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(54) **Title**: METHOD FOR PRODUCTION OF RECOMBINANT HUMAN ALPHA-GALACTOSIDASE A

(57) **Abstract**: Disclosed is a method for production of recombinant human alpha-galactosidase A (rh alpha-Gal A) in a large scale, with a high purity. The method comprises the steps of (a) culturing rh alpha-Gal A-producing mammalian cells in a serum-free medium, (b) collecting culture supernatant, (c) subjecting the culture supernatant to anion-exchange column chromatography, (d) to hydrophobic column chromatography, (e) to a column chromatography employing as solid phase a material having affinity for phosphate group, (f) to cation-exchange column chromatography, and (g) to gel filtration column chromatography, in the order.

Description

Title of Invention: METHOD FOR PRODUCTION OF RE-COMBINANT HUMAN ALPHA-GALACTOSIDASE A

Technical Field

[0001] The present invention relates to a method for production of recombinant human alpha-galactosidase A (rh alpha-Gal A). More specifically the present invention relates to a process for production of rh alpha-Gal A by culturing rh alpha-Gal A-producing mammalian cells in a serum-free medium, as well as to a process for purification of rh alpha-Gal A obtained in the culture supernatant through column chromatography, in high yield and to such a high purity as permitting direct use of the purified protein as a medical drug.

Background Art

[0002] Alpha-galactosidase A (alpha-Gal A) is a lysosomal enzyme having an activity to hydrolyze the terminal alpha-galactosyl bond of glycolipids and glycoproteins. Trihexosylceramide, which contains three hexose moieties, is one of the substrates for alpha-galactosidase A and receives hydrolysis at its terminal hexose moiety by the enzyme. Human alpha-Gal A is expressed as a precursor peptide comprising 429 amino acids, of which N-terminal 31 amino acids constitute a signal peptide. The precursor is processed to the mature peptide, which consists of 398 amino acids left behind after the removal of the signal peptide, and a pair of the mature peptide molecules readily form a homodimer, wherever condition allows, which is the enzymatically active form of human alpha-Gal A. Human alpha-Gal A possesses oligosaccharide chains containing mannose-6-phosphate residues and can be targeted to the lysosome via a mannose-6-phosphate receptor.

[0003] Fabry's disease is an X-linked inherited lysosomal storage disease, which is caused by inherited abnormality of gene encoding alpha-Gal A. Lack of the activity of this enzyme causes accumulation of trihexosylceramide in various tissues, resulting in renal impairment, angiokeratomas, and cardiovascular abnormalities, including ventricular enlargement and mitral valve insufficiency.

[0004] It had already been known in the 1960's that the enzymatic activity of alpha-galactosidase A is scarcely or only weakly detected in the tissues of patients with Fabry's disease. It thus had been speculated that some inherited abnormality of the gene encoding alpha-Gal A should be the cause of Fabry's disease. Based on this speculation, alpha-Gal A replacement therapy for Fabry's disease had been conducted by infusing alpha-Gal A extracted and purified from human plasma or spleen to the patient, resulting in the confirmation that the alpha-Gal A replacement therapy could

lower the levels of trihexosylceramide in the blood of the patients suffering from Fabry's disease (see NPL 1, NPL 2, NPL 3). Therefore it had been considered that the alpha-Gal A enzyme replacement therapy should be a promising therapy for Fabry's disease. Practical application of the alpha-Gal A replacement therapy, however, has been extremely limited due to the lack of supply of this enzyme.

[0005] In 1986, the gene encoding human alpha-Gal A was isolated (see NPL 4), and further, in 1988, a method was reported for expression and production of rh alpha-Gal A by using CHO cells transformed with a human alpha-Gal A expression vector (see NPL 5). Utilizing recombinant technology, these finding made it possible to produce human alpha-Gal A in a large scale, and to use the enzyme as a medicament for the enzyme replacement therapy for Fabry's disease. The rh alpha-Gal A has also been expressed in insect cells (PTL 1).

[0006] Further, some methods for purification of rh alpha-Gal A were reported comprising employment of hydrophobic column chromatography, heparin sepharose column chromatography, hydroxylapatite column chromatography, anion-exchange column chromatography, and gel filtration column chromatography (PTL 2, PTL 3). Although the purity of the rh alpha-Gal A obtained by the method for purification described in PTL 2 or PTL 3 has shown to be sufficiently high, it was not considered to be fully acceptable for human use. The reason is that while any medicaments to be infused to patients are required to be free of contaminants, such as, in particular, proteins derived from the host cells employed, either PTL 2 or PTL 3 fails to show whether such contaminants remain in the purified rh alpha-Gal A or not. Currently, medicaments against Fabry's disease which contain rh alpha-Gal A as an active ingredient have been on the market as medical drugs under the trade names such as Replagal™ and Fabrazyme™.

Citation List

Patent Literature

[0007] PTL 1: WO 90/11353

PTL 2: WO98/11206

PTL 3: WO00/53730

Non Patent Literature

[0008] NPL 1: Mapes et al: Science 169: 987-9 (1970)

NPL 2: Brady et al: N. Engl. J. Med. 289: 9-14 (1973)

NPL 3: Desnick et al: Proc Natl Acad Sci USA 76: 5326-30 (1979).

NPL 4: Bishop et al: Proc Natl Acad Sci USA 83: 4859-63 (1986)

NPL 5: Bishop et al: Lipid Storage Disorders - Biological and Medical Aspects, NY Plenum Press P809-823 (1988)

Summary of Invention

Technical Problem

[0009] Against the above background, it is an objective of the present invention to provide a method for production of recombinant human alpha-galactosidase A (rh alpha-Gal A) starting with culturing of rh alpha-Gal A producing mammalian cells in a serum-free medium.

[0010] It is another object of the present invention to provide a process for purification of rh alpha-Gal A obtained in the culture supernatant, through column chromatography, in high yield and to such a high purity as permits direct use of the purified protein as a medical drug.

Solution to Problem

[0011] The present inventors found that rh alpha-Gal A contained in the supernatant of a culture of rh alpha-Gal A -producing cells in a serum-free medium, can be purified to a very high purity, and in a very high yield as well, by subjecting the rh alpha-Gal A to a purification process comprising a combination of anion-exchange column chromatography, hydrophobic column chromatography, chromatography using a column having affinity for phosphate group, cation-exchange column chromatography, and gel filtration column chromatography. Alternatively, anion-exchange column chromatography above may be substituted by dye affinity column chromatography. The present invention was completed through further studies based on the finding.

[0012] Thus, the present invention provides what follows:

1. A method for production of a recombinant human alpha-Gal A comprising the steps of:

(a) culturing recombinant human alpha-Gal A-producing mammalian cells in a serum-free medium to let them secrete the recombinant human alpha-Gal A in the medium,

(b) collecting culture supernatant by removing the cells from the culture that is obtained in step (a) above,

(c) subjecting the culture supernatant collected in step (b) above to anion-exchange column chromatography or dye affinity column chromatography to collect the recombinant human alpha-Gal A -active fractions,

(d) subjecting the fractions collected in step (c) above to hydrophobic column chromatography to collect recombinant human alpha-Gal A-active fractions,

(e) subjecting the fractions collected in step (d) above to a column chromatography employing as solid phase a material having affinity for phosphate group to collect the recombinant human alpha-Gal A-active fractions,

(f) subjecting the fractions collected in step (e) above to cation-exchange column chromatography to collect the recombinant human alpha-Gal A -active fractions, and

(g) subjecting the fractions collected in step (f) above to gel filtration column chromatography to collect the recombinant human alpha-Gal A-active fractions, in the order.

2. The method according to (1) above, wherein the cation exchanger employed in the cation-exchange column chromatography is a weak cation exchanger.

3. The method according to (2) above, wherein the weak cation exchanger has a selectivity based on both hydrophobic interaction and hydrogen bond formation.

4. The method according to (2) or (3) above, wherein the weak cation exchanger has phenyl groups, amide bonds and carboxyl groups.

5. The method according to one of (1) to (4) above, wherein the dye employed in the dye affinity column chromatography is a blue triazine dye.

6. The method according to one of (1) to (5) above, wherein the material having affinity for phosphate group is selected from the group consisting of hydroxyapatite and fluoroapatite.

7. The method according to (6) above, wherein the material having affinity for phosphate group is hydroxyapatite.

8. The method according to one of (1) to (7) above, wherein the mammalian cells are CHO cells transfected with an expression vector which is designed to express the recombinant human alpha-Gal A under the regulation of EF-1(alpha) promoter.

9. A method for purification of a recombinant human alpha-Gal A having a mannose 6-phosphate residue in its oligosaccharide chain, and wherein the method comprises the steps of:

(a) applying the recombinant human alpha-Gal A with contaminants to a chromatography column which employs as solid phase a material having affinity for phosphate group,

(b) flowing a first mobile phase through the column to wash the column while letting the recombinant human alpha-Gal A be adsorbed by the column, and

(c) eluting the recombinant human alpha-Gal A from the column by flowing a second mobile phase through the column, wherein the second mobile phase contains a phosphate at higher concentration than the first mobile phase.

10. The method according to (9) above, wherein the material having affinity for phosphate group is selected from the group consisting of hydroxyapatite and fluoroapatite.

11. The method according to (10) above, wherein the material having affinity for phosphate group is hydroxyapatite.

12. A recombinant human alpha-Gal A composition produced by the method according to one of (1) to (11).

Advantageous Effects of Invention

[0013] As it has enabled to produce rh alpha-Gal A starting with serum-free culturing of cells, the present invention provides rh alpha-Gal A which is free of any serum-derived contaminants, including pathogenic agents such as viruses or prions. Therefore, the rh alpha-Gal A obtained according to the present invention can be administered into a human body as a safe medicament substantially without any risks of exposure to such pathogenic agents. Further, as it has enabled purification of rh alpha-Gal A substantially avoiding any use of organic solvents, the present invention eliminates the risk of denaturation of rh alpha-Gal A which otherwise might be caused by the exposure to an organic solvent employed. Furthermore, the present invention is favorable to the environment, for the waste fluid left after performing the purification process according to it does not contain an organic solvent, and in the economic sense as well, for it requires no facility to treat organic solvents which would otherwise be contained in the waste fluid.

[0014] Further the present invention makes it possible to selectively purify rh alpha-Gal A having mannose 6-phosphate residue in its oligosaccharide chain. To exert its enzymatic activity after administration to a human body, the rh alpha-Gal A should be taken up by relevant cells through mannose 6-phosphate receptors expressed on their surface. Therefore the efficacy of the rh alpha-Gal A as a medicament is dramatically increased through the selective purification of it according to the present invention.

Brief Description of Drawings

[0015] [fig.1]Fig. 1 shows a schematic diagram illustrating the method for construction of vector pE-neo/hGHpA.

[fig.2-1]Fig. 2-1 shows a first part of a schematic diagram illustrating the method for construction of vector pE-gs/hGHpA(alpha-Gal A).

[fig.2-2]Fig. 2-2 shows a second part of a schematic diagram illustrating the method for construction of vector pE-gs/hGHpA(alpha-Gal A).

[fig.3]Fig. 3 shows a growth curve of recombinant cells for expression of rh alpha-Gal A and the concentration of rh alpha-Gal A in the culture medium.

[fig.4]Fig. 4 shows the result of SDS-PAGE electrophoresis of the fractions obtained in the respective purification steps. In each lane, 5 micrograms of protein was applied. Lane 1: Molecular size marker, Lane 2: concentrated culture supernatant, Lane 3: Q-sepharose Fast Flow fraction, Lane 4: Phenyl sepharose Fast Flow fraction, Lane 5: Hydroxyapatite column (CHT-I) faction, Lane 6: CaptoMMC fraction, Lane 6: Superdex 200 prep grade column fraction.

[fig.5]Fig. 5 shows the SE-HPLC chart of the purified rh alpha-Gal A (Lot No. 1). The vertical and horizontal axes show absorbance at 215 nm and retention time, re-

spectively.

Description of Embodiments

[0016] In the present invention, while the recombinant human alpha-galactosidase A (rh alpha-Gal A) is preferably a recombinant protein of human wild-type alpha-Gal A, it is not excluded that the rh alpha-Gal A is a mutant-type protein having one or more substitution, deletion, and/or insertion of one or more amino acids compared with the amino acid sequence of human wild-type alpha-Gal A. The DNA sequence for human wild-type alpha-Gal A and amino acid sequence encoded by it, including N-terminal signal sequence, are shown as SEQ ID NO:13 and SEQ ID NO:14, respectively. The N-terminal signal sequence consists of 31 amino acids and is removed post-translationally.

[0017] In the present invention, the term "recombinant human alpha-Gal A-producing mammalian cells" or "rh alpha-Gal A-producing mammalian cells" means mammalian cells which have been artificially manipulated to express, or strongly express, the gene encoding human alpha-Gal A. Though in general the gene to be strongly expressed is one which is introduced to the mammalian cells (transformation) using an expression vector in which the gene is incorporated, it may also be an intrinsic gene which has been artificially modified to be strongly expressed. Examples of the means for artificially modifying an intrinsic gene to make it strongly express itself include, but not limited to, replacement of the promoter upstream of the intrinsic gene with a promoter which strongly induces expression of the gene. Such methods have been disclosed in several literatures (i.e. WO94/12650, WO95/31560). Though there is no particular limitation as to which mammalian cells are to be employed, preferred are those of human-, mouse- or Chinese hamster-origin, and, among others, CHO cells, which originate from Chinese hamster ovary cells, are particularly preferred.

[0018] In the present invention, the term "recombinant human alpha-galactosidase A" or "rh alpha-Gal A" means the human alpha-galactosidase A which is secreted by the above-mentioned recombinant human alpha-Gal A-producing mammalian cells in the medium during culture.

[0019] In the present invention, the term "oligosaccharide chain" means a chain of oligosaccharides covalently bound to the peptide chain of alpha-Gal A, including an asparagine type sugar chain, which is covalently bound to an asparagine residue of alpha-Gal A.

[0020] In the present invention, an example of preferable serum-free media in which the rh alpha-Gal A-producing mammalian cells are to be cultured is the following medium which comprises; 3-700 mg/mL of amino acids, 0.001-50 mg/L of vitamins, 0.3-10 g/L of monosaccharides, 0.1-10000 mg/L of inorganic salts, 0.001-0.1 mg/L of trace elements, 0.1-50 mg/L of nucleosides, 0.001-10 mg/L of fatty acids, 0.01-1 mg/L of

biotin, 0.1-20 micrograms/L of hydrocortisone, 0.1-20 mg/L of insulin, 0.0-10 mg/L of vitamin B_{12}, 0.01-1 mg/L of putrescine, 10-500 mg/L of sodium pyruvate, and water-soluble iron compounds. As desired, it may also include thymidine, hypoxanthine, a conventional pH indicator, and antibiotics.

[0021] Further, DMEM/F12 medium, a mixed medium consisting of DMEM and F12, may also be used as a basic serum-free medium. Both of these media are well known to those skilled in the art. As a serum-free medium, DMEM(HG)HAM modified (R5) medium may be used, too, which contains sodium hydrogen carbonate, L-glutamine, D-glucose, insulin, sodium selenite, diaminobutane, hydrocortisone, ferric (II) sulfate, asparagine, aspartic acid, serine, and polyvinyl alcohol. Furthermore, a commercially available serum-free medium may also be used as a basic medium, such as CDoptiCHO, CHO-S-SFM II or CD CHO (Invitrogen), IS CHO-V or IS CHO-V-GS (Irvine), EX-CELL302 or EX-CELL305 (JRH), or the like.

[0022] In the present invention, rh alpha-Gal A-producing mammalian cells are cultured for rh alpha-Gal A production in the culture medium for at least 5 days, more preferably for 8 to 14 days. Then the culture supernatant containing rh alpha-Gal A is collected and provided to the purification process for rh alpha-Gal A.

[0023] Each of the chromatography procedures for purification of the rh alpha-Gal A may, when needed, be carried out in the presence of a nonionic surfactant in order to prevent nonspecific binding of the protein. Though there is no particular limitation as to which nonionic surfactant is to be employed, a polysorbate-based surfactant is preferably employed, and more preferably polysorbate 80 or polysorbate 20. The concentration of such a nonionic surfactant is preferably 0.005% (w/v) to 0.05% (w/v), more preferably about 0.01% (w/v).

[0024] The process for purification of the rh alpha-Gal A may be carried out at room temperature or at lower temperatures, but preferably at lower temperatures, particularly at 1-10 deg C.

[0025] In the first chromatography step for the purification process, the pH of the culture supernatant is adjusted preferably to 7.0-8.0, more preferably to pH 7.2-7.8, and further preferably to about pH 7.5. Then the culture supernatant is applied to the anion-exchange column that has been equilibrated with a phosphate buffer supplemented with a salt to let rh alpha-Gal A bind to the column. The pH of this phosphate buffer is preferably to 7.0-8.0, more preferably about 7.2-7.8, and still more preferably about 7.5. Though there is no particular limitation as to which salt is to be added to the phosphate buffer, sodium chloride and potassium chloride are preferred. In the case where sodium chloride is used, its concentration is preferably in the range of 5-100 mM, more preferably in the range of 10-60 mM, and still more preferably about 50 mM.

[0026] After the anion-exchanger column to which rh alpha-Gal A is bound is washed, the rh alpha-Gal A is eluted using a phosphate buffer with increased salt concentration. Though there is no particular limitation as to which salt is to be used, sodium chloride and potassium chloride are preferred. In the case where sodium chloride is used, its concentration is preferably in the range of 125-500 mM, more preferably 175-350 mM, and still more preferably about 225 mM.

[0027] Further, though there is no particular limitation as to which anion-exchanger resin is to be employed, strong anion-exchanger resin may be used preferably. Commercially available resins, such as Q Sepharose Fast Flow (GE Healthcare), may be used preferably.

[0028] Alternatively, the dye affinity chromatography may be used as the first chromatography step for the purification process. In this case the pH of the culture supernatant is adjusted preferably to 6.5-7.5, more preferably to 6.8-7.2, and still more preferably to about 7.0, then the culture supernatant is applied to the dye affinity column that has been equilibrated with a phosphate buffer supplemented with a salt. The pH of this phosphate buffer is preferably 6.5-7.5, more preferably 6.8-7.2, and still more preferably about 7.0. Concurrently, Tris buffer may be added to the phosphate buffer at the concentration of preferably 30-70 mM, more preferably about 50 mM.

[0029] After the column to which rh alpha-Gal A is bound is washed, the rh alpha-Gal A is eluted. Elution may be done by a phosphate buffer with an increased pH. The concentration of a salt in the buffer may be increased at the same time. The pH of this phosphate buffer is preferably 7.0-7.8, more preferably 7.3-7.7, and still more preferably about 7.5. In the case where the concentration of a salt is also increased, sodium chloride and potassium chloride are preferably used for this purpose. When sodium chloride is chosen, its concentration is preferably in the range of 400-800 mM, more preferably in the range of 500-700 mM, and still more preferably about 600 mM. Concurrently, Tris buffer may be added to the phosphate buffer at the concentration of preferably 30-70 mM, more preferably about 50 mM.

[0030] Further, though there is no particular limitation as to which resin is to be employed for dye affinity chromatography, blue triazine dye is preferably used, but other triazine dyes are also suitable. A particularly preferred column material for this is Blue Sepharose 6 Fast Flow (GE Healthcare) in which the dye, Cibacron™ Blue F3GA, is covalently immobilized to Sepharose 6 Fast Flow matrix.

[0031] The hydrophobic column chromatography, the second step of the purification process, is a step for removing contaminants making use of the hydrophobic interaction between rh alpha-Gal A and hydrophobic ligand attached to the resin. Though there is no particular limitation as to which hydrophobic ligand is to be employed in the hydrophobic column chromatography, preferable is a ligand containing phenyl

group, and more preferable a ligand containing phenyl group attached to the resin via a spacer arm, such as represented by the formula R-O-CH_2-CH(OH)-CH_2-O-C_6H_5 where R denotes the resin. A particularly preferred column material for this is Phenyl Sepharose 6 Fast Flow (GE Healthcare) in which phenyl group is covalently immobilized to Sepharose 6 Fast Flow matrix via a certain spacer arm.

[0032] The hydrophobic column chromatography column is equilibrated with a phosphate buffer supplemented with a salt. The pH of this phosphate buffer is preferably 6.5-7.5, more preferably about 6.7-7.3, and still more preferably about 7.0. Though there is no particular limitation as to which salt is to be added to the phosphate buffer, sodium chloride and potassium chloride are preferred. In the case where sodium chloride is used, its concentration is preferably in the range of 500 mM -1 M, and more preferably in the range of 700-800 mM, and still more preferably about 750 mM. Concurrently, Tris buffer may be added to the phosphate buffer at the concentration of preferably 30-70 mM, more preferably about 50 mM.

[0033] Then, rh alpha-Gal A-containing fractions of the eluate obtained in the first step are applied to the column. After the column to which rh alpha-Gal A is bound is washed, the rh alpha-Gal A is eluted. Elution may be done by a phosphate buffer not supplemented with salt, where the concentration of phosphate buffer is preferably 3-10 mM, and still more preferably about 5mM. Concurrently, Tris buffer may be added to the phosphate buffer at the concentration of preferably 30-70 mM, more preferably about 50 mM. Alternatively elution may be done by pure water.

[0034] The column chromatography employing a solid phase having affinity for phosphate group, the third step of the purification, is a step to eliminate contaminant proteins, such as carboxyl peptidases. Though there is no particular limitation as to which solid phase is to be used having affinity for phosphate group, hydroxyapatite and fluoroapatite may preferably be used, and hydroxyapatite is particularly preferred.

[0035] The hydroxyapatite column is equilibrated with a phosphate buffer supplemented with a salt. The pH of this phosphate buffer is preferably 6.5-7.5, more preferably about 6.8-7.2, and still more preferably about 7.0, and the concentration of phosphate preferably 3.0-20 mM, more preferably about 3.0-15 mM, and further preferably about 10 mM. Though there is no particular limitation as to which salt is to be added to the phosphate buffer, sodium chloride and potassium chloride are preferred. In the case where sodium chloride is used, its concentration is preferably lower than 800 mM, and more preferably in the range of 10-200 mM, and further preferably about 50 mM. Concurrently, Tris buffer may be added to the phosphate buffer at the concentration of preferably 30-70 mM, more preferably about 50 mM.

[0036] Then rh alpha-Gal A-containing fractions of the eluate obtained in the second step are applied to the column. After the column to which rh alpha-Gal A is bound is

washed, the rh alpha-Gal A is eluted. Elution may be done by a phosphate buffer containing a salt. The pH of this phosphate buffer is preferably 6.5-7.5, more preferably about 6.7-7.3, and still more preferably about 7.0, and the concentration of phosphate is preferably to 30-80 mM, more preferably about more than 40-60 mM, and still more preferably about 45 mM. Though there is no particular limitation as to which salt is to be used, sodium chloride and potassium chloride are preferred. In the case where sodium chloride is used, its concentration is preferably lower than 300 mM, more preferably in the range of 10-200 mM, and still more preferably of about 50mM.

[0037] The cation-exchange column chromatography, the forth step of the purification process, is a step to eliminate contaminant proteins. Though there is no particular limitation as to which cation-exchanger is to be employed in the cation-exchange column chromatography, a weak cation exchanger is preferred, and more preferred is a weak cation exchanger having a selectivity based on both hydrophobic interaction and hydrogen bond formation. For example, a weak cation exchanger having phenyl groups, amide bonds and carboxyl groups and having a selectivity based on both hydrophobic interaction and hydrogen bond formation, such as Capto MMC (GE Healthcare), etc., may be employed

[0038] In the cation-exchange column chromatography, the column is equilibrated with a phosphate buffer supplemented with a salt. The pH of this phosphate buffer is preferably 4.5-6.5, more preferably about 5.0-6.0, and still more preferably about 5.5. Though there is no particular limitation as to which salt is to be added to the phosphate buffer, sodium chloride, potassium chloride and calcium chloride are preferred. In the case where sodium chloride is used, its concentration is preferably in the range of 50-250 mM, and more preferably in the range of 100-200 mM, and still more preferably about 150 mM.

[0039] Then rh alpha-Gal A-containing fractions of the eluate obtained in the third step are applied to the column. After the column to which rh alpha-Gal A is bound is washed, the rh alpha-Gal A is eluted using a phosphate buffer with an increased pH, which is preferably in the range of about 5.5-7.5, more preferably in the range of about 6.0-7.0 and still more preferably about 6.5

[0040] The gel filtration column chromatography, the fifth step of the purification process, is a step for elimination of low molecular-weight impurities, such as endotoxins, as well as multimeric complexes or decomposition products of rh alpha-Gal A. Thus, substantially pure rh alpha-Gal A is obtained through the steps 1st to 5th.

[0041] Additionally one more column chromatography may be incorporated into the purification process mentioned above, comprising anion-exchange or dye affinity column chromatography, hydrophobic column chromatography, column chromatography employing a solid phase having affinity for phosphate group, cation-exchange column

chromatography, and gel filtration column chromatography in the order. Such the additional column chromatography is preferably dye affinity column chromatography and may be employed between any two adjacent steps of the purification process mentioned above.

[0042] A step for virus inactivation may optionally be added to the purification process of the present invention. Though there is no particular limitation as to which process for virus inactivation is to be applied, a solvent-detergent method may preferably be applied. For this, a nonionic surfactant is added to a solution containing rh alpha-Gal A, and the mixture is incubated for more than 3 hours. Though there is no particular limitation as to which surfactant is to be used, polysorbate 20, polysorbate 80, tritonX-100, and tri(n-butyl)phosphate may preferably be used alone or in any combinations, and more preferably the combination of polysorbate 80 and tri(n-butyl)phosphate may be used.

[0043] Such an additional step for virus inactivation may be employed either previous to the purification process or between any two adjacent steps of the purification process mentioned above.

[0044] Further, the present invention also provides a method for purification of rh alpha-Gal A having a mannose 6-phosphate residue in its oligosaccharide chain. The method includes a step in which a solution containing rh alpha-Gal A is subjected to column chromatography employing a solid phase having affinity for phosphate group. Preferred examples of such a solid phase include hydroxyapatite and fluoroapatite, and hydroxyapatite is particularly preferred. The purpose of this step employing a solid phase having affinity for phosphate group is to purify rh alpha-Gal A containing mannose 6-phosphate residues in its oligosaccharide chain. The step can be performed in combination with at least one other purification step, selected from cation-exchange column chromatography, hydrophobic column chromatography, dye-affinity column chromatography, anion-exchange column chromatography, and gel filtration column chromatography, in any order. Preferably, the combination is anion-exchange column chromatography, hydrophobic column chromatography, column chromatography employing a solid phase having affinity for phosphate group, cation-exchange column chromatography, and gel filtration column chromatography in the order.

[0045] The present invention is described in further detail below with reference to examples. However, it is not intended that the present invention be limited to the examples.

Example 1

[0046] Construction of pE-gs/hGHpA(alpha-GalA), a rh alpha-Gal A expression vector

pEF/myc/nuc vector (Invitrogen) was digested with KpnI and NcoI to cut out a DNA fragment including the EF-1(alpha) promoter and its first intron, which then was blunt-

ended with T4 DNA polymerase. Then the DNA fragment thus prepared was inserted into pC1-neo vector (Invitrogen) which had been digested with BglII and EcoRI and then blunt-ended with T4 DNA polymerase. Inserted into this was the above-mentioned blunt-ended fragment including the EF-1(alpha) promoter and its first intron to give pE-neo vector (Fig. 1).

[0047] A DNA fragment containing poly adenylation signal sequence of human growth hormone gene was synthesized by annealing four synthetic oligonucleotides: (a) hGH-f1 (ID NO:1), (b) hGH-r1 (SEQ ID NO:2), (c) hGH-f2 (SEQ ID NO:3), and (d) hGH-r2 (SEQ ID NO:4). The DNA fragment thus prepared was inserted between the MluI and KpnI sites of pE-neo to give pE-neo/hGHpA vector (Fig. 1).

[0048] A 5' half of Chinese hamster Glutamine synthetase ("Ch GS" or "GS") coding region was amplified by PCR using a primer set, GS-1 (SEQ ID NO:5) and GS-3 (SEQ ID NO:6), and, as a template, ssDNA prepared from RNA extracted from CHO cells. A 3' half of Ch GS coding region was amplified by PCR using a primer set, GS-2 (SEQ ID NO:7) and GS-4 (SEQ ID NO:8), and, as a template, ssDNA prepared from RNA extracted from CHO cells. Then the amplified 5' half of Ch GS coding region was ligated into pT7blue-T vector (EMD Millipore). The vector thus obtained was digested with EcoRV and BamHI, and then the amplified 3' half of CH GS coding region, after digestion with EcoRV and BamHI, was inserted into the digested vector to obtain a plasmid vector containing the full length cDNA for Ch-GS. The plasmid obtained was designated pT7blue(GS) (Fig. 2-1).

[0049] A 5' half of human alpha-GalA coding region was amplified by PCR using a primer set, GAL-Mlu (SEQ ID NO:9) and GAL-Sca2 (SEQ ID NO:10), and, as a template, human placental Quick-Clone cDNA (Clontech). A 3' half of human alpha-GalA coding region was amplified by PCR using a primer set, GAL-Sca1 (SEQ ID NO:11) and GAL-Xba (SEQ ID NO:12), and, as a template, human placental Quick-Clone cDNA (Clontech). Then both PCR products were respectively ligated into the EcoRV site of pBluescriptSK(+) vector. The 5' half of human alpha-GalA coding region then was excised from the vector thus obtained, by digestion with MluI and ScaI, and the 3' half of human alpha-GalA coding region was excised from the vector by digestion with ScaI and NotI Then the excised fragments were concomitantly inserted into the MluI and NotI sites of the multi cloning site in pE-neo vector to obtain full length cDNA for human alpha-GalA. The plasmid thus obtained was designated pE-neo(alpha-GalA) (Fig.2-2).

[0050] pE-neo/hGHpA was digested with AfIII and BstXI, and into the vector thus digested was inserted Ch-GS cDNA which had been excised out from pT7blue(GS) by digestion with AfIII and BstXI. The plasmid obtained was designated pE-gs/hGHpA (Fig. 2-1). pE-gs/hGHpA vector was digested with MluI and NotI, and into the vector

thus digested was inserted human alpha-GalA cDNA which had been excised out from pE-neo(alpha-GalA) by digestion with MluI and NotI. The plasmid obtained, designated pE-gs/hGHpA(alpha-GalA), was used as rh alpha-Gal A expression vector in further experiments (Fig.2-2).

Example 2

[0051] Production of Recombinant Cells for Expression of rh alpha-Gal A

CHO cells (CHO-K1: purchased from American Type Culture Collection) was transfected with the above-mentioned expression vector pE-gs/hGHpA(alpha-Gal A) using Lipofectamine 2000 (Invitrogen) according to the following method. Briefly, two days before transfection, 2.5 x 10^5 CHO-K1 cells were seeded in a 3.5-cm culture dish containing 3 mL of D-MEM/F12 medium containing 5 % FCS (D-MEM/F12/5%FCS), and the cells were cultured at 37 deg C in a humidified atmosphere of 5% CO_2 and 95% air. After two days of culture, the cells were washed with PBS, and 1 mL of fresh serum-free D-MEM/F12 was added. Then the cells were transfected by addition of 200 microliters of a 1:1 mixture solution consisting of Lipofectamine 2000 solution diluted 20 times with Opti-MEM I medium (Invitrogen) and a pE-gs/hGHpA(alpha-GalA) solution diluted to 20 micrograms/mL with Opti-MEM I medium, followed by incubation at 37 deg C in a humidified atmosphere of 5% CO_2 and 95% air for 5 hours. After transfection, the medium was replaced with D-MEM/F12 / 5 % FCS and cultivation followed for 24 hours.

[0052] Then, the medium was replaced with glutamine-free GMEM-S medium (JRH Biosciences) supplemented with 1 X GS supplement (JRH Biosciences), 10% dialyzed FBS and 30 micromole/L methionine sulfoximine (MSX), and a selective culture was carried out at 37 deg C in a humidified atmosphere of 5% CO_2 and 95% air. Cells that had grown in the medium for selective culture were subjected to several successive rounds of subculture in the medium to give recombinant cells. In the course of the selective culture, concentration of MSX in the medium was elevated step by step to amplify the inserted recombinant genes. Finally bulk cells that stably grew in the medium with 300 micromole/L MSX were obtained after two steps subculture at elevated MSX concentrations (100 and 300 micromole/L MSX).

[0053] Then, according to the limiting dilution technique, the cells having the amplified recombinant gene were seeded on a 96-well plate in such a manner that not more than one cell might be seeded per well. The cells then were cultured for about 10 days to let each of them form a monoclonal colony. The culture supernatant in the wells where a monoclonal colony was formed was each sampled and examined for human alpha-Gal A activity as described below, and cell lines which were found expressing a high activity for human alpha-Gal A were selected.

[0054] For adaptation to serum-free suspension cell culture, the selected cell lines were each cultured in a commercially available serum-free medium, CD-Opti CHO medium (Invitrogen) supplemented with 10 mg/L insulin, 40 mg/L thymidine, 100 mg/L hypoxantine and 100 micromole/L MSX, at 37 deg C in a humidified atmosphere of 5% CO_2 and 95% air until the cells stably grew. The cells then were suspended in CD-Opti CHO medium (Invitrogen) supplemented with 10 mg/L insulin, 40 mg/L thymidine, 100 mg/L hypoxantine, 100 micromole/L MSX, and 10% DMSO, and stored as seed cells in liquid nitrogen.

Example 3

[0055] Culture of Recombinant Cells for Expression of rh alpha-Gal A

The above seed cells were thawed and diluted to a density of 4 x 10^5 cells/mL and cultured for 3 to 4 days with CD-Opti CHO medium supplemented with 10 mg/L insulin, 40 mg/L thymidine, 100 mg/L hypoxantine and 100 micromole/L MSX (CD medium), and then diluted to a density of 2 x 10^5 cells/mL with CD medium and cultured for 3 to 4 days. These cells were again diluted to a density of 2 x 10^5 cells/mL with CD medium and further cultured for 3 to 4 days.

[0056] The number of the cells thus cultured was counted, and the cell culture was diluted with CD medium to a density of 2 x 10^5 cells/mL, and then the cells in 1L of the diluted culture were cultured with shaking for a few days. The scale of culture was escalated until the culture volume reached 200 L.

[0057] Then the number of the cells was counted, and the cells were diluted to a density of 2 x 10^5 cells/mL with 1800 L of CD-Opti CHO medium supplemented with 10 mg/L insulin, 40 mg/L thymidine, 100 mg/L hypoxantine and 0.1% HY soy (Invitrogen), and cultured for 11 days. 22.4 L of feed solution which contained 8.84 g insulin, 35.2 mmol hypoxantine, 5.6 mmol thymidine and 1.8 kg HY soy in CD-Opti CHO medium was added on the 3rd, 5th, 7th, and 9th days. Sampling was made every day during the culture, and measurement was performed for cell number, survival rate, glucose concentration, and amount of expressed rh alpha-Gal A. In the case where glucose concentration became lower than 3 g/L, 30% glucose solution was added so that the concentration reached 3.5 g/L. During the 11 days of culture, the viable cell density and the concentration of rh alpha-Gal A were daily monitored. The viable cell density reached about 6 x 10^6 cells/mL on days 6-7 of culture, indicating that high-density cell culture was successfully achieved (Fig 3). As measured by ELISA as described below, the concentration in the medium of rh alpha-Gal A secreted by the cells increased over time and reached almost 140 mg/L on day 6 (Fig 3).

[0058] After completion of the culture above, the cell culture was collected and filtered successively through Millistak+ HC Pod Filter grade D0HC, (Millipore), Millistak+ HC

Pod Filter grade X0HC (Millipore), and then Durapore Opticap XLT20 (0.22 micrometer, Millipore), to give a culture supernatant. The obtained culture supernatant was concentrated by a hollow fiber module equipped with PS membrane to one 13th of the original volume.

Example 4

[0059] Virus Inactivation

Tri-n-butyl phosphate (TNBP) and polysorbate 80 were added to the approximately 75 L of the concentrated culture supernatant described above so that their final concentrations would be 0.3 %(v/v) and 1 %(w/v), respectively, and this mixture solution then was gently stirred for 3 to 4 hours at room temperature.

Example 5

[0060] Method for Purification of rh alpha-Gal A

The pH of the above virus-inactivated solution then was adjusted to 7.5 by addition of 1 M Tris-buffer (pH 8.8), and subsequently the conductivity was adjusted to that of the 10 mM phosphate buffer (pH 7.5) containing 50 mM NaCl, by addition of 50 mM phosphate buffer (pH 7.0) containing 1 M NaCl. Then the solution was filtered through Durapore Opticap XL10 (Millipore), and then was loaded on a Q Sepharose Fast Flow column (column volume: about 19.2 L, bed height: about 20 cm, GE Healthcare), an anion-exchange column, which had been equilibrated with 10 mM phosphate buffer (pH 7.5) containing 50 mM NaCl at a linear flow rate of 150 cm/hr to let rh alpha-Gal A be adsorbed by the column. Then, the column was washed with a threefold column volume of the same buffer supplied at the same flow rate, and the adsorbed rh alpha-Gal A then was eluted with a sixfold column volume of 10 mM phosphate buffer (pH 7.5) containing 225 mM NaCl, subsequently with a threefold column volume of 50 mM phosphate buffer (pH 7.0) containing 1 M NaCl, and the fractions containing rh alpha-Gal A were collected.

[0061] To the fractions collected at the step of Q Sepharose Fast Flow column chromatography above, one fourth volume of 2 M NaCl was added to adjust the concentration of NaCl to 500 mM, and subsequently acetic acid was added to adjust the pH to 7.0. The solution obtained was loaded on a Phenyl Sepharose 6 Fast Flow column (column volume: 19.2 L, bed height: about 20 cm, GE Healthcare), a hydrophobic column, which had been equilibrated with 50 mM Tris-HCl, 5 mM phosphate buffer (pH 7.0) containing 750 mM NaCl, at a linear flow rate of 150 cm/hr to let rh alpha-Gal A be adsorbed by the column. Then after the column was washed with a threefold column volume of the same buffer supplied at the same flow rate, the adsorbed rh alpha-Gal A was eluted with a ninefold column volume of a buffer containing 50 mM Tris-HCl and 5 mM phosphate (pH 7.0), and subsequently with a

threefold column volume of pure water, and the fractions containing rh alpha-Gal A were collected.

[0062] The fractions collected at the step of Phenyl Sepharose 6 Fast Flow column chromatography above, was loaded on a CHT-I ceramic hydroxyapatite column (column volume: 19.2 L, bed height: about 20 cm, Bio-Rad Laboratories), a hydroxyapatite column, which had been equilibrated with 50 mM Tris-HCl, 10 mM phosphate buffer (pH 7.0) containing 50 mM NaCl, at a linear flow rate of 150 cm/hr to let rh alpha-Gal A be adsorbed by the column. Then, the column was washed with a fivefold column volume of the same buffer supplied at the same flow rate, and the adsorbed rh alpha-Gal A then was eluted with a ninefold column volume of a buffer containing 50 mM Tris-HCl, 45 mM sodium phosphate and 50 mM NaCl (pH 7.0), and subsequently with a threefold column volume of 200 mM phosphate buffer (pH 7.0), and the fractions containing rh alpha-Gal A were collected.

[0063] To the fractions collected at the step of CHT-I ceramic hydroxyapatite column chromatography above, acetic acid was added to adjust the pH to 5.5. A precipitate formed by this was removed by filtration through Opticap SHC XL5 (0.22 micrometer, Millipore). The solution obtained was loaded on a Capto MMC column (column volume: 19.2 L, bed height: about 20 cm, GE Healthcare), a cation-exchange column having a selectivity based both on hydrophobic interaction and on hydrogen bond formation, which had been equilibrated with 50 mM acetate buffer (pH 5.5) containing 150 mM NaCl, at a linear flow rate of 150 cm/hr to let rh alpha-Gal A be adsorbed by the column. Then after the column was washed with a fourfold column volume of the same buffer supplied at the same flow rate, the adsorbed rh alpha-Gal A was eluted with a fivefold column volume of 50 mM phosphate buffer (pH 6.5), and subsequently with a threefold column volume of 50 mM phosphate buffer (pH 7.0) containing 1 M NaCl, and the fractions containing rh alpha-Gal A were collected.

[0064] The fractions collected at the step of Capto MMC column chromatography above was concentrated using Pellicon 3 Cassettes equipped with Biomax™ 30 membrane (Millipore) until the total volume became less than 1 L. One third of the concentrated solution was loaded on a Superdex 200 prep grade column (column volume: about 58 L, bed height: about 30 cm x 2, GE Healthcare) which had been equilibrated with 20 mM phosphate buffer (pH 5.8) containing 137 mM NaCl. The same buffer then was supplied at a linear flow rate of 25 cm/hr, and the fractions which corresponded to the main peak observed by absorbance at 280 nm were collected as fractions containing purified rh alpha-Gal A. This chromatography was carried out three times to purify all the concentrated solution above, and the fractions collected by each run were pooled.

[0065] The fractions pooled at the step of Superdex 200 prep grade column chromatography above were concentrated by Hollow fiber module equipped with PS membrane, and to

the concentrated solution, polysorbate 80 was added at the final concentration of 0.02%. The obtained solution was filtered through Viresolve Pro Modus1.3 (0.07 m^2 size, Millipore) to avoid any possible contamination with viruses and microorganisms in the final product.

[0066] The amount of rh alpha-Gal A after each step was quantified using ELISA as described below. The recovery rate of rh alpha-Gal A of each purification step is shown in Table 1, in which "rh alpha-Gal A recovery rate/step" means the proportion of the amount of recovered rh alpha-Gal A to that of the loaded rh alpha-Gal A in each step, and "rh alpha-Gal A recovery rate/total" the proportion of the amount of recovered rh alpha-Gal A in each process to the initial amount of rh alpha-Gal A subjected to the purification process. The amount of rh alpha-Gal A subjected to the above purification process was 133.800 g, of which 78.5 g of rh alpha-Gal A was finally recovered, thus giving the rh alpha-Gal A recovery rate/total as high as 58.7%. These results show that the method for purification described above enables to purify rh alpha-Gal A in very high yield and in a large production scale.

[0067] [Table 1]

Table 1. Recovery rate of rh alpha-Gal A in each purification step (Lot No. 1)

Purification process	rh alpha-Gal A loaded (g)	rh alpha-Gal A recovered (g)	rh alpha-Gal A recovery rate/step (%)	rh alpha-Gal A recovery rate/total (%)
Anion-exchange column (Q Sepharose FF)	133.8	139.4	104.2	104.2
Hydrophobic column (Phenyl Sepharose FF)	123.3	112.8	91.5	84.3
Hydroxyapatite column (CHT-I)	112.2	106.6	94.1	78.9
Cation-exchange column (Capto MMC)	105.0	100.1	95.4	74.8
Gel filtration column (Superdex 200pg)	94.2	78.5	83.3	58.7

Example 6

[0068] Analysis of Human alpha-Gal A by ELISA

To each well of a 96-well microtiter plate (Nunc) was added 100 microliters of mouse anti-human alpha-Gal A monoclonal antibody diluted to 0.25 micrograms/mL with 0.05 M carbonate-bicarbonate buffer (pH 9.6), and the plate was let stand for at least 1 hour at room temperature or overnight at 2-8 deg C to let the antibody be absorbed by the wells. Then, after each well was washed with phosphate buffered saline, pH 7.4 (PBS) containing 0.05% Tween 20 (PBS-T), 300 microliters of PBS-T

containing 1% BSA was added, and the plate was let stand for at least one hour at room temperature. Then, after each well was washed three times with PBS-T, 100 microliters of the test sample or human alpha-Gal A standard, which had been diluted as desired with PBS containing 0.5% BSA and 0.05% Tween 20 (PBS-BT), was added to the well, and the plate was let stand for at least one hour at room temperature. Then, after each well was washed three times with PBS-T, 100 microliters of horseradish peroxidase-labeled rabbit anti-human alpha-Gal A polyclonal antibody diluted with PBS-BT was added and the plate was let stand for at least one hour. Then, after each well was washed three times with PBS-T, 100 microliters of 0.4 mg/mL o-phenylenediamine with phosphate-citrate buffer (pH 5.0) containing 0.009% hydrogen peroxide was added to the well, and the plate was let stand for 10 to 25 minutes at room temperature. Then 0.1 mL of 1 mol/L H_2SO_4 was added to each well to stop the reaction, and the optical density at 490 nm was measured for the well on a 96-well plate reader.

Example 7

[0069] Measurement of the Activity of rh alpha-Gal A

Standard solutions were prepared by dissolving 4-methylumbelliferone in Dilution Buffer (26.7mM Citrate-44.8 mM phosphate buffer, pH 4.6 containing 0.1 mg/mL BSA,) at concentrations of 0 to 200 micromole/L. A substrate solution was prepared by dissolving 4-methylumbelliferyl-alpha-D-galactopyranoside (SIGMA) in Dilution Buffer at the final concentration of 5 mM. 10 microliters of each of the standard solutions or the test sample diluted with Dilution Buffer was added to each well of a 96-well microtiter plate (FluoroNunc Plate, Nunc). 75 microliters of the substrate solution was added to each well containing the diluted test sample or any of the standard solutions, and the plate was let stand for one hour at 37 deg C in the dark. After this incubation, 200 microliters of Stop Buffer (0.2 M glycine-NaOH, pH 10.6) was added to each well. Then fluorescent intensity of the solution in each well was measured by a 96-well plate reader with excitation light at the wavelength of 335 nm and fluorescent light at the wavelength of 460 nm.

[0070] A standard curve was produced by plotting fluorescence intensity measured of the standard solutions and interpolating between the plotted data points. The fluorescence intensity of each sample was corresponded to the concentration of the released 4-MUF using the standard curve. The specific activity of human alpha-Gal A was calculated as Units/mg, where one Unit of activity was equal to one micromole of 4-MUF produced per minute at 37 deg C. A published US patent application (publication No. 2004-0229250) was referred to for conducting the above measurement of the activity of rh alpha-Gal A. The specific activity of rh alpha-Gal A purified by the process

described above was found to be about 50 U/mg (46.7 to 54.5 U/mg).

Example 8

[0071] Analysis of purity of rh alpha-Gal A

The rh alpha-Gal A purified above was subjected to SDS-PAGE electrophoresis under a reductive, heating (70 deg C 10 min.) condition. The gel-stained with Coomassie brilliant blue revealed a single band at the position of molecular weight of about 55 kD in Superdex 200pg fraction (Fig. 4, lane 7).

[0072] Further, rh alpha-Gal A purified above was analyzed by Size-elimination HPLC (SE-HPLC). HPLC was performed using LC-20A System, SPD-20AV UV/VIS Detector (Shimazu Corp.). For SE-HPLC analysis, 10 microliters of a sample solution containing 2 mg/mL of rh alpha-Gal A purified above was applied onto TSKgel G3000SW$_{XL}$ column (7.8 mm I.D. x 30 cm, TOSOH) equilibrated with 25 mM phosphate buffered saline (PBS) at a flow rate of 0.5 ml/min. Elution profile was produced by monitoring absorbance at 215 nm.

[0073] The SE-HPLC analysis of the above purified rh alpha-Gal A revealed a single peak alone (Fig. 6). The result showed that the rh alpha-Gal A obtained above was highly purified and free of any detectable contaminants. This result demonstrates that the rh alpha-Gal A purified above is of such a high purity as may be directly used as a medical drug.

Industrial Applicability

[0074] The present invention is utilized for production of rh alpha-galactosidase A (rh alpha-Gal A) in a large scale, with a sufficiently high purity to be administered as a medicament to a patient suffering from Fabry's syndrome.

Sequence Listing Free Text

[0075] SEQ ID NO:1 = hGH-f1, synthetic sequence

SEQ ID NO:2 = hGH-r1, synthetic sequence

SEQ ID NO:3 = hGH-f2, synthetic sequence

SEQ ID NO:4 = hGH-r2, synthetic sequence

SEQ ID NO:5 = Primer GS-1, synthetic sequence

SEQ ID NO:6 = Primer GS-3, synthetic sequence

SEQ ID NO:7 = Primer GS-2, synthetic sequence

SEQ ID NO:8 = Primer GS-4, synthetic sequence

SEQ ID NO:9 = Primer GAL-Mlu, synthetic sequence

SEQ ID NO:10 = Primer GAL-Sca2, synthetic sequence

SEQ ID NO:11 = Primer GAL-Sca1, synthetic sequence

SEQ ID NO:12 = Primer GAL-Xba, synthetic sequence

Claims

[Claim 1] A method for production of a recombinant human alpha-Gal A comprising the steps of:
(a) culturing recombinant human alpha-Gal A-producing mammalian cells in a serum-free medium to let them secrete the recombinant human alpha-Gal A in the medium,
(b) collecting culture supernatant by removing the cells from the culture that is obtained in step (a) above,
(c) subjecting the culture supernatant collected in step (b) above to anion-exchange column chromatography or dye affinity column chromatography to collect the recombinant human alpha-Gal A -active fractions,
(d) subjecting the fractions collected in step (c) above to hydrophobic column chromatography to collect recombinant human alpha-Gal A-active fractions,
(e) subjecting the fractions collected in step (d) above to a column chromatography employing as solid phase a material having affinity for phosphate group to collect the recombinant human alpha-Gal A-active fractions,
(f) subjecting the fractions collected in step (e) above to cation-exchange column chromatography to collect the recombinant human alpha-Gal A -active fractions, and
(g) subjecting the fractions collected in step (f) above to gel filtration column chromatography to collect the recombinant human alpha-Gal A-active fractions, in the order.

[Claim 2] The method according to claim 1, wherein the cation exchanger employed in the cation-exchange column chromatography is a weak cation exchanger.

[Claim 3] The method according to claim 2, wherein the weak cation exchanger has a selectivity based on both hydrophobic interaction and hydrogen bond formation.

[Claim 4] The method according to claim 2 or 3, wherein the weak cation exchanger has phenyl groups, amide bonds and carboxyl groups.

[Claim 5] The method according to one of claims 1 to 4, wherein the dye employed in the dye affinity column chromatography is a blue triazine dye.

[Claim 6] The method according to one of claims 1 to 5, wherein the material

having affinity for phosphate group is selected from the group consisting of hydroxyapatite and fluoroapatite.

[Claim 7] The method according to claim 6, wherein the material having affinity for phosphate group is hydroxyapatite.

[Claim 8] The method according to one of claims 1 to 7, wherein the mammalian cells are CHO cells transfected with an expression vector which is designed to express the recombinant human alpha-Gal A under the regulation of EF-1(alpha) promoter.

[Claim 9] A method for purification of a recombinant human alpha-Gal A having a mannose 6-phosphate residue in its oligosaccharide chain, and wherein the method comprises the steps of:

(a) applying the recombinant human alpha-Gal A with contaminants to a chromatography column which employs as solid phase a material having affinity for phosphate group,

(b) flowing a first mobile phase through the column to wash the column while letting the recombinant human alpha-Gal A be adsorbed by the column, and

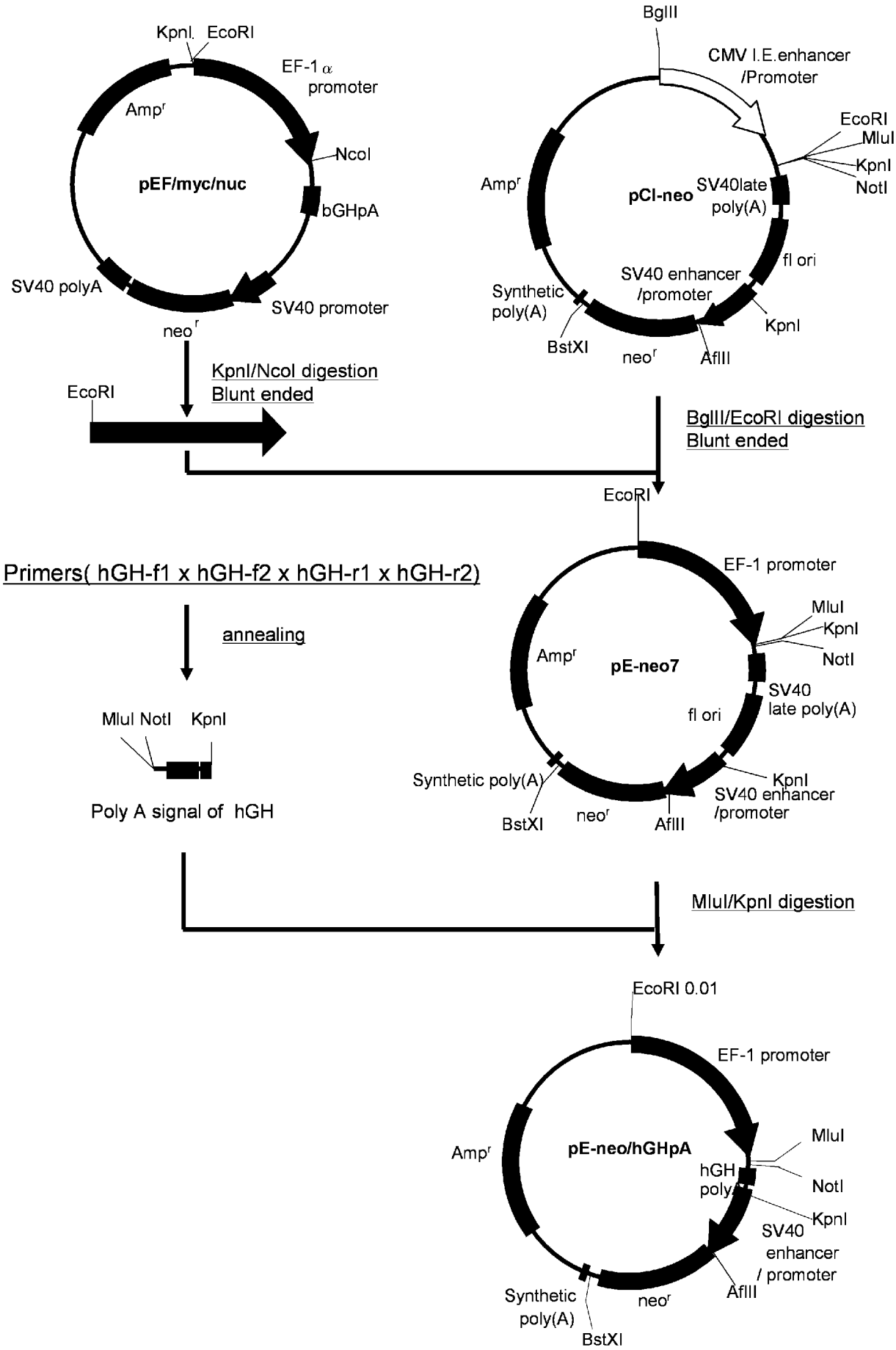
(c) eluting the recombinant human alpha-Gal A from the column by flowing a second mobile phase through the column, wherein the second mobile phase contains a phosphate at higher concentration than the first mobile phase.

[Claim 10] The method according to claim 9, wherein the material having affinity for phosphate group is selected from the group consisting of hydroxyapatite and fluoroapatite.

[Claim 11] The method according to claim 10, wherein the material having affinity for phosphate group is hydroxyapatite.

[Claim 12] A recombinant human alpha-Gal A composition produced by the method according to one of claims 1 to 11.

[Fig. 1]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BglII
CMV I.E.enhancer /Promoter
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KpnI
BstXI
neo^r
AflII
BglII/EcoRI digestion
Blunt ended
EcoRI
Primers(hGH-f1 x hGH-f2 x hGH-r1 x hGH-r2)
annealing
MluI NotI KpnI
Poly A signal of hGH
EF-1 promoter
MluI
KpnI
NotI
Amp^r
pE-neo7
SV40 late poly(A)
fl ori
Synthetic poly(A)
KpnI
SV40 enhancer /promoter
BstXI
neo^r
AflII
MluI/KpnI digestion
EcoRI 0.01
EF-1 promoter
Amp^r
pE-neo/hGHpA
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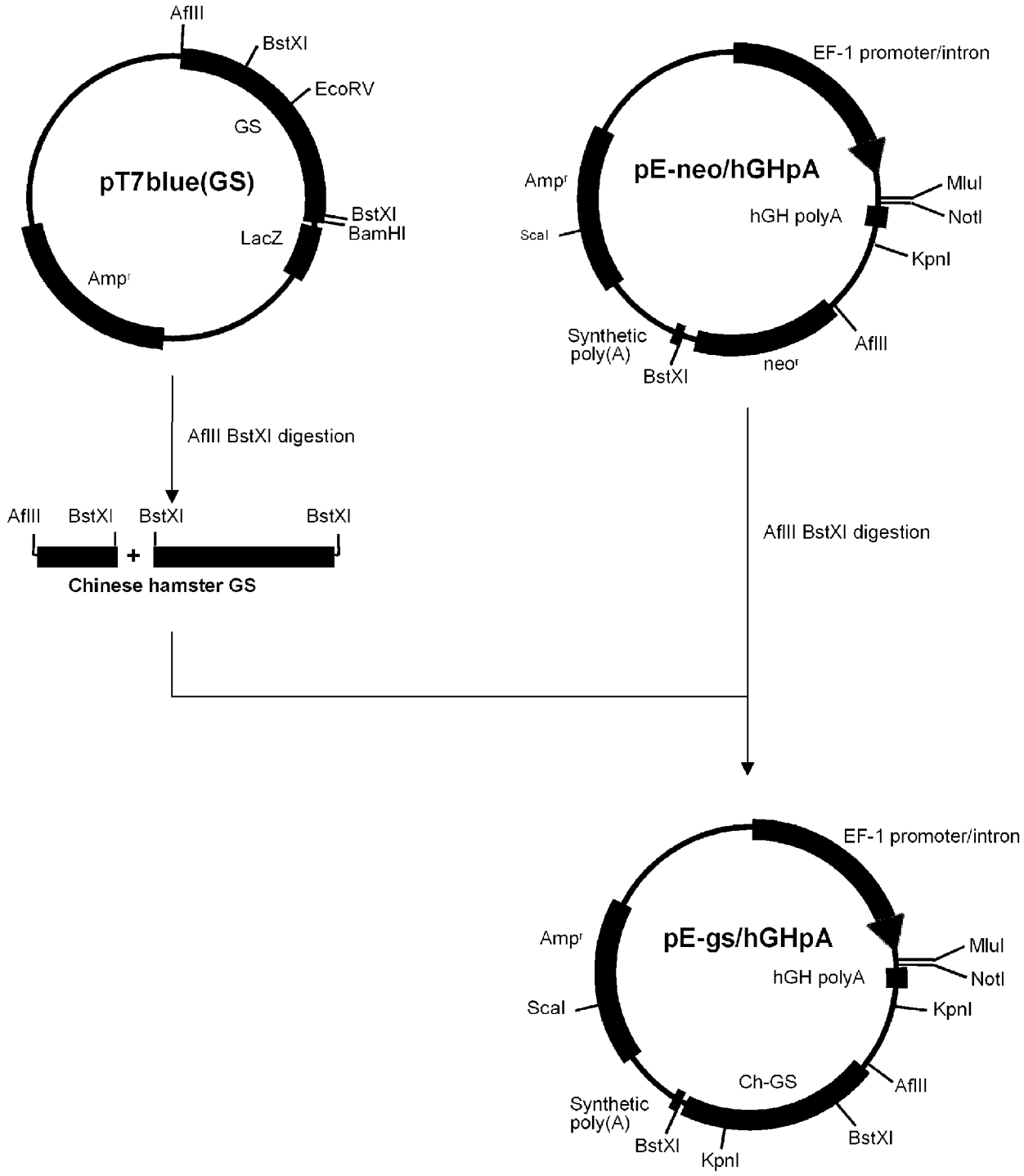
[Fig. 2-1]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GS
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Ampr
MluI
hGH polyA
NotI
ScaI
KpnI
Synthetic
poly(A)
AflII
BstXI
neor
AflII BstXI digestion
AflII BstXI BstXI BstXI
Chinese hamster GS
AflII BstXI digestion
EF-1 promoter/intron
pE-gs/hGHpA
Ampr
MluI
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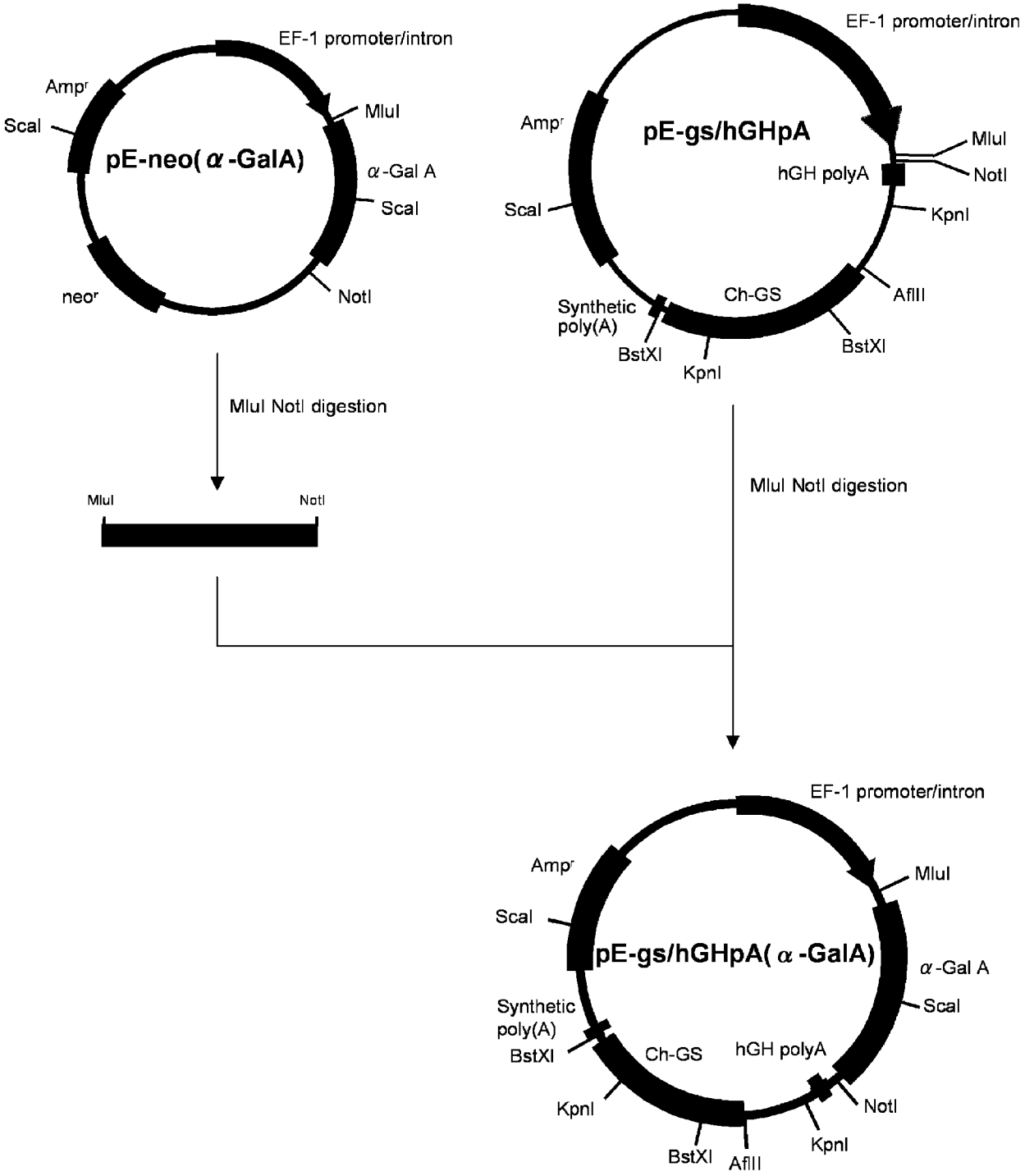
BstXI
KpnI

[Fig. 2-2]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ScaI
pE-neo(α-GalA)
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ScaI
neo^r
NotI
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MluI
NotI
EF-1 promoter/intron
Amp^r
pE-gs/hGHpA
MluI
NotI
hGH polyA
ScaI
KpnI
Synthetic poly(A)
Ch-GS
AflII
BstXI
KpnI
BstXI
MluI NotI digestion
EF-1 promoter/intron
Amp^r
MluI
ScaI
pE-gs/hGHpA(α-GalA)
α-Gal A
ScaI
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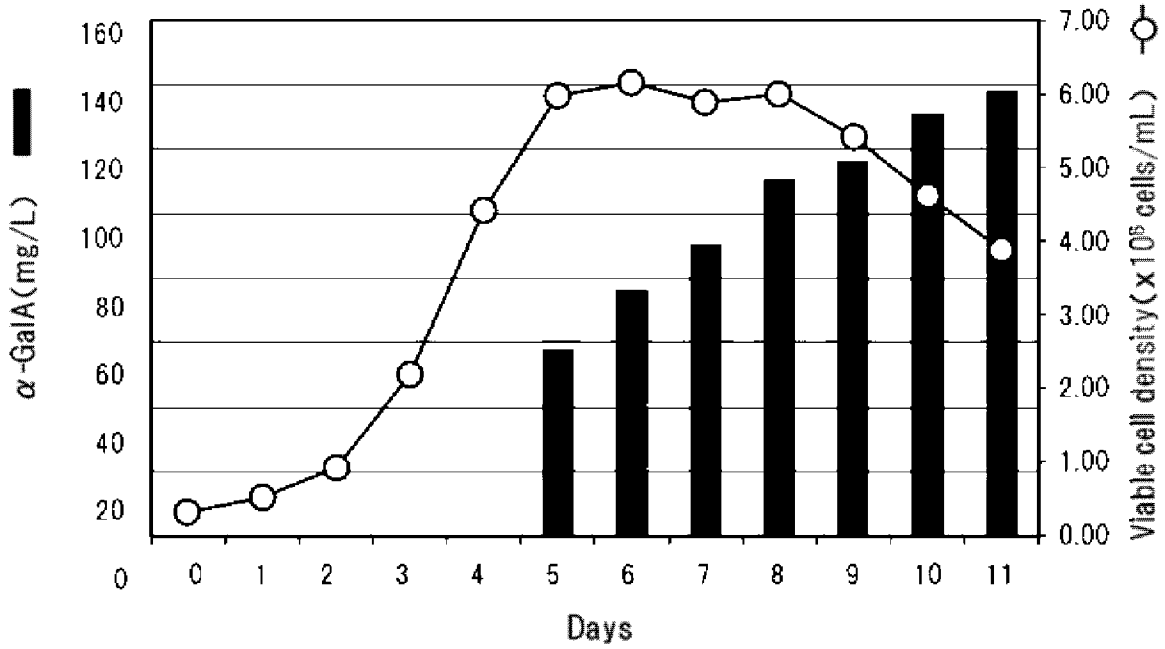
BstXI
Ch-GS
hGH polyA
KpnI
NotI
BstXI
AflII
KpnI

[Fig. 3]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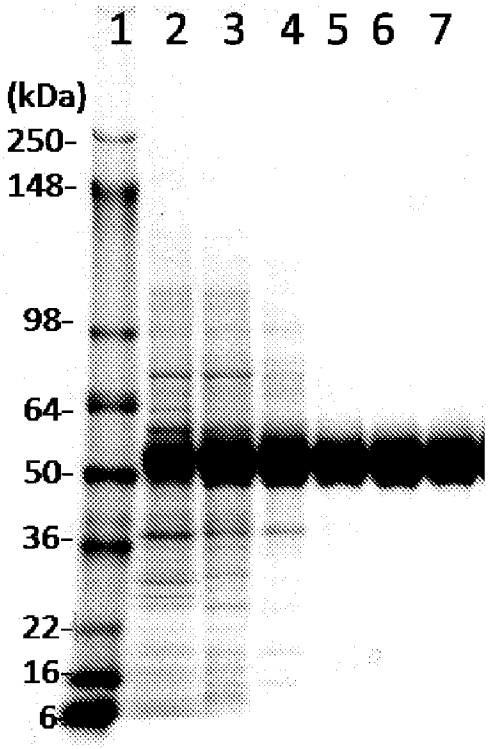
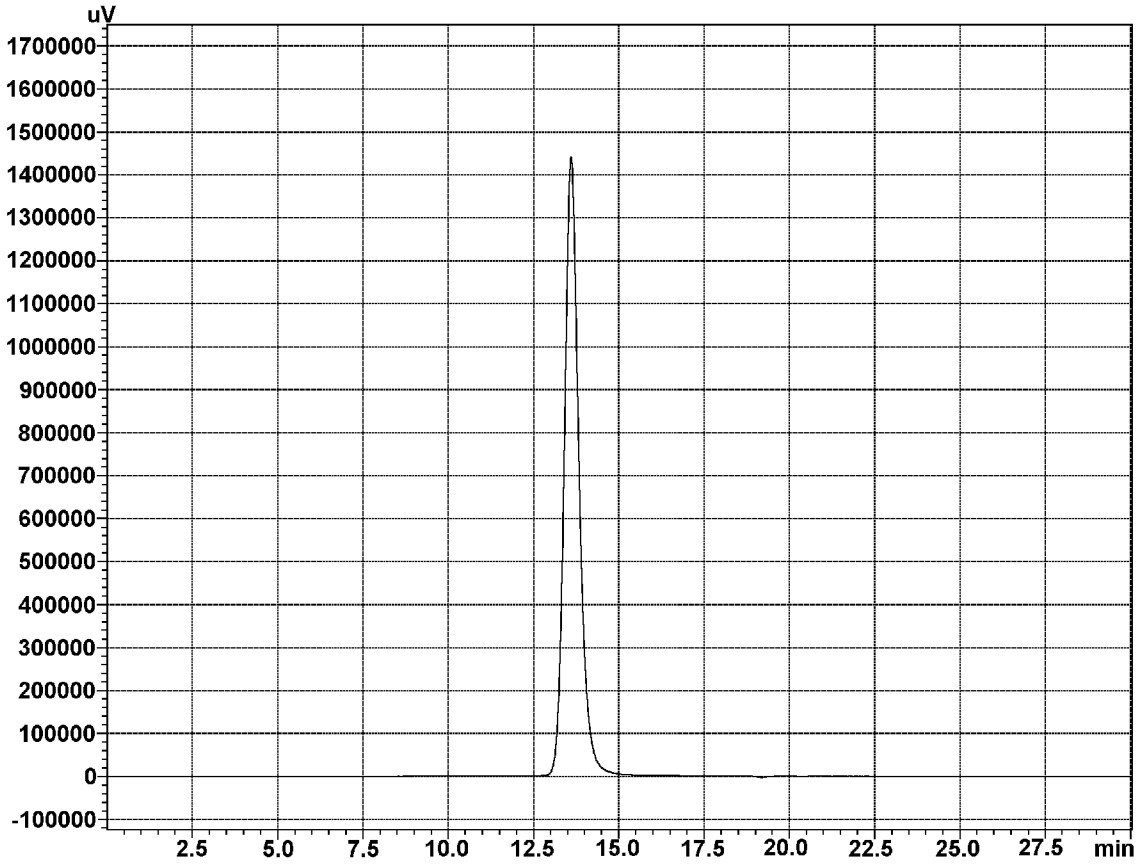
Viable cell density(x10^6 cells/mL)
Days
0
20
40
60
80
100
120
140
160
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
0 1 2 3 4 5 6 7 8 9 10 11

[Fig. 4]


1 2 3 4 5 6 7
(kDa)
250-
148-
98-
64-
50-
36-
22-
16-
6-

[Fig. 5]


uV
1700000
1600000
1500000
1400000
1300000
1200000
1100000
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
-100000
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
min

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2013/004510

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl. C12N9/40(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl. C12N9/40

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Published examined utility model applications of Japan 1922-1996
Published unexamined utility model applications of Japan 1971-2013
Registered utility model specifications of Japan 1996-2013
Published registered utility model applications of Japan 1994-2013

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAplus/MEDLINE/EMBASE/BIOSIS(STN), JSTPlus/JMEDPlus/JST580(JDreamIII), GenBank/EMBL/DDBJ/GeneSeq, UniProt/GeneSeq, PubMed, Science Direct, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X/Y	WO 1994/012628 A1 (THE MOUNT SINAI SCHOOL OF MEDICINE OF THE CITY UNIVERSITY OF NEW YORK) 1994.06.09, 5.2.3., 7.2.1., Fig.6 & US 5401650 A1 & EP 0670896 A & JP 8-503615 A	12/1-11
X/Y	WO 1998/011206 A2 (TRANSKARYOTIC THERAPIES, INC.) 1998.03.19, ExamplesIIA-IIE & US 2003-0077806 A1 & EP 0935651 A & JP 2001-504324 A	9-12/1-8
Y	WO 2000/053730 A2 (TRANSKARYOTIC THERAPIES, INC.) 2000.09.14, CLAIMS, p.18 L5-20 & US 2003-0077806 A1 & EP 1163349 A & JP 2002-538183 A	1-12

☑ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26.08.2013	03.09.2013

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4N 4868

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2013/004510

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2011/108451 A1 (JCR PHARMACEUTICALS CO., LTD.) 2011.09.09, [0031], [0032] (No Family)	1-12