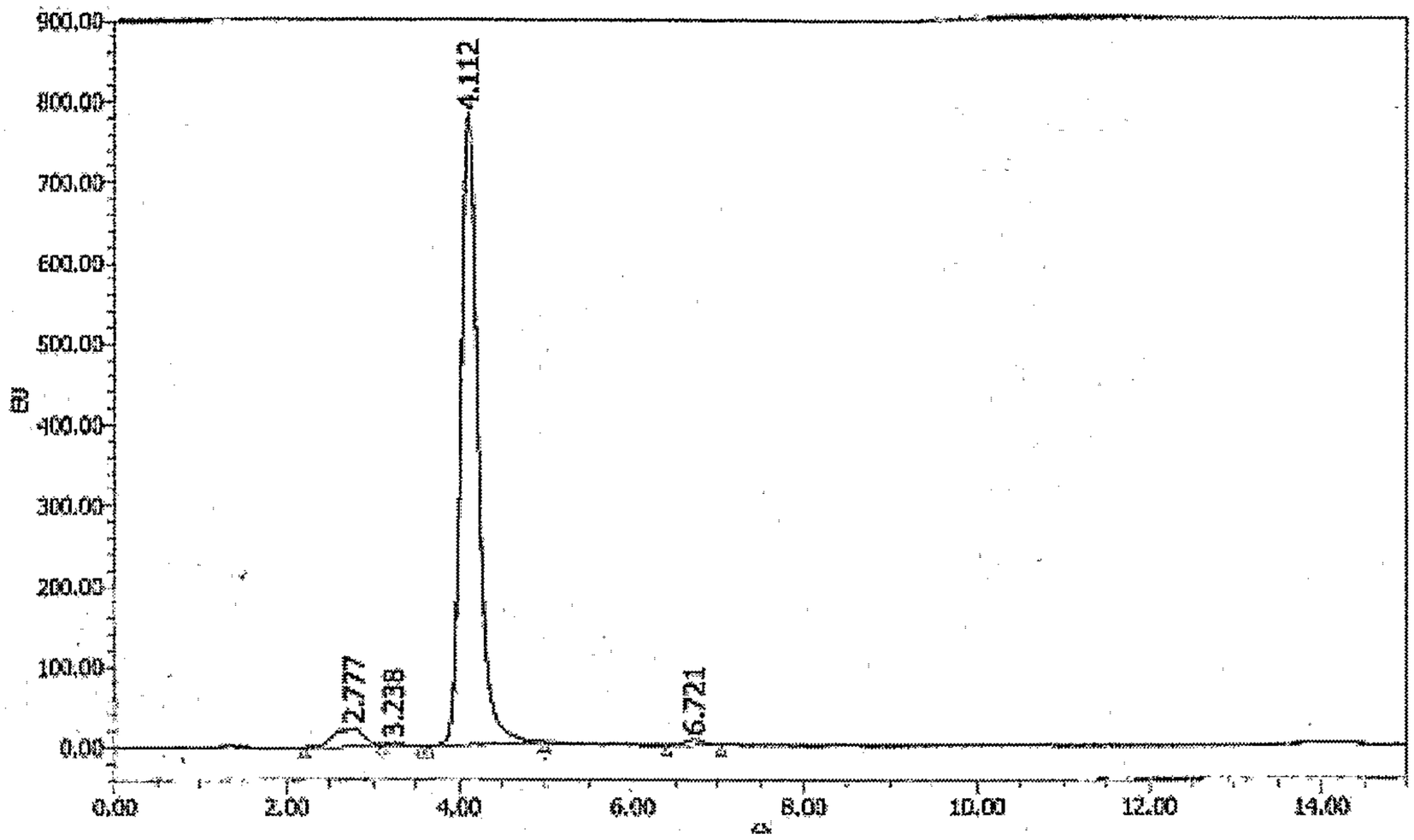
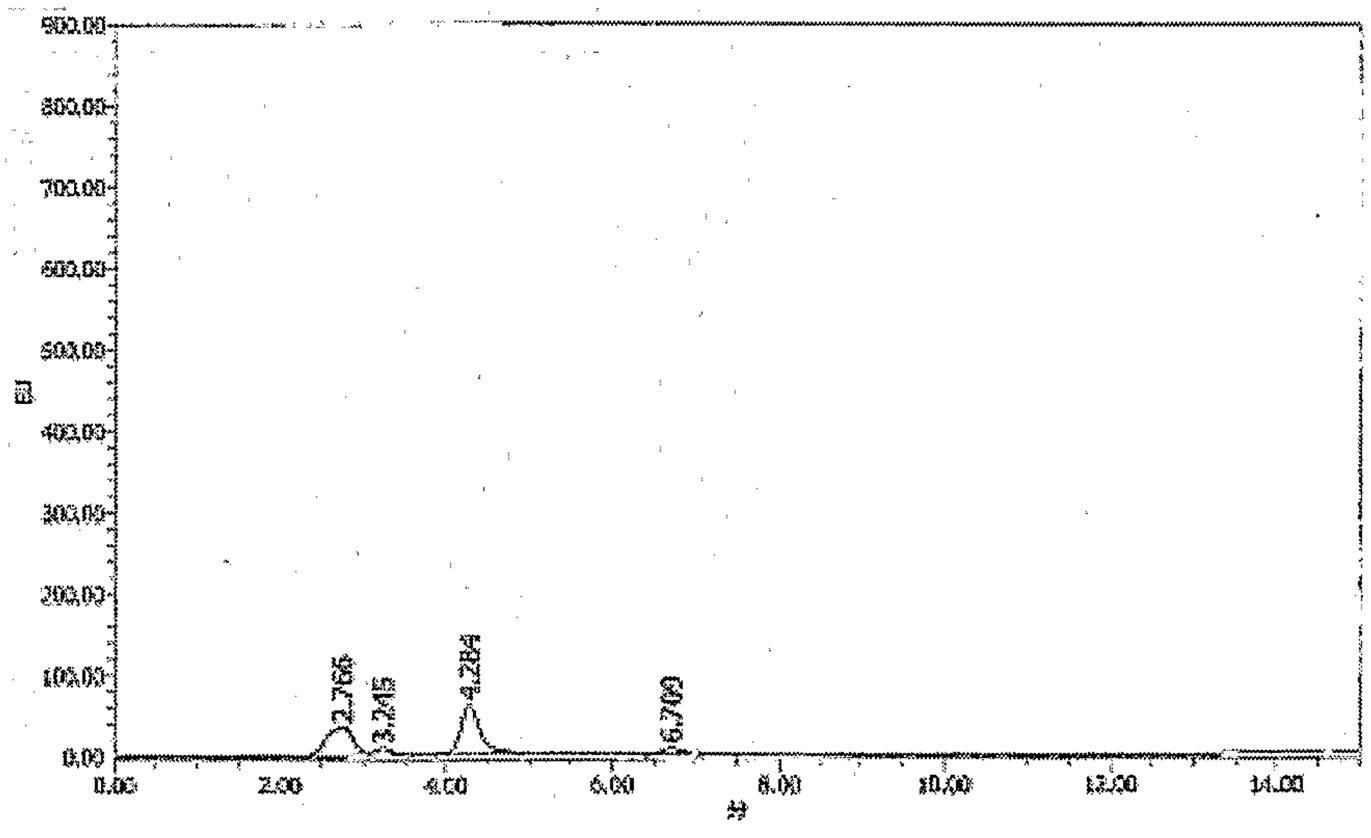

mV
2.763
6.717
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
分

Fig. 4

(A)


900.00
800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
EU
4.112
2.777
3.238
6.721
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
分

(B)


900.00
800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
EU
2.765
3.245
4.284
6.700
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
分

(C)

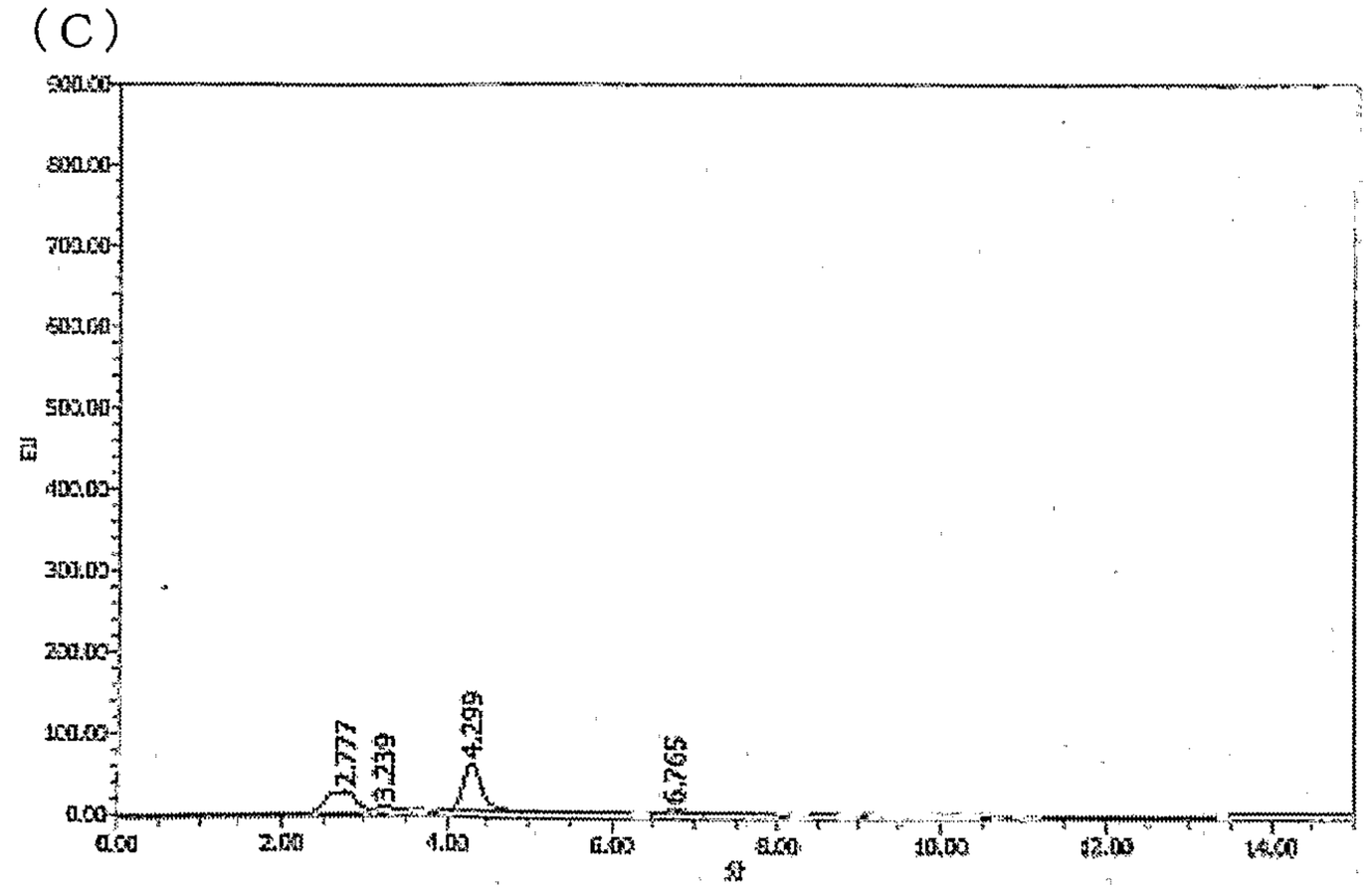
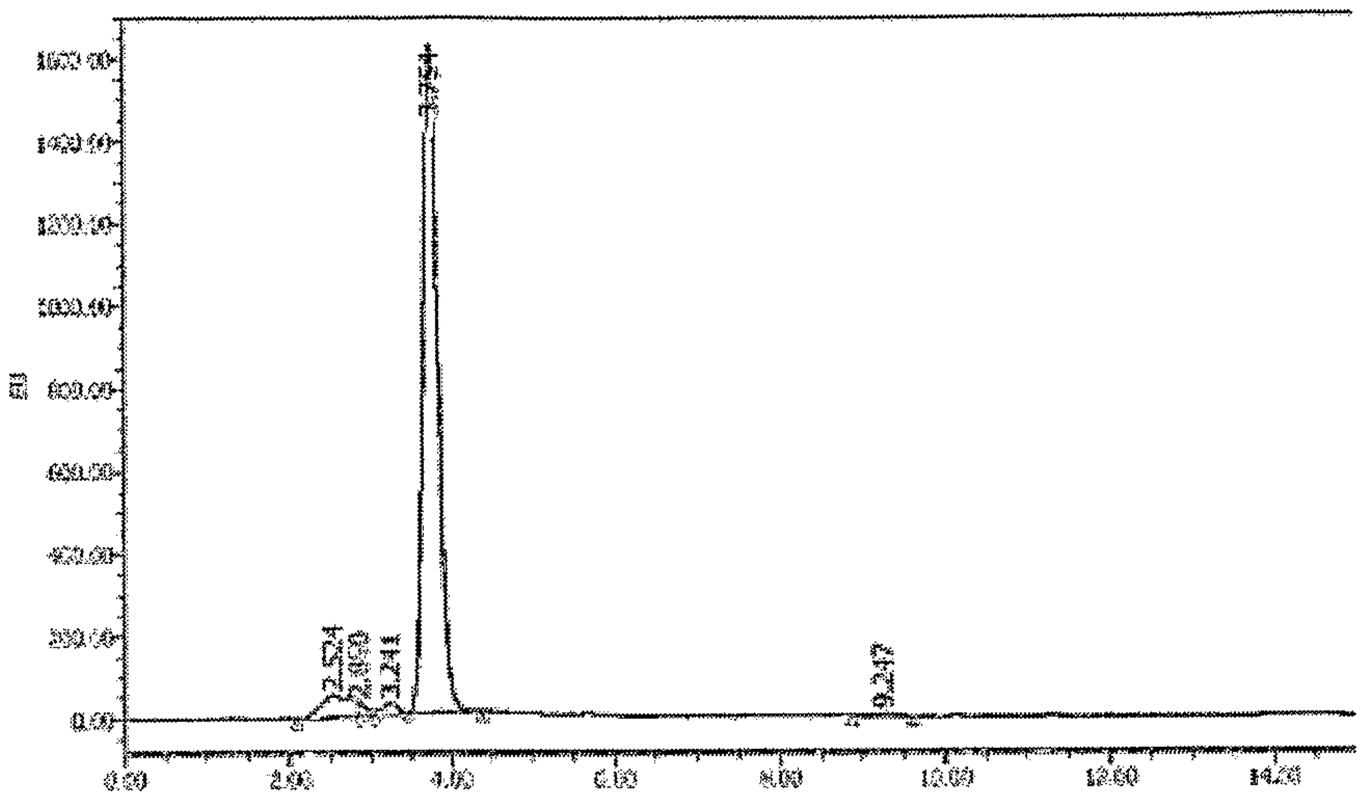
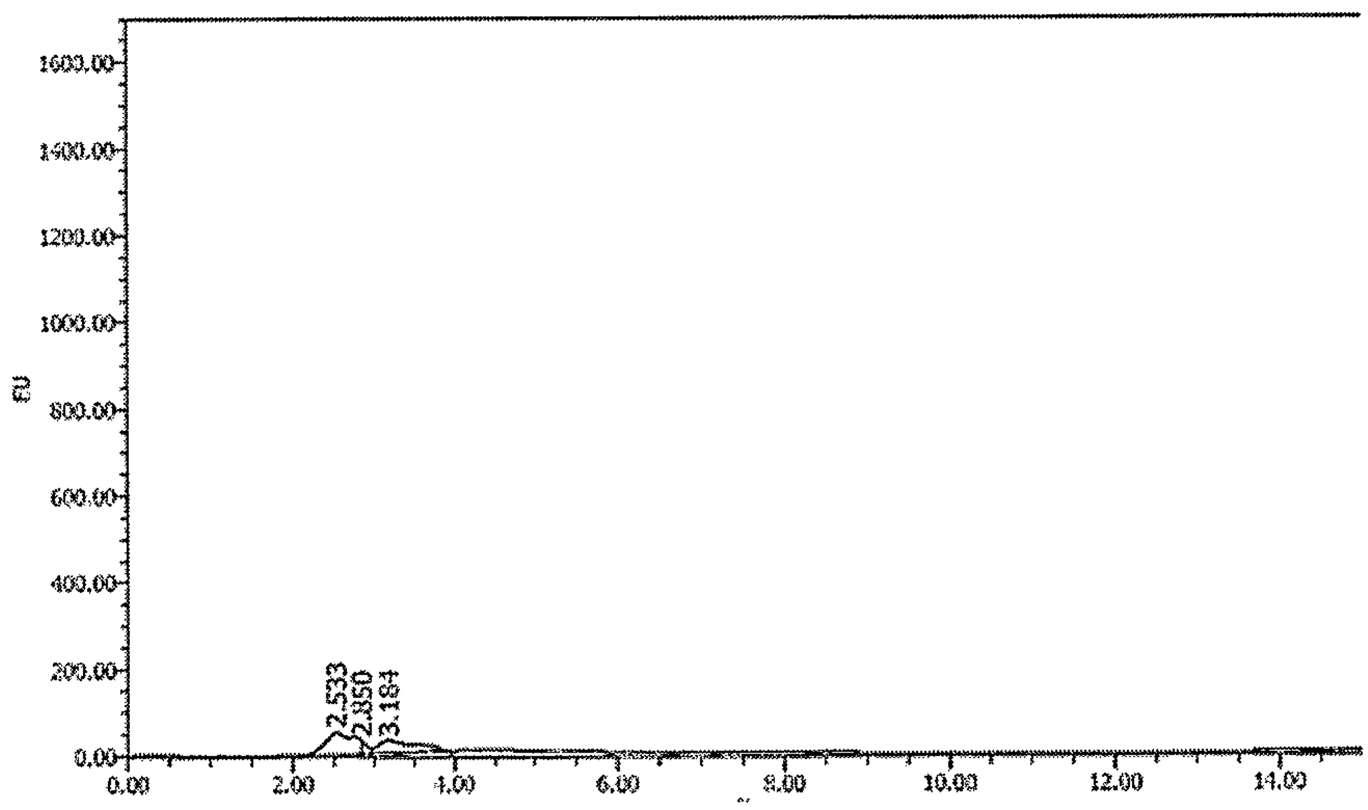

900.00
800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
EU
2.777
3.239
4.299
6.765
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
分

Fig. 5

(A)


EU
2.524
2.650
3.241
9.247
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00

(B)


EU
1600.00
1400.00
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
2.533
2.850
3.184
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00

(C)

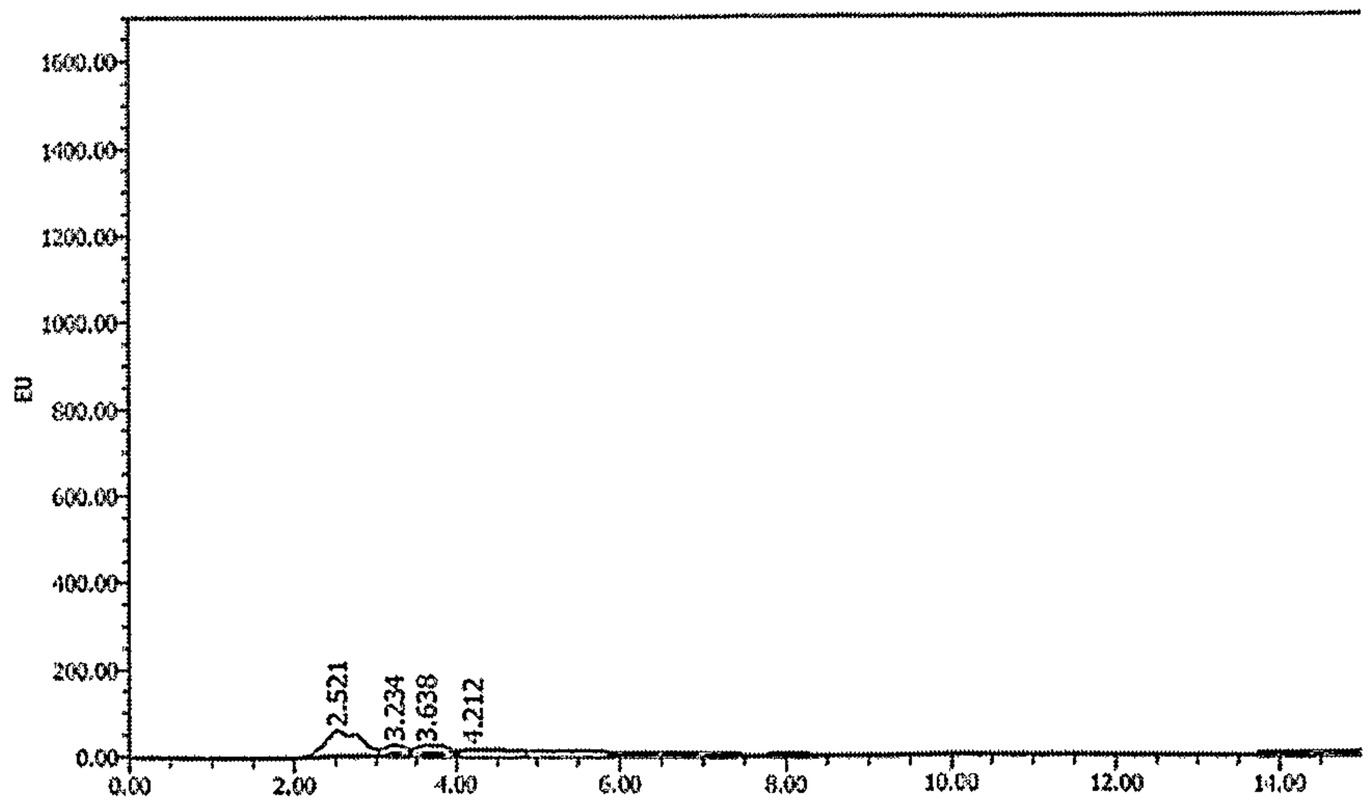
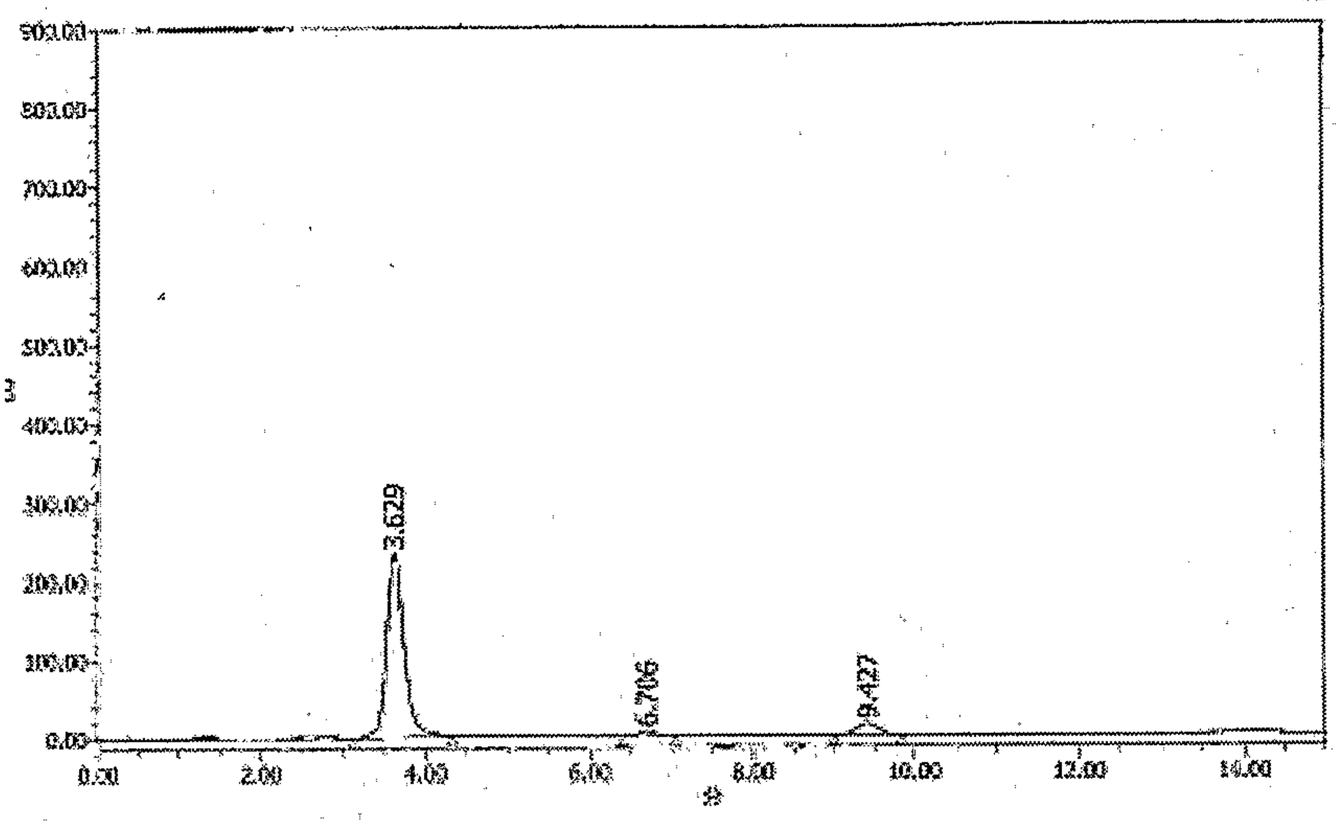

EU
1600.00
1400.00
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
2.521
3.234
3.638
4.212
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00

Fig. 6

(A)


3.629
6.706
9.427
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
min

(B)

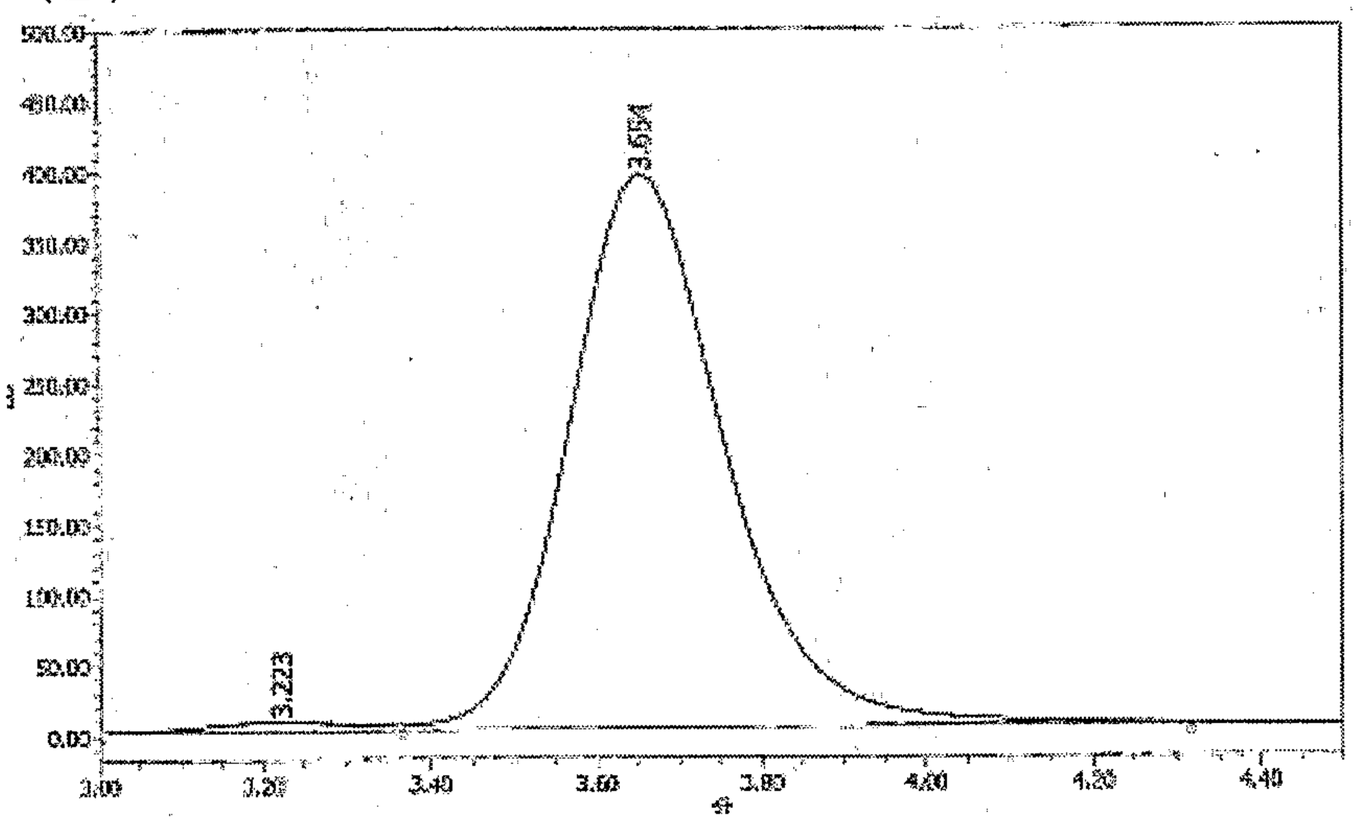
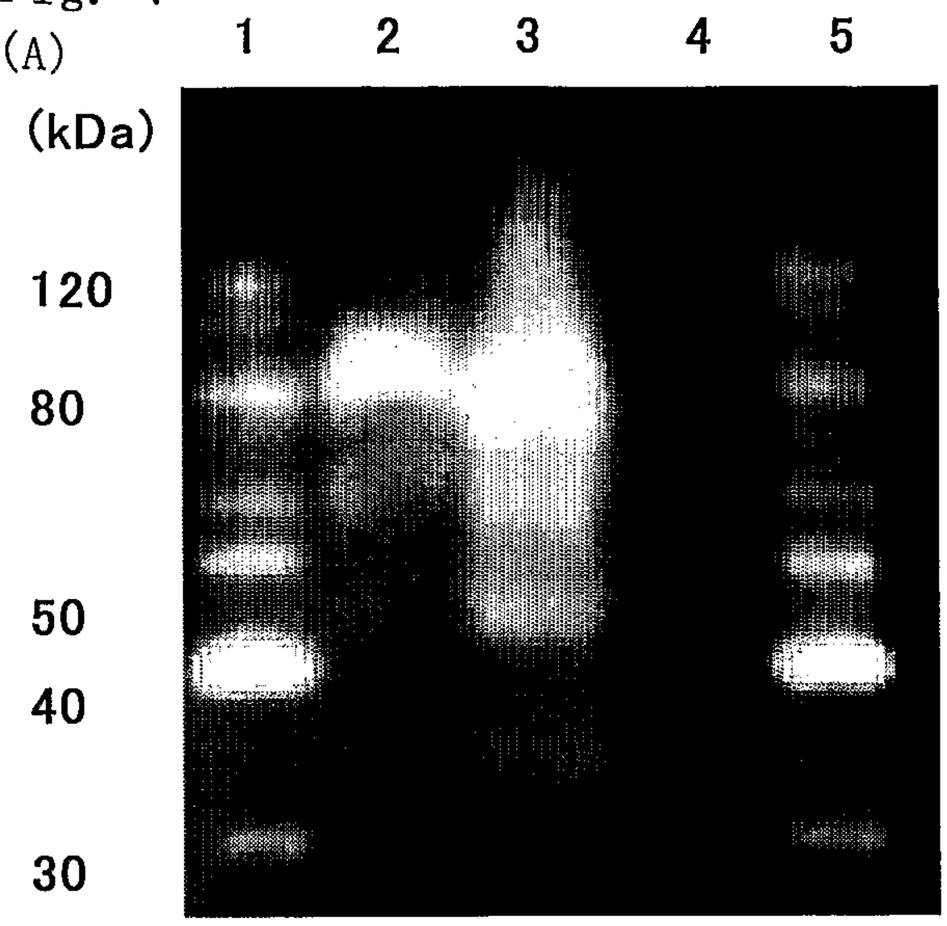
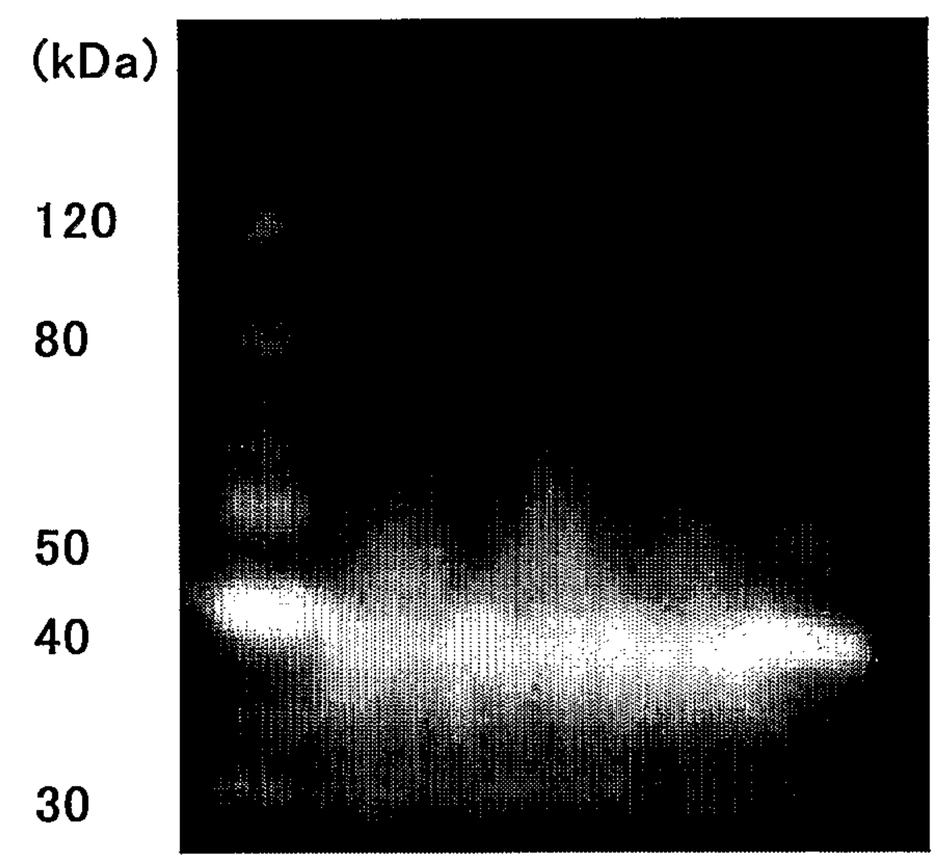

3.654
3.223
0.00
3.00
3.20
3.40
3.60
3.80
4.00
4.20
4.40
min

Fig. 7

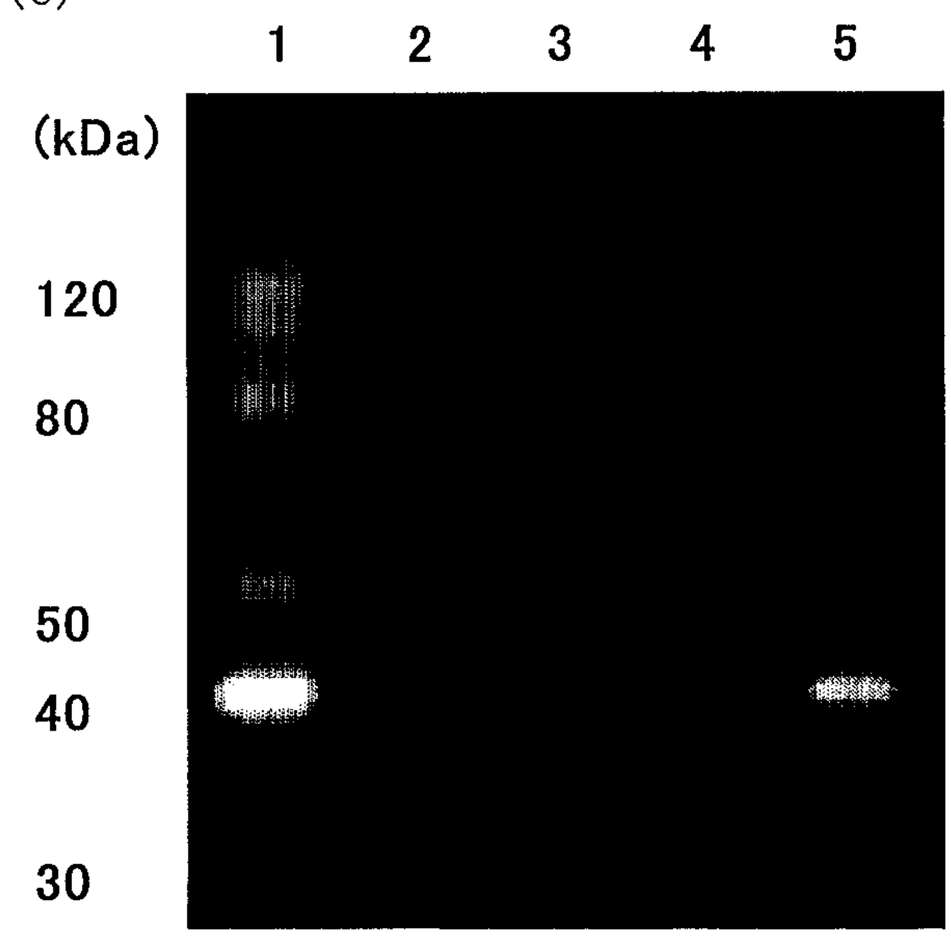
(A)


1
2
3
4
5
(kDa)
120
80
50
40
30

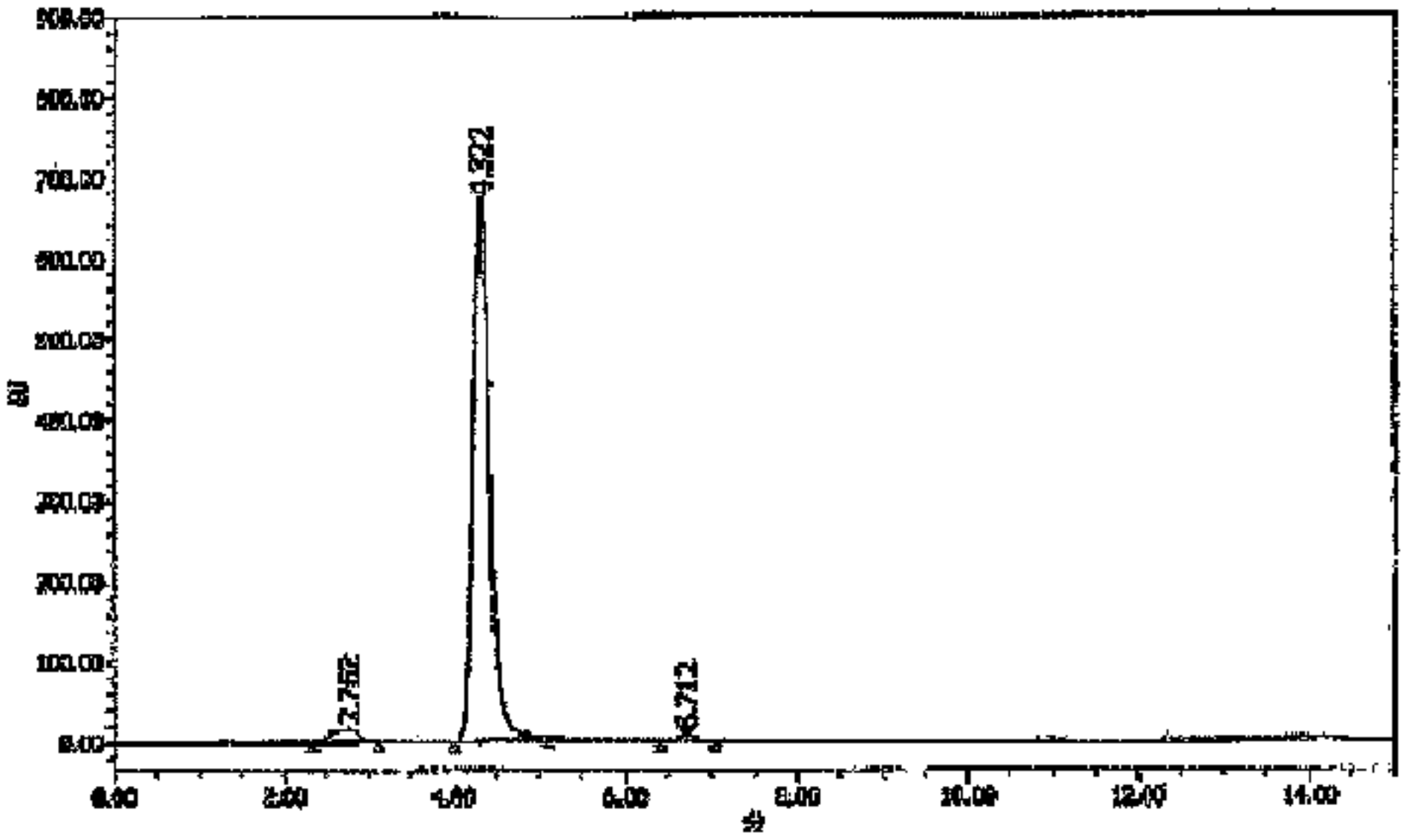
(B)


1
2
3
4
5
(kDa)
120
80
50
40
30

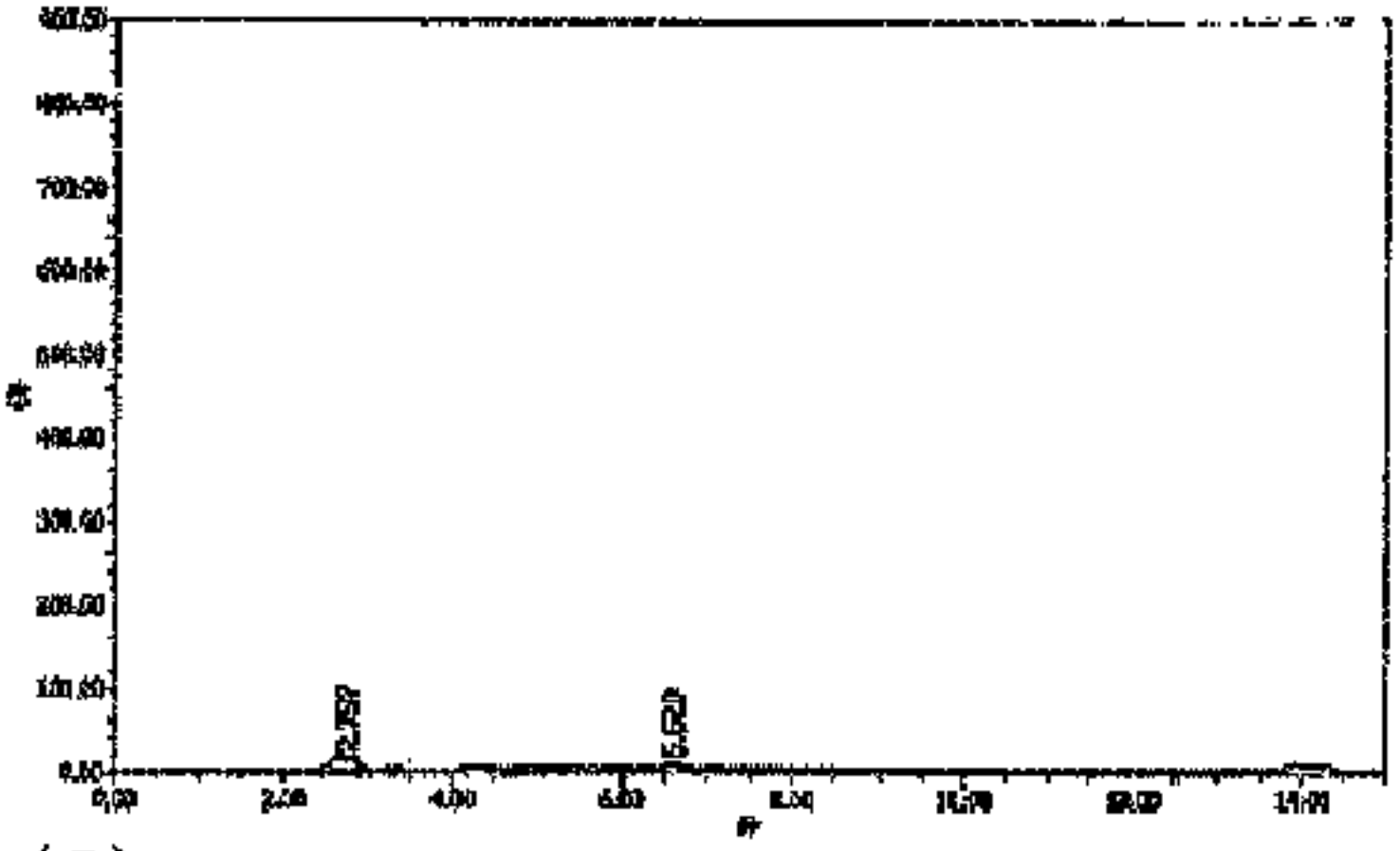
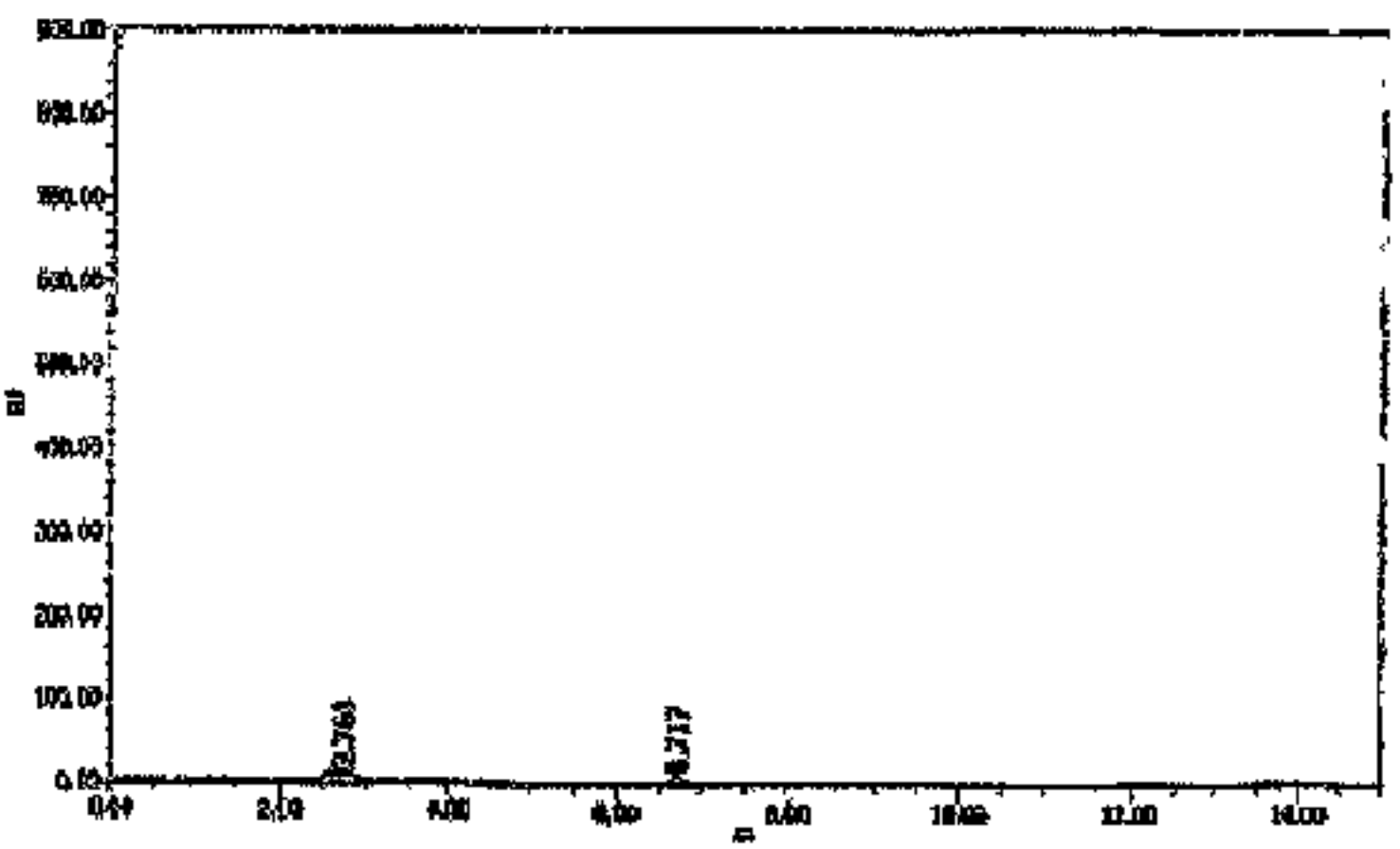
(C)


1
2
3
4
5
(kDa)
120
80
50
40
30

(A)



(B)



(C)