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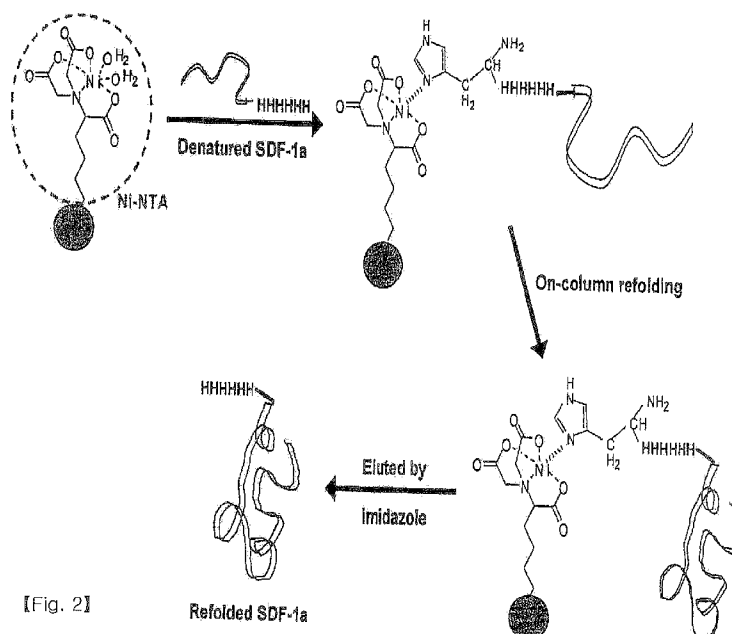
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(54) Title: A METHOD FOR PRODUCING STROMAL CELL-DERIVED FACTOR-1A AS AN ACTIVE SOLUBLE PROTEIN FORM



[Fig. 2]

(57) Abstract: Disclosed is a method for the production of an active form of human stromal-derived factor (SDF-I a), featuring the renaturation of an inclusion body of proteins expressed in a large quantity in E. coli into an active form using a column. Also, an active, solubilized SDF- I a is provided. The protein refolding system is simpler and faster than conventional methods using dialysis or chaperones, thus having useful applications to other inclusion bodies.

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A METHOD FOR PRODUCING STROMAL CELL-DERIVED FACTOR-1A AS AN ACTIVE SOLUBLE PROTEIN FORM

【Technical Field】

5 The present invention relates to a method for the production of an active form of human stromal-derived factor (SDF-1a). More particularly, the present invention relates to a method for renaturing an inclusion body of proteins expressed in a large quantity in *E. coli* into an active form using a column.

【Background Art】

10 Proteins are large organic compounds made of amino acids arranged in a linear chain and joined together by peptide bonds between the carboxyl and amino groups of adjacent amino acid residues. After being translated, a protein undergoes a change in three-dimensional conformation to a biologically active form or a thermodynamically stable form through a folding process in which relatively
15 weak forces such as hydrogen bonds, ionic bonds, etc. are involved. In order to be used for therapeutic treatment, proteins are isolated or extracted in thermodynamically stable or biologically active forms from various animals and plants.

 With a great advance in genetic recombination technology, extensive
20 attempts have recently been made to produce industrially useful gene products on a mass scale using microorganisms. Among microorganisms for use in the production of recombinant proteins, *E. coli* is most widely used, having the most gene information available and various expression vectors developed therefor. In addition, cell culture techniques for the mass production of proteins are well
25 established for *E. coli*. Further, these techniques are now most widely applied to the production of recombinant proteins. When recombinant proteins are produced in large quantities from microorganisms, however, there are problems with the tertiary structure thereof. In detail, the recombinant proteins synthesized from *E. coli* are frequently secreted as inclusion bodies, which are an inactive form. The term
30 “inclusion body” refers to an insoluble aggregate of proteins in which the native three-dimensional structure thereof is not formed due to protein misfolding.

Typically, an inclusion body is formed as a result of misfolding due to non-covalent interactions between hydrophobic residues exposed from dynamic intermediates in folding process (Cleland, Tl. L., Wang, I. C., Biochemistry, 29, 11072-11078, 1990).

An inclusion body, which is a protein aggregate with a modified three-dimensional structure, can be converted into a native form, which has biological activity. In this regard, typically, the protein aggregate is solubilized in a buffer containing a denaturant to unfold the polypeptide chain and the denaturing condition is removed through dilution or dialysis to refold the protein into a native form.

A refolding process by which a recombinant protein existing as an inclusion body can be recovered in an active form generally comprises solubilizing an inclusion body with a denaturant and removing the denaturant to induce correct folding of the protein. The removal of denaturants may be implemented by simple dilution, serial dilution, dialysis, gel filtration chromatography, etc. Simple dilution, a typical process for protein refolding, suffers from the following disadvantages. A large reactor is required for reaction because a sample must be diluted to 4 to 100 volumes. With a limitation only to a very low concentration of proteins, a simple dilution method shows low efficiency. Further, a concentration process is required due to the large volume to be treated, increasing a cost for the production and purification of the recombinant protein. Moreover, disulfide bonds may be formed randomly during simple dilution.

In order to overcome the problems with simple dilution, a protein refolding method using size exclusion batch chromatography was developed and utilized. Based on column chromatography, this protein refolding method is advantageous in that a buffer can be easily exchanged with a desired one and the protein denaturant used in protein refolding can be readily removed. In addition, size exclusion batch chromatography allows a refolding process to be applied to a protein concentration of 80 mg/ml or more, whereas simple dilution is impossible to apply to a concentration of 10 mg/ml. Particularly, a size exclusion batch chromatography method can be automated for the full process thereof, thereby affording process standardization and effective management.

The refolding process, whether based on simple dilution or batch chromatography, employs a urea-containing buffer. Urea plays an important role in refolding proteins. As described above, when synthesized in large quantities in microorganisms, the recombinant proteins are aggregated to form inactive inclusion bodies in many cases. Thus, there is a need for a process of unfolding inclusion bodies in the presence of a denaturant before refolding them into a native form. For this, urea is widely used as a denaturant. For example, 8 M urea is used for unfolding inclusion bodies produced in recombinant *E. coli* and 2 M urea is used for refolding the denatured polypeptides at a high yield.

SDF-1a (stromal cell-derived factor 1a) is a small cytokine belonging to the chemokine family, known as CXC chemokine, which was first isolated from a mouse bone marrow stromal cell line (Nagasawa T. et al., *Nature* 1996, 382, 635-638). SDF-1a is involved in diverse functions including hematopoiesis, cardiogenesis, B cell lymphopoiesis, and cell migration (Doitsidou M. et al., *Cell*, 2002, 111, 647-659). Also, SDF-1a is known to play an important role in the mechanism of embryo development and division by specifically binding to the membrane receptor CXCR4 (Ma Q., *Proc. Natl. Acad. Sci. USA*, 1998, 95, 9448-9453).

It is reported that SDF-1a is expressed as an insoluble form in bacterial expression systems. Many research groups have made extensive effort to obtain SDF-1a in an active form.

For example, chemical synthesis or eukaryotic expression has been attempted to give an active form of SDF-1a.

Leading to the present invention, intensive and thorough research into the renaturation of an inclusion body of proteins into an active form, conducted by the present inventors, resulted in the finding that after being denatured, mistolded proteins can be easily refoled into an active form when renaturation is carried out on a column.

【Disclosure】

【Technical Problem】

It is therefore an object of the present invention to provide a method for the

production of recombinant human stromal cell-derived factor 1A as an active form.

It is another object of the present invention to provide a recombinant human stromal cell-derived factor, expressed through an E. coli expression system.

5 It is a further object of the present invention to provide a method for renaturing an insoluble form of a protein into a soluble form.

It is still a further object of the present invention to provide a recombinant human stromal cell-derived factor refolded into an active form.

10 It is still another object of the present invention to provide a recombinant human stromal cell-derived factor capable of binding to a column.

【Technical Solution】

The present invention pertains to a method for the renaturation of an inclusion body of arecombinant protein into an activated, soluble form.

15 In greater detail, when a recombinant SDF-1a protein is expressed in the form of an inclusion body, it is renatured into an active form through a protein refolding system using a column.

In accordance with the present invention, SDF-1a is cloned into an expression vector containing a 6x His-tag and a tobacco etch virus (TEV) protease recognition site so that a recombinant SDF-1a is expressed, with 6x His residues
20 tagged at the N terminus thereof and a TEV recognition site subsequent to the tag. The recombinant protein is immobilized on a column due to interaction with the 6x His tag before the protein refolding.

As for the TEV protease recognition site, it is digested with the enzyme so as to remove the 6x His residues attached thereto, leaving activated, soluble SDF-1a
25 alone.

Also, the present invention pertains to an activated, soluble SDF-1a protein produced by the method. The SDF-1a produced by the method was found to be activated, as assayed for the characteristic cell chemotaxis thereof.

30 In the present invention, a recombinant SDF-1a protein is expressed. In this regard, a gene coding for SDF-1a may be obtained by PCR amplification from a human fibroblast cDNA library. The SDF-1a gene is cloned into an expression vector containing a 6x His tag and a tobacco etch virus (TEV) protease recognition

site so that a modified SDF-1a protein containing 6x His residues at an N-terminal region thereof followed by a tobacco etch virus protease recognition site is expressed.

5 Useful as the expression vector in the present invention is a pET-28a vector. In order to join the synthesized SDF-1a gene to the T7 promoter of pET-28a, both the genetic materials are digested with BamHI and XhoI, and then react with each other in the presence of a ligase to afford a recombinant expression vector.

10 Then, the recombinant expression vector thus obtained is transformed into an ER2566 competent cell (Novagen) to produce a transformant which anchors the expression vector therein.

15 In order to over-express the protein of interest, the recombinant pET-28a vector carrying an SDF-1a gene is introduced into E. coli BL21 (DE3). For comparison, a mutant SDF-1a (K1A) gene is synthesized by polymerase chain reaction using site-directed mutagenesis. The mutant gene is transformed into E. coli BL21 (DE3) to express the mutant protein.

20 For protein refolding, an affinity column, such as a HiTrap Chelating HP column (GE Healthcare), may be used. After being denatured with 8M urea, the SDF-1a protein is immobilized on a HiTrap Chelating HP column resin via the binding of the 6x His tag to the Ni ions of the resin. While being sustained by the column, the SDF-1a is depleted of the urea at a very low rate (0.2 ml/min), with protein refolding taking place gradually. Then, the column onto which the protein is immobilized is sufficiently washed with a 45 mM imidazole buffer, followed by 300 mM imidazole to completely elute the refolded SDF-1a from the column.

25 Subsequently, the 6x His-tag is deleted from the protein by cleavage with TEV protease, followed by removing remaining 6x His-tagged SDF-1a by chromatography with a HiTrap Chelating HP column. Finally, the eluate thus obtained is purified with a Superdex Peptide Column (GE healthcare) to afford the protein of interest at a purity of 95% or higher.

30 Generally, a protein may be produced *in vivo* using a recombinant expression vector carrying a gene encoding the protein. In the present invention, the gene is a nucleotide sequence coding for SDF-1a (stromal cell-derived factor-

1a) and the expression vector is preferably based on pET-28a containing a 6x His tag and a tobacco etch virus (TEV) protease recognition site.

Further, the present invention pertains to an E. coli strain anchoring the
5 expression vector therein.

Preferably, the E. coli is based on BL21(DE3).

Furthermore, the present invention pertains to a method for producing SDF-1a in an active form in a large quantity, comprising: (a) constructing a recombinant expression vector carrying a nucleotide sequence coding for SDF-1a, a 6x His tag
10 and a tobacco etch virus protease (TEV) protease recognition site; (b) transforming the recombinant expression vector into E. coli and culturing the E. coli to express a recombinant SDF-1a protein; (c) unfolding the recombinant SDF-1a protein under denaturing conditions to give a denatured SDF-1a solution; and (d) refolding the unfolded SDF-1a protein into an active form with the use of column chromatography.
15 In this method, the unfolding is carried out by lysing the E. coli of step (b) in the presence of a protease inhibitor and treating the resulting cell lysate with a denaturant to give a denatured SDF-1a solution. Preferably, the denaturant is urea.

Preferably, the recombinant expression vector is based on pET-28a.

The E. coli useful in the present invention is BL21 (DE3).

20 In an embodiment of the method, the refolding is carried out by loading the denatured SDF-1a solution on an affinity chromatography column pre-equilibrated with Ni and a denaturing buffer, renaturing the SDF-1a protein immobilized on the column with a renaturing buffer substituting for the denaturing solution, washing the renatured SDF-1a protein with an imidazole solution, and eluting an active SDF-1a
25 protein having a 6x His tag with an imidazole buffer.

Also, the present invention is concerned with the active SDF-1a protein having a 6x His tag, produced by the method of the present invention.

In accordance with another embodiment of the present invention, the method further comprises removing the 6x His tag from the active SDF-1a by use of
30 TEV protease after the eluting step. In a further embodiment, the method further comprises filtering out SDF-1a having a 6x His tag through affinity chromatography after the removing step. In still a further embodiment, the method further comprises

purifying SDF-1a free of a 6x His tag using peptide column chromatography after the filtering.

Moreover, the present invention pertains to an active form of the SDF-1a
5 which is free of a 6x His tag.

In accordance with an aspect thereof, the present invention provides a method for renaturing an inactive form of protein into an active form, comprising: expressing a protein containing a group through which the protein can bind to a column; unfolding the protein and immobilizing the protein onto a column; refolding
10 the immobilized protein; and separating the refolded protein from the column.

In the method of the present invention, the protein may be one that is changed in activity through protein unfolding and refolding processes. The method according to the present invention may be applicable to a variety of insoluble proteins which show no activity without renaturation. In a preferred embodiment of
15 the present invention, the protein is SDF-1a, which is produced in an insoluble form upon expression in an E. coli system using an expression vector carrying an SDF-1a gene, and can be refolded into an active, solubilized form through renaturation.

In accordance with the present invention, the protein preferably contains an attachment group which allows the protein to bind to a column for use in protein
20 unfolding and refolding. In a preferable embodiment of the present invention, the attachment group may be added to a protein of interest by inserting a nucleotide sequence coding for the attachment group, along with a nucleotide sequence coding for the protein, into an expression vector. In another embodiment of the present invention, the attachment group may be introduced into a protein of interest
25 through a chemical reaction therebetween.

In accordance with the present invention, the method may further comprise removing the attachment group from the protein. The removal of the attachment group may be achieved by introducing a nucleotide sequence coding for an enzyme cleavage site between a protein of interest and the attachment group, expressing the
30 recombinant protein, and cleaving the cleavage site with a suitable enzyme. In a preferred embodiment of the present invention, the expression vector comprises a nucleotide sequence encoding a tobacco etch virus (TEV) recognition site so that a

6x His tag, serving as the attachment group, can be removed after protein refolding.

In accordance with another aspect thereof, the present invention provides a method for producing SDF-1a in an active form, comprising:
5 constructing a recombinant expression vector containing nucleotide sequences respectively coding for SDF-1a, 6x His-tag, and tobacco etch virus (TEV) protease; expressing a recombinant SDF-1a protein in an E. coli strain transformed with the expression vector; unfolding an aggregate of expressed proteins under a denaturing condition; and refolding the recombinant protein on a column.

10 In a preferred embodiment of the present invention, the expression vector is pET-28a and the E. coli strain is based on BL21(DE3), in which the protein is over-expressed in an insoluble form.

The bacteria is lysed in the presence of a protease inhibitor, and the cell lysate thus obtained is treated with a denaturant, preferably with urea, so as to
15 unfold protein aggregates.

Then, the cell lysate is loaded on a HiTrap Chelating HP column to immobilize the unfolded protein onto the column, followed by refolding the protein thereon. Then, the histidine residues tagged to the N-terminal of the refolded SDF-1a protein are removed by cleaving the TEV protease recognition site with TEV
20 protease.

【Advantageous Effects】

As described above, an SDF-1a gene amplified from a human fibroblast cDNA library is introduced into a recombinant E. coli system for the overexpression thereof, and an inclusion body of the SDF-1a protein thus expressed can be
25 renatured into a soluble form using an on-column protein refolding system. The protein refolding system according to the present invention is simpler and faster than conventional methods using dialysis or chaperones, thus having useful applications to other inclusion bodies.

【Description of Drawings】

30 FIG. 1 shows the expression of stromal cell-derived factor-1a (SDF-1a) in a soluble form and an insoluble complex form in an E. coli expression system, wherein

lane M shows a marker with a lowest band of 14.4 kDa, lane BF shows a state before IPTG treatment, lanes 2h and 4h show states after treatment with 0.4 mM IPTG for 2 and 4 hours, respectively, lane “soluble” shows proteins existing in an aqueous layer after cell lysis, and lane “insoluble” shows insoluble protein aggregates, obtained after cell lysis, at the point indicated by the arrow.

FIG. 2 is a diagram illustrating a protein refolding process wherein denatured SDF-1a contained in a cell lysate is immobilized onto Ni-NTA (nitrilotriacetic acid) resins in a pre-equilibrated HiTrap Chelating HP column, treated with a refolding buffer for 6 – 10 hrs, and washed with 45 mM imidazole buffer, followed by elution with 300 mM imidazole buffer.

FIG. 3 is an absorbance profile upon the refolding of SDF-1a on a HiTrap Chelating HP column.

FIG. 4 is a photograph taken after the SDS electrophoresis of SDF-1a purified to a purity of 95% or higher, which is detected at 8 kDa as indicated by the arrow, along with a standard protein marker (left lane) with a lowest band detected at 14.4 kDa.

FIG. 5 is a graph showing the cell chemotaxis of the refolded SDF-1a according to the present invention in a dose-dependent manner upon the treatment of the human T cell leukemia CCRF-CEM with 10, 50, 100 and 250 nM of SDF-1a, wherein the same results are obtained for migrated cells upon treatment with a mutant (K1A) of SDF-1a and without treatment (Con).

【Best Mode】

A better understanding of the present invention may be grasped with reference to the following examples, which are set forth to illustrate, but are not to be construed to limit the present invention.

EXAMPLE 1

Cloning of SDF-1a Gene

A human stromal cell-derived factor 1a (SDF-1a) gene was amplified from a human fibroblast cDNA library by PCR using a pair of primers (Primer-5' : 5' –

GCGGATCCAAGCCCGTCAGCCTGAG-3' , SEQ ID NO.: 1; Primer-3' : 5' - CGCTCGAGTTACTTGTTTAAAGCTTTCTCCAG-3' , SEQ ID NO: 2), which are modified from the nucleotide sequence of SDF-1a. After being purified, the synthesized SDF-1a gene was digested with BamHI and XhoI and jointed into the T7 promoter of pET-28a in the presence of a ligase to construct a recombinant expression vector.

EXAMPLE 2

Preparation of Transformant Anchoring SDF-1a Expression Vector

The recombinant expression vector constructed in Example 1 was transformed into E. coli. For this, ER2566 cells (Novagen) were first inoculated into an LB broth, incubated overnight at 37°C and then amplified to an OD₆₀₀ of 0.4 in an LB broth. After being harvested by centrifugation, the cells were suspended in 500 ml of sterile deionized water and centrifuged at 2,500 rpm and 4°C for 20 min. The cell pellet thus obtained was suspended in 250 ml of 10% glycerol, followed by centrifugation at 4°C and 2,500 rpm for 20 min. The suspension and centrifugation were repeated, with the exception that 10 ml, instead of 250 ml, of 10% glycerol was used, to afford competent cells. The ER2566 competent cell pellet was suspended in 40 µl of 10% glycerol to which the constructed recombinant expression vector was then added, followed by transformation using an electroporation method. Thereafter, the cells were incubated in 1 ml of a fresh SOC broth (0.5% yeast extract, 2% tryptophan, 8.5 mM NaCl, 2.5 mM KCl, 20 mM glucose, 10 mM MgCl₂) at 37°C with shaking at 250 rpm. Subsequently, the cell culture was spread over an LB plate containing 50 µg/ml of kanamycin to select transformants anchoring wild-type SDF-1a or mutants thereof. For the overexpression of SDF-1a, the pET-28a carrying an SDF-1a gene was transformed into E. coli BL21 (DE3). For comparison, a mutant SDF-1a (K1A) gene was synthesized through polymerase chain reaction using site-directed mutagenesis. The mutant gene was transformed into E. coli BL21 (DE3) in the same manner as described above.

EXAMPLE 3

Expression of SDF-1a Protein

5 The E. coli BL21(DE3) into which the expression vector pET-28a carrying an SDF-1a gene prepared in Example 1 was cultured at 37°C to the degree of an OD₆₀₀ of 0.6 in 2 liters of LB broth, after which wild-type and mutant proteins were induced in the presence of 0.4 mM at 37°C express them. After 4 hours of the induction, the cells were harvested by centrifugation and resuspended in 100 ml of a
10 buffer (10 mM sodium phosphate, 150 mM NaCl, pH7.4) containing a protease inhibitor. To the suspension was added lysozyme (1.0 mg/ml) to destroy cell membranes, followed by sonication to rupture cells. The insoluble complex obtained by centrifuging the cell lysate thus obtained was subjected to solubilization with a denaturing buffer (8 M urea, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.5 mM
15 b-mercaptoethanol, 3% glycerol) for 2 hrs at room temperature. Centrifugation at 15,000 rpm for 30 min afforded a denatured extract.

EXAMPLE 4

On-Column Refolding of SDF-1a

20

 The extract obtained in Example 2 was applied to the HiTrap Chelating HP column (GE Healthcare) pre-equilibrated with Ni ions and a denaturant buffer. After the extract was loaded at a rate of 3 ml per min on the column, a refolding solution was slowly poured at a rate of 0.2 ml per min for 6 – 10 hrs to replace the pre-
25 existing buffer therewith.

 Subsequently, the column was washed with 45 mM imidazole buffer, followed by elution with 300 mM imidazole buffer. The recombinant SDF-1a having a 6x His tag thus eluted was treated with TEV protease to delete the His tag therefrom. Using a HiTrap Chelating HP column, the SDF-1a tagged with 6x His
30 residues was removed again. The SDF-1a protein was purified to a purity of 95% or higher using a Superdex peptide column (GE Healthcare) equilibrated with a buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 3% glycerol, 1 mM DTT).

EXAMPLE 5

Assay for Biological Activity of SDF-1a

5

The SDF-1a prepared in Example 3 was assayed for cell chemotaxis using the human leukemia cell line CCRF-CEM on which CXCR4, a receptor of SDF-1a, is expressed, commercially available from the Korean Cell Line Bank (KCLB). The CCRF-CEM cell line was maintained in an RPMI 1640 medium supplemented with 25 mM Hepes and 10% FBS. The CCRF-CEM cells were plated at a density of 2×10^6 cells/ml and washed with an assay solution (RPMI 1640, 25 mM Hepes, pH 7.5, 0.1% BSA). The chemotactic ability of SDF-1a was analyzed using a 48-well ChemoTx chamber (NeuroProbe, Gaithersburg, MD) in which 27 μ l of the SDF-1a was placed in each of the lower wells while 50 μ l of CCRF-CEM cells was placed in each of the upper wells. After the chamber was incubated at 37°C for 3 hrs in a 5% CO₂ incubator, the CCRF-CEM cells that migrated into the lower wells were counted to determine cell chemotaxis, compared with that of the wild-type SDF-1a or the mutant (K1A).

【Claims】**【Claim 1】**

A method for renaturing an inactive form of a protein into an active form,
5 comprising steps of:

(1) expressing a protein containing an attachment group through which the
protein can bind to a column;

(2) unfolding the protein and immobilizing the protein onto a column;

(3) refolding the immobilized protein; and

10 (4) detaching the refolded protein from the column.

【Claim 2】

The method according to claim 1, wherein the unfolding step (2) is carried
out by denaturing the protein.

【Claim 3】

15 The method according to claim 1, wherein the protein is denatured using
urea.

【Claim 4】

The method according to claim 1 or 2, wherein the attachment group is
expressed simultaneously with the protein or is introduced into the protein after
20 protein expression.

【Claim 5】

The method according to claim 4, wherein the attachment group is one
histidine residue or a set of histidine residues.

【Claim 6】

25 The method according to claim 1 or 2, wherein the refolding step is carried
out by loading a
refolding buffer on the column.

【Claim 7】

The method according to claim 1 or 2, further comprising removing the

attachment group after the detaching step.

【Claim 8】

The method according to claim 1 or 2, wherein the expressing step is
5 carried out by transforming an expression vector carrying an SDF-1a (stromal cell-
derived factor-1a) gene into an E. coli strain and culturing the E. coli strain.

【Claim 9】

The method according to claim 8, wherein the expression vector contains a
nucleotide sequence coding for 6x His residues.

10 **【Claim 10】**

The method according to claim 9, wherein the expression vector contains a
nucleotide sequence coding for a tobacco etch virus (TEV) protease recognition site.

【Claim 11】

The method according to claim 8, wherein the expression vector is based on
15 pET-28a.

【Claim 12】

The method according to claim 8, wherein the E. coli strain is BL21 (DE3).

【Claim 13】

A method for producing SDF-1a in an active form, comprising the steps of:
20 (a) constructing a recombinant expression vector containing nucleotide
sequences respectively coding for SDF-1a, 6x His-tag, and tobacco etch virus
(TEV) protease;
(b) expressing a recombinant SDF-1a protein in an E. coli strain
transformed with the expression vector;
25 (c) unfolding an aggregate of expressed proteins under denaturing
conditions; and
(d) refolding the recombinant protein on a column.

【Claim 14】

The method according to claim 13, wherein the recombinant expression

vector is based on pET-28a.

【Claim 15】

The method according to claim 13, wherein the E. coli strain is BL21(DE3).

5 **【Claim 16】**

The method according to claim 13, wherein the unfolding step is carried out by rupturing the E. coli strain transformed with the recombinant expression vector in the presence of a protease inhibitor to give a cell lysate, and denaturing the cell lysate with a denaturant.

10 **【Claim 17】**

The method according to claim 16, wherein the denaturant is urea.

【Claim 18】

The method according to claim 13, further comprising removing the 6x His tag from the SDF-1a protein group after the refolding step.

15 **【Claim 19】**

The method according to claim 18, wherein the 6x His tag is removed by TEV protease.

【Claim 20】

20 A soluble SDF-1a protein containing a 6x His tag, produced by the method of one of claims 13 to 18.

【Claim 21】

A soluble SDF-1a, free of a 6x His tag, produced by the method of claim 19 or 20.

【Claim 22】

25 A recombinant expression vector, carrying nucleotide sequences coding for SDF-1a, a 6x His tag, and a tobacco etch virus (TEV) protease recognition site, respectively, capable of expressing SDF-1a in an E. coli strain.

【Claim 23】

The recombinant expression vector according to claim 22, wherein the recombinant expression vector is based on pET-28a.

【Claim 24】

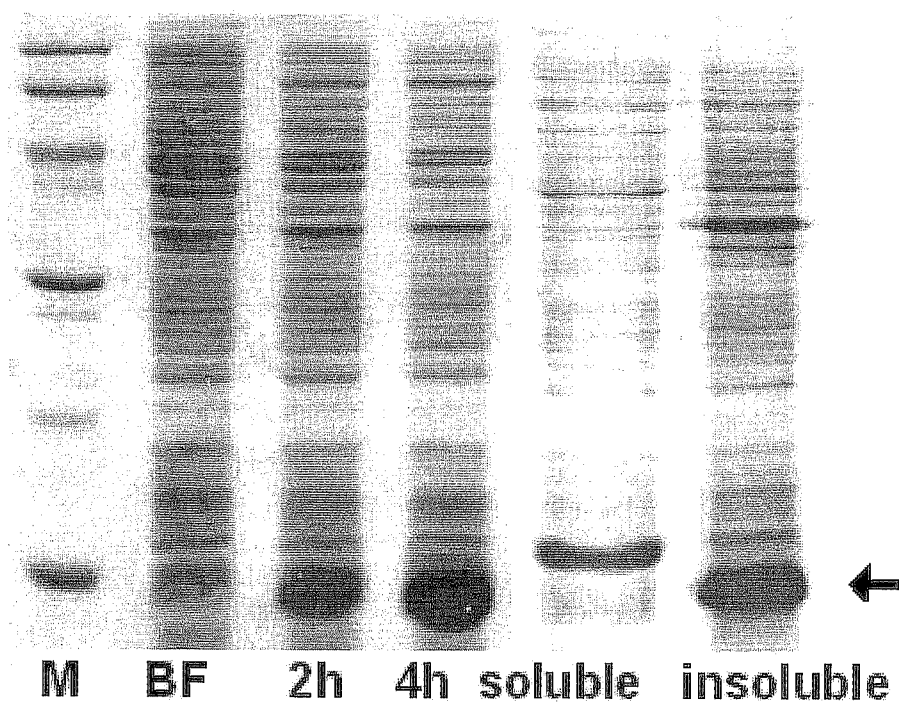
- 5 An E. coli strain, transformed with the recombinant expression vector of claim 22 or 23.

【Claim 25】

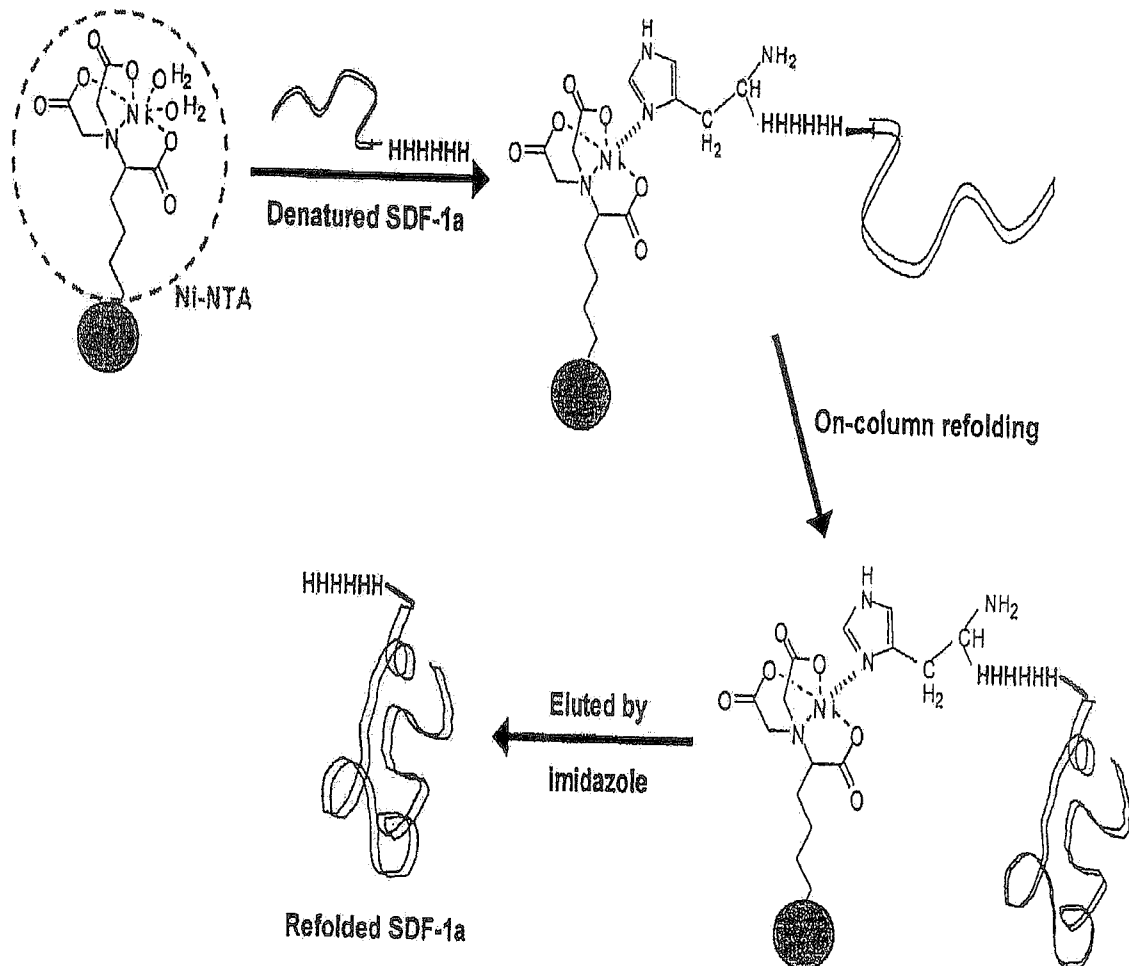
The E. coli strain according to claim 24, wherein the E. coli strain is based on BL21 (DE3).

【DRAWINGS】

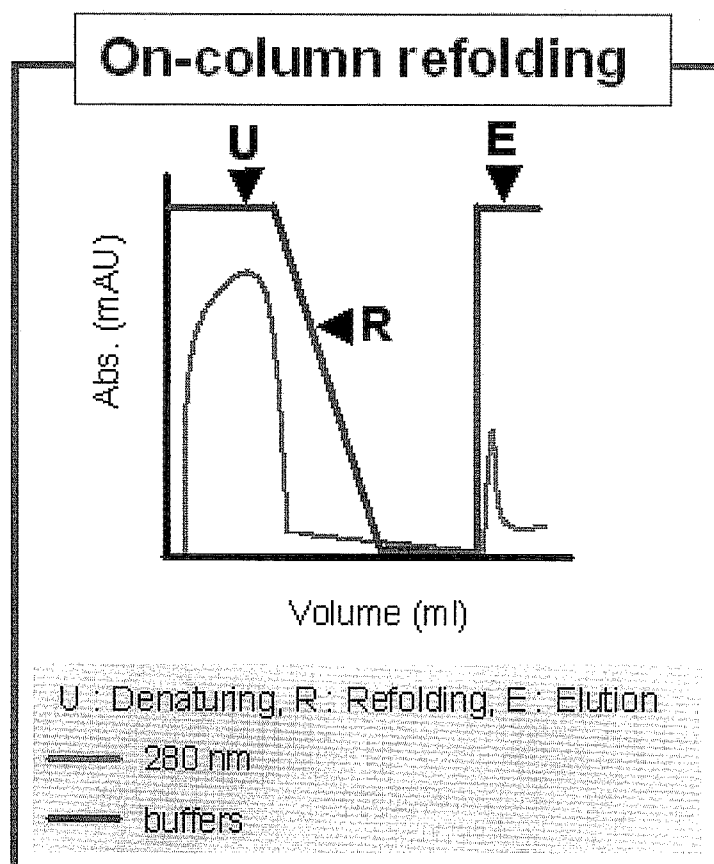
【Fig. 1】



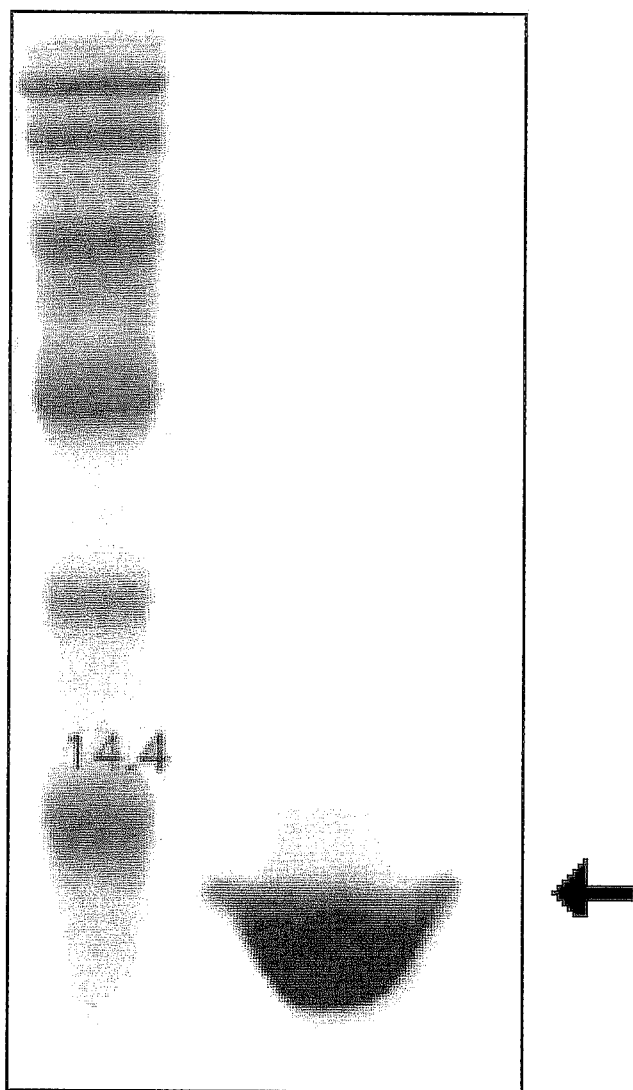
【Fig. 2】



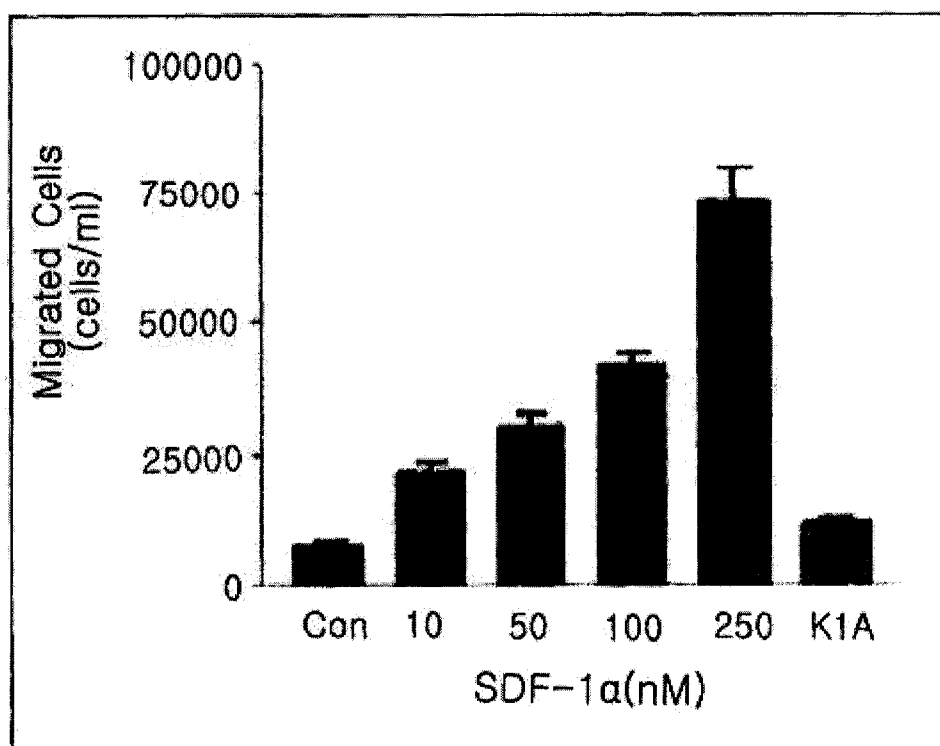
【Fig. 3】



【Fig. 4】



【Fig. 5】



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2008/000265**A. CLASSIFICATION OF SUBJECT MATTER****C07K 1/14(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)

IPC 8 C07K 1/14

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKIPASS, NCBI PubMed database, Delphion Research Intellectual Property Network database, google scholar, BLAST database
"protein", "purification", "unfolding", "denaturation", "refolding", "renaturation", "column", "SDF-1"**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RYU E.K. et al. 'Crystal Structure of Recombinant Human Stromal Cell-Derived Factor-1 α ' Structure, Function, and Bioinformatics, March 2007, Vol. 67, pages 1193-97. See Materials and Methods.	1-20,22-25
X	VELDKAMP C.T. et al. 'On-column Refolding of Recombinant Chemokines for NMR Studies and Biological Assay' Protein Experimental Purification, March 2007, Vol. 52, No. 1, pages 202-209. See the abstract and Methods.	1-20,22-25
A	KR 10-0409092 B1 (GREEN CROSS CORP.) 11 Decemberr 2003 See the abstract and claims.	3
A	KR 10-0482190 B1 (LG LIFE SCIENCE CORP.) 13 April 2005 See the abstract and claims.	5,9

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

23 MAY 2008 (23.05.2008)

Date of mailing of the international search report

23 MAY 2008 (23.05.2008)

Name and mailing address of the ISA/KR

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gu, Daejeon 302-701, Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

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Telephone No. 82-42-481-5975



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2008/000265

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

a. type of material



a sequence listing



table(s) related to the sequence listing

b. format of material



on paper



in electronic form

c. time of filing/furnishing



contained in the international application as filed



filed together with the international application in electronic form



furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2008/000265**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 21
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2008/000265

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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