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(54) Title: PREPARATION OF PEPTIDES BY USE OF HUMAN GLUCAGON SEQUENCE AS A FUSION EXPRESSION PARTNER		
(57) Abstract The present invention relates to a method for preparation of recombinant proteins or peptides expressed in form of X_G-L-Y containing strong self-association peptide as fusion partner. X_G: is fusion partner (polypeptide containing whole or partial amino acid sequence of human glucagon or its derivatives), L: linker (sequence for in-frame expression or recognition by peptidase), Y recombinant protein or peptide. Particularly, the present invention relates to a gene encoding fusion protein which comprises polypeptide containing whole or partial amino acid sequence of human glucagon or its derivatives as a fusion partner, a vector comprising the gene, a microorganism transformed with the vector, and a method for preparation of the fusion protein.		

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Preparation of Peptides by Use of Human Glucagon
Sequence as a Fusion Expression Partner

FIELD OF THE INVENTION

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The present invention relates to a method for preparation of recombinant proteins or peptides expressed in form of X_G -L-Y containing strongly self-associating peptide as fusion partner.

10 X_G : fusion partner (polypeptide containing whole or partial amino acid sequence of human glucagon or its derivatives)

L : linker (sequence for in-frame expression or in-vitro enzymatic cleavage)

15 Y : recombinant protein or peptide

Paticulary, the present invention relates to genes encoding fusion proteins each of which comprises polypeptide containing whole or partial amino acid sequence of human glucagon or its derivatives as a fusion partner, vectors comprising the genes, microorganisms transformed with the
20 vectors, and methods for preparation of the fusion proteins.

BACKGROUND

25

For heterologous expression by genetic recombination in microorganisms of many pharmaceutically important proteins which were obtained with difficulty in organ of human or

animals, a gene of target protein is inserted into an expression vector, and an appropriate host microorganism is transformed with this recombinant vector, and followed by culturing. *E.coli* has been used as a host for production of useful foreign proteins by use of genetic recombinant technique because more informations about its genes and metabolic system are known than other microorganisms. In direct expression of target proteins in *E.coli*, expressed proteins must be transformed into natural proteins owing to addition of methionine to their N-terminus. When target proteins containing methionine at their N-terminus are applied for human or other animals, immunogenecity can be induced, or structural instability of the proteins can cause their malfunction. In addition, in expression of very toxic proteins, such as antibacterial peptide, growth of host cells may be inhibited and stable large-scale production of small polypeptides less than 10,000 Da, is difficult owing to degradation by protenases in host cells.

To overcome these problems, it is used to express the target proteins containing fusion proteins at N-terminus of target proteins. Particulary, the target polypeptides could be produced with fusion partner which is expressed as soluble form in a selected. Until now, those soluble proteins produced stably in *E.coli* are β -galactosidase, maltose binding protein (45 kDa), glutathione-S-transferase (26 kDa), etc. Fusion proteins using the above proteins as fusion partners, can be purified easily by affinity chromatography,

whereas recovery yield of polypeptides is decreased remarkably since the size of fusion partners are relatively larger than the desired polypeptides. Additionally, the above method cannot be used effectively in case that expressed fusion proteins maintain their cell toxicity.

Another method using the fusion partner is that target polypeptides are expressed in form of inclusion bodies in cells (Ulman, A. et al., *Gene*, 29, 27-31, 1984; Nisson, B. et al., *EMBO J.* 4, 1075-1080, 1985; Di Guan et al., *Gene* 67, 21-30, 1988; La Vallie, E.R. et al., *Bio/Technology* 11, 187-193, 1993). This method has an advantage in that the desired recombinant proteins can be isolated easily from cell culture in early state of isolation procedure. However, the inclusion bodies is sometimes so non-specific that coaggregation of various host proteins (i.e. chaperon, ribosome initial factor, etc.) by intermolecular disulfide bond or hydrophobic interaction significantly lowers the inclusion body purity of target recombinant protein (Anna Mituraki et al., 1989, *Bio/Technol.* 7, 690-697).

When proteins are expressed in form of insoluble inclusion bodies, denaturants, such as guanidine hydrochloride, urea, etc., are generally used to purify active proteins from the insoluble inclusion bodies. Proteins in form of inclusion bodies must be dissolved using the denaturants, and refolded through dilution. Since inclusion bodies formed by disulfide bond require reducing agents for dissolving and there are many problems in refolding process, production yield can be decreased (Marston,

F. A. O. et al., 1986, *Biochem. J.*, 240, 1-12; Mitraki, A. and King, J. et al., *Bio/Technology* 7, 690-697, 1989).

Human glucagon is a peptide hormone that antagonizes insulin action and thereby leads to a stimulation of hepatic glucose production. The glucagon hormone is already commercialized as therapeutics for hypoglycemia or auxiliary for X-ray diagnosis and endoscopy in America, Japan, etc. It is known that glucagon has 29 amino acids and is stably produced in form of fusion protein at high expression rate in *E.coli* (Shin et al., *Appl. Microbiol. Biotechnol.* 49, 364-370, 1998). Although glucagon is small peptide (3.5 kDa), X-ray analysis has demonstrated that in crystal, the peptide adopts a mainly α -helical conformation, and in dilute solution, the glucagon molecules have strong tendency to stabilize the α -helical conformation by hydrophobic interactions as an oligomer by self-association between molecules (Sasaki, K. et al., *Nature*, 257, 751-757, 1979). These properties of small-sized glucagon regarding intermolecular association provide a rationale for using glucagon as fusion expression partner for production of inclusion bodies containing the desired proteins at high purity as well as for increment of production yield in cells.

For large-scale production of foreign proteins in form of insoluble inclusion bodies from recombinant *E.coli*, the present inventors developed a method for maximizing the production yield of recombinant proteins in cells, and purity in inclusion bodies by using polypeptide of human glucagon or

its derivatives having strong tendency of self-association as an N-terminal fusion partner. The method of the present invention enables inclusion bodies to solubilize easily in alkaline solution without using denaturants and reducing agents.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a method for preparing recombinant proteins in form of fusion proteins. In case of producing the desired proteins in form of inclusion bodies from *E. coli*, the method of the present invention makes it possible to recover and purify active proteins without using denaturants or reducing agents as well as to increase production yield in culture and purity of the desired proteins in inclusion bodies.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 represents a procedure for preparing expression vector, pT7IL-2,

FIG. 2 represents a procedure for preparing expression vector, pT1GIL-2,

FIG. 3 represents a procedure for preparing expression vector, pT2GIL-2,

FIG. 4 represents a procedure for preparing expression vector, pG1IL-2,

FIG. 5 represents a procedure for preparing expression vector, pG2IL-2,

FIG. 6 represents a procedure for preparing expression vector, pG3IL-2,

5 FIG. 7 represents intracellular expression level(%) of recombinant proteins produced from *E.coli* transformed with each vector,

10 FIG. 8 represents content (purity, %) of recombinant proteins contained in insoluble inclusion bodies recovered from culture of each *E.coli* transformants,

FIG. 9 represents solubility (%) of inclusion bodies recovered from culture of each *E.coli* transformants in alkaline solution,

15 FIG. 10 represents results of western blotting after non-reducing SDS-PAGE of fusion proteins in inclusion bodies recovered from culture of each *E.coli* transformants,

A) lane 1: a marker of molecular size

lane 2: inclusion bodies produced from *E.coli* transformed with expression vector, pT7IL-2

20 B) lane 1: molecular size marker

lane 2: inclusion bodies produced from *E.coli* transformed with expression vector, pT1GIL-2

lane 3: inclusion bodies produced from *E.coli* transformed with expression vector, pT2GIL-2

25 lane 4: inclusion bodies produced from *E.coli* transformed with expression vector, pG1IL-2

lane 5: inclusion bodies produced from *E.coli* transformed with expression vector, pG2IL-2

lane 6: inclusion bodies produced from *E.coli*
transformed with expression vector, pG3IL-2

FIG. 11 represents percentage of proteins formed by
self-association between the recombinant protein molecules,
as analyzed from results of western blotting after non-
reducing SDS-PAGE of fusion proteins in inclusion bodies
recovered from culture of each *E.coli* transformant.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

For achievement of the above-mentioned objects, the
present invention provides a method for preparing the desired
proteins of high purity at high yield from *E.coli* by using
polypeptide containing amino acid sequence of human glucagon
or its derivatives having strong tendency for self-
association as a fusion partner. Particularly, the present
invention provides a method for large-scale production of
recombinant proteins in form of inclusion bodies by using
heterologous polypeptides comprising hybrid peptide derived
from human glucagon and tumor necrosis factor, or polypeptide
containing 1 to 3-consecutive copies of human glucagon amino
acid sequences as a fusion partner.

Partially modified glucagon derivatives as well as whole
or partial amino acids sequence of glucagon can be used for a
fusion partner.

Whole or partial amino acids sequence of tumor necrosis
factor or its mutant can be used as a fusion partner with
glucagon, and polyhistidine sequence can be contained in the

fusion partner to isolate easily recombinant proteins.

In addition, specific peptide sequence recognized by enterokinase can be used as a linker for the above fusion partner proteins.

5

On the other hand, the present invention provides a method for preparing human proteins or its derivatives using microorganisms transformed with recombinant plasmids containing the above fusion partners sequence. Especially,
10 the present invention provides a method for purifying recombinant proteins by renaturing the synthesized recombinant proteins through simply pH shift: the insoluble inclusion bodies of recombinant proteins produced by the above method in alkaline solution, followed by restoring pH
15 to neutral condition.

Hereinafter, the present invention is described in detail.

20

The present invention produces recombinant proteins in form of fusion proteins using human glucagon peptide hormone (molecular weight 3.5 kDa) expressed stably in *E.coli* as a soluble protein.

25

Glucagon is a 29-residue peptide hormone having α -helical conformation, and expressed stably in *E.coli*. Moreover, glucagon has strong self-association tendency to stabilize the α -helical conformation by hydrophobic interaction forming an oligomer between molecules, and

thereby leads to a effective use as a fusion partner. In detail, the present invention produces recombinant proteins by using polypeptides containing whole or partial human glucagon described by SEQ ID No: 1 as fusion partners.

5

More particular, as follows, the present invention provides a method for producing recombinant proteins using various peptides/polypeptides containing whole or partial amino acid sequence of glucagon (G peptide) described by SEQ ID No: 1 as fusion partners.

10

1) From N-terminus, a polypeptide composed of peptide of 1th to 57th residues from N-terminus of human tumor necrosis factor (T1 peptide), G-peptide and polyhistidine [(His)₆] in order (**a fusion partner T1G**)

15

2) From N-terminus, a polypeptide composed of peptide of 8th to 12th residues from N-terminus of human tumor necrosis factor (T2 peptide), polyhistidine [(His)₁₀] and G-peptide in order (**a fusion partner T2G**)

20

3) From N-terminus, a polypeptide composed of G-peptide and polyhistidine [(His)₆] in order (**a fusion partner G1**)

4) From N-terminus, a polypeptide composed of G2-peptide which G-peptide is repeated sequentially twice, and polyhistidine [(His)₆] in order (**a fusion partner G2**)

25

5) From N-terminus, a polypeptide composed of G3-peptide which G-peptide is repeated sequentially three times, and polyhistidine [(His)₆] in order (**a fusion partner G3**)

The polyhistidine sequence enables fusion proteins to be

purified easily by use of metal chelating resin or cation exchange resin by using its properties having metal affinity and positive charge under low pH.

5 The present invention produces human interleukin-2 as a model protein in form of inclusion bodies at high expression level and high purity by using the above five fusion partners. Follows are a method for construction of expression vectors producing the fusion proteins.

10 1) The nucleotide sequence encoding human interleukin-2 is amplified by PCR (Polymerase Chain Reaction),

 2) The amplified DNA is inserted into pT7-7 vector containing T7 promoter to construct expression vector pT7IL-2 capable of expressing the human interleukin-2,

15 3) Sequences encoding polypeptides, which are composed of the five fusion partners (T1G, T2G, G1, G2, G3) having C-terminal Asp Asp Asp Asp Lys (DDDDK, hereinafter, referred as "D4K"), amino acid sequence recognized by enterokinase, are amplified by PCR, and

20 4) Expression vectors (pT1GIL-2, pT2GIL-2, pGIL-2, pG2IL, pG3IL-2) are constructed by insertion of the amplified DNA fragments into 5'-end of the interleukin-2 in pT7IL-2. The expression vectors are capable of expressing fusion proteins composed of interleukin-2, D4K and the each five
25 fusion partner, and the produced fusion proteins enables only interleukin-2 to be separated through digestion with enterokinse during purification procedure.

Besides, the DNAs encoding the polypeptides of the fusion partners having specific amino acid sequence for enzymatic cleavage sequence by enterokinase at its C-terminus, are inserted into vectors containing appropriate promoter, such as, lac, trp, tac, pL, T3, T7, SP6, SV40, λ (pL/pR), etc., or the above DNAs (fusion partner-D4K) are ligated to DNAs having these promoters and ribosomal binding site, followed by construction of vector system through inserting them into various plasmids.

DNAs encoding the desired proteins or peptides are inserted into 3'-terminus of these vectors, the resulting expression vectors are introduced into appropriate hosts, and the constructed transformants are cultured to produce the desired fusion proteins.

The transformants were prepared by introducing the expression vectors producing the interleukin-2 fusion proteins into suitable hosts by method of Hanahan (Hanahan, D. 1985, *DNA cloning* 1, 109-135, IRS press).

Particularly, *E.coli* BL21(DE3) were transformed with the expression vector pT1GIL-2, pT2GIL-2, pGIL-2, pG2IL-2 and pG3IL-2, and the resulting transformants were deposited in Korean Collection for Type Cultures (KCTC) on November 13, 1998 (Accession No.: KCTC 0555BP, KCTC 0556BP, KCTC 0552BP, KCTC 0553BP, KCTC 0554BP).

The fusion proteins containing interleukin-2 can be produced by culturing the transformed recombinant *E.coli* under suitable conditions. Cell extracts are obtained by

treatment with lysozyme digestion, freezing and thawing, ultrasonication or french press, followed by centrifugation or filtration. The fusion proteins can be purified by general purification methods, such as solubilization of
5 extracts, ultrafiltration, dialysis, ion exchange chromatography, gel filtration, electrophoresis and affinity chromatography. Interleukin-2 is isolated by digestion with enterokinase.

10 The expression level of interleukin-2 using fusion partners comprising human glucagon of the present invention, is more excellently improved than the direct expression of interleukin-2 (50-60% of total proteins in *E.coli*), and insoluble inclusion bodies containing foreign protein of high
15 purity are obtained (about 80% of total proteins composing inclusion body). This insoluble inclusion bodies is solubilized more easily in alkaline solution than general insoluble inclusion bodies. The general insoluble inclusion bodies needs solubilization using denaturants or reducing
20 agent, followed by refolding procedure, whereas the fused recombinant proteins of the present invention are able to overcome the problems of refolding by simply solubilizing the inclusion bodies in alkaline solution without using denaturants or reducing agents.

25 This property is due to that the N-terminal fusion partner causes binding of highly pure recombinant protein molecules by hydrophobic interaction between the same recombinant molecules.

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1: Construction of pT7IL-2 directly expressing interleukin-2

For large-scale expression of interleukin-2 and in *E.coli* by using T7 promoter expression system, *E.coli* (KCTC 8258P) producing interleukin-2 was obtained from Korean Collection for Type Culture. Plasmid pNKM21 in this *E.coli* strain contains a structural gene for human interleukin-2 mutant which has 125-cysteine substituted for serine, and this structural gene is regulated by PL promoter. The sequence encoding 133 amino acids of interleukin-2 gene in plamid pNKM21 was amplified by PCR using the following primers.

- 1) GCA CAT ATG GCA CCT ACT TCA AGT TCT (pNIL)
- 2) GCA AAG CTT CTA TTA AGT TAG TGT TGA GAT GAT (pHIL)

100 ng of template DNA and 50 pmol of each primer was
5 added to PCR buffer (0.25 mM dNTPs; 50 mM KCl; 10 mM
(NH₄)₂SO₄; 20 mM Tris-HCl (pH 8.8); 2 mM MgSO₄; 0.1% Triton X-
100), and PCR was performed by adding Taq DNA polymerase to
the above PCR mixture. PCR condition was 95°C/30 sec
(denaturation), 52°C/30 sec (annealing), and 72°C/60 sec
10 (elongation) (hereinafter, all PCRs were performed by this
condition except specially mentioned PCR). PCR was repeated
by 30 cycles. The amplified DNA fragments were analyzed on
1% agarose gel, purified by use of Quiagen Gel extraction kit,
digested with *Nde*I and *Hind*III, and the resulting DNA
15 fragments having *Nde*I site at 5'-end and *Hind*III site at 3'-
end were inserted into *Nde* I and *Hind* III sites of expression
vector pT7-7. The constructed recombinant plasmid was
designated as pT7IL-2 (FIG. 1).

20 **Example 2: Construction of interleukin-2 expressing vector
using human glucagon gene as a fusion partner**

A. Construction of plasmid pT1GIL-2

The coding sequence of T1 peptide (1th to 57th residue of
25 human tumor necrosis factor) was amplified by PCR using
mononuclear cell cDNA library (Clontech) constructed from
U937 cell line as a template, and the amplified DNA fragments
were purified by Qiagen Gel extraction kit. The purified DNA

fragments were digested with *Bam*HI and *Nde*I to have *Nde*I site at 5'-end and *Bam*HI sites at 3'-end, and inserted into *Nde*I and *Bam*HI sites of a vector pT7-7. This resulting recombinant plasmid was designated as pT7-T1.

5 Fragment-1 was amplified by PCR using the glucagon gene synthesized by DNA synthesizer as a template and the following primers.

- 1) GCA GGA TCC GAC GAC GAC AAA CAC TCT CAG GGT (BGD4K)
- 2) TTT GTC GTC GTC GTC CTC GAG AGT GTT CAT CAG CCA (XGD4K)

10 The amplified DNA fragments were analyzed on 1% agarose gel, and purified by Quiagen Gel extraction kit.

Fragment-2 was amplified by PCR using the interleukin-2 gene in pT7IL-2 as a template and the following primers.

- 15
- 1) GAC GAC GAC GAC AAA GCA CCT ACT TCA AGT TCT (XID4K)
 - 2) GCA AAG CTT CTA TTA AGT TAG TGT TGA GAT GAT (HIL)

The amplified DNA fragments were analyzed on 1% agarose gel, and purified by Quiagen Gel extraction kit.

20 Second PCR was performed by using fragment-1 and fragment-2 as templates and the following primers.

- 1) GCA GGA TCC GAC GAC GAC GAC AAA CAC TCT CAG GGT (BGD4k)
- 2) GCA AAG CTT CTA TTA AGT TAG TGT TGA GAT GAT (HIL)

25 The amplified DNA fragments were analyzed on 1% agarose gel, and purified by gel extraction kit. The purified DNA fragments were digested with *Bam*HI and *Hind*III to have *Bam*HI site at 5'-end and *Hind*III site at 3'-end, and inserted into

*Bam*HI and *Hind*III sites of pT7-T1. This resulting recombinant plasmid was designated as pT7-T1GIL-2.

XH6IL-2 fragment was amplified by PCR using plasmid pT7-T1GIL as a template and the following primers.

- 1) GCA CTC GAG CAT CAT CAT CAT CAT AGC AGC GAC GAC GAC
GAC AAA GCA (XH6)
- 2) GCA AAG CTT CTA TTA AGT TAG TGT TGA GAT GAT (HIL)

The amplified DNA fragments were analyzed on 1% agarose gel, and purified by gel extraction kit. The purified DNA fragments were digested with *Xho*I and *Hind*III, respectively, to have *Xho*I site at 5'-end and *Hind*III site at 3'-end, and inserted into *Xho*I and *Hind*III sites of pT7-T1GIL-2. This resulting recombinant plasmid was designated as pT1GIL-2 (FIG. 2).

B. Construction of plasmid pT2GIL-2

*Nde*I site and polyhistidine sequence [(His)₁₀]/*Bam*HI site were introduced at 5' and 3' ends of the coding sequence of Pro-Ser-Asp-Lys-Pro (8th to 12th residue of human tumor necrosis factor), respectively, and the constructed DNA fragment was inserted into *Nde*I and *Bam*HI sites of pT7-7. This resulting recombinant plasmid was designated as pT7-T2.

GIL-2 (BH) fragment was amplified by PCR using the gene of interleukin-2 fusion protein fused with glucagon in pT7-T1GIL-2 as a template and the following primers.

- 1) GCA GGA TCC CAC TCT CAG GGT ACT TTC (GBAM)
- 2) GCA AAG CTT CTA TTA AGT TAG TGT TGA GAT GAT (HIL)

The amplified DNA fragments were analyzed on 1% agarose gel, and purified by gel extraction kit (Quiagen). The purified DNA fragments were digested with *BamH* I and *Hind* III to have *BamHI* site at 5' end and *HindIII* site at 3' end, and inserted into *BamHI* and *HindIII* sites of pT7-T2. This resulting recombinant plasmid was designated as pT2GIL-2 (FIG.3).

C. Construction of plasmid pG1IL-2

GH6IL-2 (NH) fragment was amplified by PCR using the gene of interleukin-2 fusion protein fused with glucagon in pT1GIL-2 as a template and the following primers.

- 1) GCA CAT ATG CAC TCT CAG GGT ACT (GNDE)
- 2) GCA AAG CTT CTA TTA AGT TAG TGT TGA GAT GAT (HIL)

The amplified DNA fragments were analyzed on 1% agarose gel, and purified by gel extraction kit (Quiagen). The purified DNA fragments were digested with *Nde*I and *HindIII* to have *Nde*I site at 5' end and *HindIII* site at 3' end, and inserted into *Nde*I and *HindIII* sites of pT7-7. This resulting recombinant plasmid was designated as pG1IL-2 (FIG.4).

D. Construction of plasmid pG2IL-2.

GH6IL-2 (BH) fragment was amplified by PCR using the gene of interleukin-2 fusion protein fused with glucagon in pGIL-2 as a template and the following primers.

- 1) GCA GGA TCC CAC TCT CAG GGT ACT TTC (GBAM)
- 2) GCA AAG CTT CTA TTA AGT TAG TGT TGA GAT GAT (HIL)

The amplified DNA fragments were analyzed on 1% agarose gel, and purified by gel extraction kit. The purified DNA fragments were digested with *Bam*HI and *Hind*III to have *Bam*HI site at 5' end and *Hind*III site at 3' end, and inserted into *Bam*HI and *Hind*III sites of pT7-7. This resulting recombinant plasmid was designated as pGIL-2 (BH).

G(NB) fragment was amplified by PCR using the gene of glucagon synthesized by DNA synthesizer as a template and the following primers.

- 1) GCA CAT ATG CAC TCT CAG GGT ACT (GNDE)
- 2) GCA GGA TCC AGT GTT CAT CAG CCA (GBAM-3)

The amplified DNA fragments were analyzed on 1.5% agarose gel, and purified by gel extraction kit (Quiagen). The purified DNA fragments were digested with *Nde*I and *Bam*HI to have *Nde*I site at 5' end and *Bam*HI site at 3' end, and inserted into *Nde*I and *Bam*HI sites of pGIL-2 (BH). This resulting recombinant plasmid was designated as pG2IL-2 (FIG. 5).

E. Construction of pG3IL-2

G(EB) fragment was amplified by PCR using the gene of glucagon in pGIL-2 as a template and the following primers.

- 1) GCA GAA TCC CAC TCT CAG GGT ACT (GECO)
- 2) GCA GGA TCC AGT GTT CAT CAG CCA (GBAM-3)

The amplified DNA fragments were analyzed on 1.5% agarose gel, and purified by gel extraction kit. The purified DNA fragments were digested with *Eco*RI and *Bam*HI to

have *EcoRI* site at 5' end and *BamHI* site at 3' end, and inserted into *EcoRI* and *BamHI* sites of pGIL-2(BH). This resulting recombinant plasmid was designated as pG2IL-2 (EH).

5 Also, G(NE) fragment was amplified by PCR using the gene of glucagon in pGIL-2 as a template and the following primers.

3) GCA CAT ATG CAC TCT CAG GGT ACT (GNDE)

4) GCA GAA TTC AGT GTT CAT CAG CCA (GECO-3)

10 The amplified DNA fragments were analyzed on 1.5% agarose gel, and purified by gel extraction kit. The purified DNA fragments were digested with *NdeI* and *EcoRI* to have *NdeI* site at 5' end and *EcoRI* site at 3' end, and inserted into *NdeI* and *EcoRI* sites of pG2IL-2 (EH). This
15 resulting recombinant plasmid was designated as pG3IL-2 (FIG. 6).

Example 3: Construction of *E. coli* transformants

20 *E. coli* BL21(DE3) (Studier, F. A. and Moffatt, B. A. 1986, 189, 113-130) was transformed with the expression vectors pT7IL-2, pT1GIL-2, pT2GIL-2, pG1IL-2, pG2IL-2 and pG3IL-2 by using the method described by Hanahan D. (Hanahan D. 1985, *DNA Cloning* 1, 109-135, IRS press), followed by
25 selection of colonies having ampicillin resistance.

 The selected recombinant *E. coli* strains were deposited in Korean Collection for Type Cell (KCTC) on November 23, 1998 (Accession No: pT7IL-2, KCTC 0555BP; pT2GIL-2, KCTC

0556BP; pG1IL-2, KCTC 0552BP; pG2IL-2, KCTC 0553BP; pG3IL, KCTC 0554BP).

Example 4: Culture of E.coli transformants and production of fusion proteins

20 μ L of the culture solution cultivated for 1 day was inoculated into 2 mL of LB (ampicillin 100 mg/ L), and cultured at 37°C and 250 rpm. When the culture O.D.₆₀₀ reached 0.4, the gene expression was induced by adding IPTG (isopropylthio- β -D-galactosidase, 0.5 mM), and the induced cultures were cultivated for further 3-4 hours under the same condition.

Example 5: Selection of E.coli having high productivity

After transforming *E.coli* BL21(DE3) with the each expression vector, 10 colonies were selected from each plate. 20 μ L of each subculture solution of the selected colonies was inoculated into 2 mL of LB media containing 100 mg ampicillin per liter, and cultured at 37°C and 200 rpm. When the culture O.D.₆₀₀ reached 0.4, the gene expression was induced by adding IPTG (isopropylthio- β -D-galactosidase, 0.5 mM), and the induced cultures were cultivated for further 3-4 hours under the same condition. The recombinant cells in culture were centrifuged at 6000 rpm, and the cell pellets were resuspended in distilled water. The cells were disrupted by using Branson Sonifier (Branson Ultrasonics

Corp., Danbury, CT). After separation of proteins in the lysates by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (14% Tris-Glycine gel), the expression level of each protein was analyzed by using Densitometer (Bio-Rad). From the result, colony showing the highest expression level was inoculated into LB and cultured. 15% glycerol was added to the culture in order to store at -70°C. The result of analysis of expression level demonstrated that expression of all fusion proteins was far superior in expression level to direct expression of only interleukin-2. Especially, in use of T2G and G3 as fusion partners, the expression level in *E.coli* was high, and in use of G3 as a fusion partner, amounts of fusion proteins was more than 60% of total amounts of proteins in *E.coli* (FIG. 7).

Example 6: Isolation of insoluble inclusion bodies from cell culture

The recombinant cells in culture were centrifuged at 6000 rpm and the cell pellets were resuspended in distilled water. The cells were disrupted by using Branson Sonifier, centrifuged at 5000 g for 10 min, and the supernants and pellets were analyzed by SDS-PAGE (14% Tris-Glycine), respectively, in order to confirm recovery of proteins. Insoluble protein aggregates were washed twice with 0.5% Triton X-100, and subject to necessary analyses.

Example 7: Construction of vectors expressing interleukin-2

using a human glucagon-derived gene as a fusion partner,
and expression of fusion proteins by transformants

To elucidate the effect of glucagon mutation on
5 expression of interleukin-2, partial sequences of glucagon
were changed (⁶Phe→⁶Lys; ¹⁰Tyr→¹⁰Lys; ¹³Tyr→¹³Lys).
Recombinant interleukin-2 (GK3IL-2) fused with glucagon
mutant was constructed by the following method.
Oligonucleotide GK3-1 and GK3-2 represented by SEQ ID No.: 2
10 and 3, respectively, were amplified by PCR using primers GNDE
and GXHO.

1) GCA CAT ATG CAC TCT CAG GGT ACT (GNDE)

2) GAC CTC GAG AGT GTT CAT CAG CCA (GXHO)

15 The amplified DNA fragments were analyzed on 2% agarose
gel, and purified by gel extraction kit (Quiagen). The
purified DNA fragments were digested with *Nde*I and *Xho*I to
have *Nde*I site at 5' end and *Xho*I site at 3' end, and
inserted into *Nde*I and *Xho*I sites of pIL-2(NX). This
20 resulting recombinant plasmid was designated pGK3IL-2. pIL-2
was constructed by removing G3 sequence through digestion
with *Nde*I and *Xho*I. *E.coli* BL(DE3) was transformed with
pGK3IL-2, and the resulting *E.coli* transformant was deposited
in Korean Collection for Type Cell (KCTC) on February 24,
25 1999 (Accession NO : KCTC 0582BP).

The expression level of recombinant interleukin-2
(GK3IL-2) fused with glucagon mutant was compared with that

of GIL-2 by using method of the above-mentioned Example. As indicated in Table 1, the high expression level was shown in case of fusion with specific glucagon.

5

<Table 1>

	GIL-2	GK3IL-2
Expression level (%)	25.02	32.89

Experiment 1: Purity of the expressed recombinant proteins

10

The colonies selected from the Example 5 were subcultured in 100 mL of LB media containing 100 mg ampicillin per liter. When the culture O.D.₆₀₀ reached 0.4, the gene expression was induced by adding IPTG (isopropylthio- β -D-galactosidase, 0.5 mM), and the induced

15 cultures were cultivated for further 3-4 hours under the same condition. The insoluble protein inclusion bodies produced from the culture were recovered by using the method of the Example 6. After the recovered inclusion body was washed twice with 0.5% Triton X-100 and analyzed by

20 SDS-PAGE (sodium dodecyl sulfate-polyacryamide gel electrophoresis) (14% Tris-Glycine gel). Purity of the recombinant protein in each inclusion body was estimated by using densitometer (Bio-Rad).

25

The result demonstrated that in direct expression of interleukin-2 without fusion partners, purity of proteins in the inclusion bodies was about 40%, whereas in

expression in form of fusion proteins of interleukin-2 with the five fusion partners, purity of recombinant proteins in inclusion bodies was highly increased (FIG. 8). Especially, when G3 was used as a fusion partner, purity of recombinant proteins in inclusion bodies was increased to about 80%.

Experiment 2: Solubilization of inclusion bodies

Insoluble proteins obtained from each recombinant strain were dried by using freeze-drying method, resuspended in alkaline solution at pH 12 to the concentration of 50 mg/mL, and the solubility was estimated during sequential dilution (2X) using alkaline solution at pH 12). By every step, the sample was separated into a supernant and pellet through centrifugation (5000 g, 10 min), and respectively quantitated by using Bradford method. The solubility was determined by the following mathematical formula. The pellets were dissolved in solution at pH 12, and quantitated.

$$\text{Solubility (\%)} = A \times 100 / A + B$$

A: total amounts (μg) of proteins in supernant

B: total amounts (μg) of proteins in pellet

As shown in Figure 9, in case of protein in inclusion body co-expressed with the above fusion partners when compared with the direct expression of interleukin-2, the its solubility was remarkably increased. Especially when G3

was used as fusion partner, solubility of the inclusion body was about 100% at its concentration of above 8 g/L. From the result, much insoluble protein inclusion bodies were able to be dissolved by change of pH without using
5 denaturants or reducing agents, and this special property identified from the present invention can be used effectively in the purification process. In addition, the recombinant proteins easily dissolved in alkaline solution at pH 12, can be reactivated by decrement of pH to 8.

10

Experiment 3: Binding property between protein molecules composing inclusion bodies

Analysis by Western blotting was performed to examine
15 binding property among protein molecules composing inclusion body. First, the proteins in each inclusion body were separated by non-reducing SDS-PAGE (14% Tris-Glycine gel), followed by Western blotting. Mouse monoclonal IgG1, anti-human interleukin-2 (BioSource International, Inc.,
20 Camarillo, CA) and goat anti-mouse IgG conjugated to alkaline phosphatase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used as primary and secondary antibodies, respectively. As indicated in Figure 10, in the direct expression mode of interleukin-2 in inclusion bodies,
25 various heterogeneous multimers were significantly formed via disulfide bridge with other host proteins. However, in interleukin-2 fusion proteins, almost recombinant protein molecules were bound via hydrophobic interaction among the

same molecules.

The ratio of hydrophobically-bound homologous multimer in each protein inclusion body was analyzed by using densitometer, and the result of analysis was shown in Figure 11. The result showed that in direct expression of only interleukin-2, hydrophobically-bound homologous multimer was 32%, however in expression in form of the fusion protein of the present invention, hydrophobically-bound homologous multimer was more than 60%, and plentifully above 90%. Inclusion bodies in form of hydrophobic-bound homologous multimer were solubilized easily by using the above purification method.

INDUSTRIAL APPLICABILITY

15

By using the polypeptides comprising amino acid sequence of human glucagon provided by the present invention as fusion partners, recombinant proteins in inclusion bodies can be produced at high expression level, and simultaneously purity of recombinant proteins is able to be remarkably improved.

20

Additionally, large amounts of the produced inclusion bodies together with expression of the fused recombinant proteins are solubilized easily in alkaline solution. Generally, the inclusion bodies must be dissolved by denaturants or reducing agents, and followed by refolding step, whereas in use of the fusion partners of the present invention, the problems of refolding can be overcome by simply solubilizing the inclusion bodies in alkaline solution.

25

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

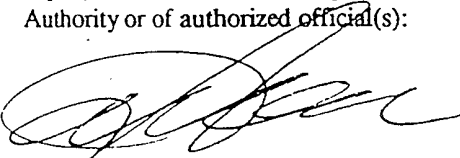
INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO: LEE Jeewon

Expo Apt. 107-1205, Jeonmin-dong, Yusong-ku, Taejon 305-390,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: <i>Escherichia coli</i> BL21(DE3) /pG IL-2	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0552BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☒ a scientific description ☐ a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on November 23 1998	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on _____ and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  PARK Yong-Ha, Director Date: November 30 1998

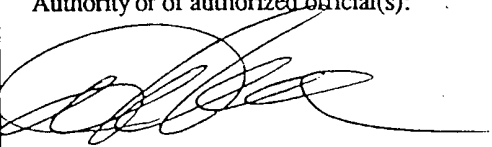
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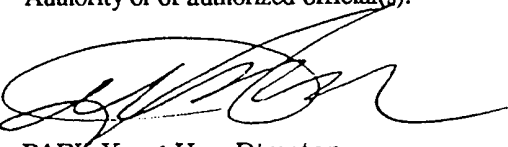
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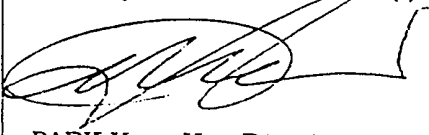
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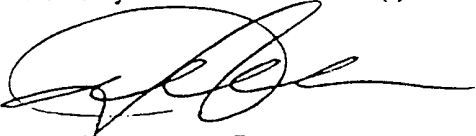
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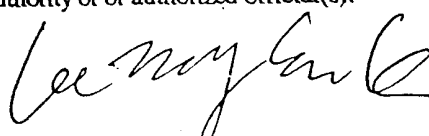
BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO: KIM, Dae-Young
Samsungpureun Apt. 113-1003, Jeonmin-dong, Yusong-ku, Taejon 305-390,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: <i>Escherichia coli</i> BL21(DE3) / pGK3IL-2	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY KCTC 0582BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
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III. RECEIPT AND ACCEPTANCE	
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Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  PARK Yong-Ha, Director Date: February 26 1999

What is Claimed is

1. A method for preparing recombinant proteins or peptides expressed in form of X_G -L-Y containing self-associating peptides as fusion partners by culturing transformed microorganisms.

X_G : fusion partner (polypeptide containing whole or partial amino acid sequence of hydrophobic self-association human glucagon or its derivatives)

L : linker (sequence for in-frame expression, or in-vitro enzymatic cleavage)

Y : recombinant protein or peptide

2. The method for preparing recombinant proteins or peptides according to claim 1, wherein the fusion partners are polypeptide containing whole or partial amino acid sequences of human glucagon described by SEQ ID No.: 1.

3. The method for preparing recombinant proteins or peptides according to claim 1, wherein the fusion partners are polypeptides containing whole or partial amino acid sequences of human glucagon derivatives described by SEQ ID No.: 2 or 3.

4. The method for preparing recombinant proteins or peptides according to claim 1, wherein a fusion partner contains whole or partial amino acid sequence of human tumor necrosis factor or its derivatives.

5. The method for preparing recombinant proteins or peptides according to claim 1, wherein the linker is a peptide containing a amino acid sequence of Asp Asp Asp Asp Lys (DDDDK, D4K) recognized by enterokinase.

6. The method for preparing recombinant proteins or peptides according to claim 1, wherein the fusion partners containe polyhistidine sequence.

7. A method for preparing human interleukin-2 or its derivatives in microorganisms according to the method of claim 1.

8. The method for preparing human interleukin-2 or its derivatives according to claim 7, which comprises the steps of:

- 1) culturing *E.coli* transformants producing human interleukin-2 or its derivatives fused with fusion partners;
- 2) obtaining protein aggregates from the cell lysates;
- 3) solubilizing the protein aggregates in alkaline solution; and
- 4) renaturing recombinant protein by restoring neutral pH.

9. The method for preparing human interleukin-2 or its derivatives according to claim 8, wherein the purified fusion proteins are digested with enterokinase during purification

process.

10. Expression vectors according to claims 1, 2, 3, 4, 5,
and 6, which contain nucleotide sequences of a fusion partner
5 and a linker.

11. Expression vectors, which are constructed by
insertion of the gene encoding human interleukin-2 or its
derivatives into the expression vectors of claim 10.
10

12. Expression vectors, which are pT1GIL-2, pT2GIL-2,
pGIL-2, pG2IL-2, pG3IL-2 or pGK3IL-2.

13. Microorganisms transformed with the expression
15 vectors of claim 12 (Accession No.: KCTC 0555BP, KCTC 0556BP,
KCTC 0552BP, KCTC 0553BP, KCTC 0554BP or KCTC 0582BP).

FIG. 1

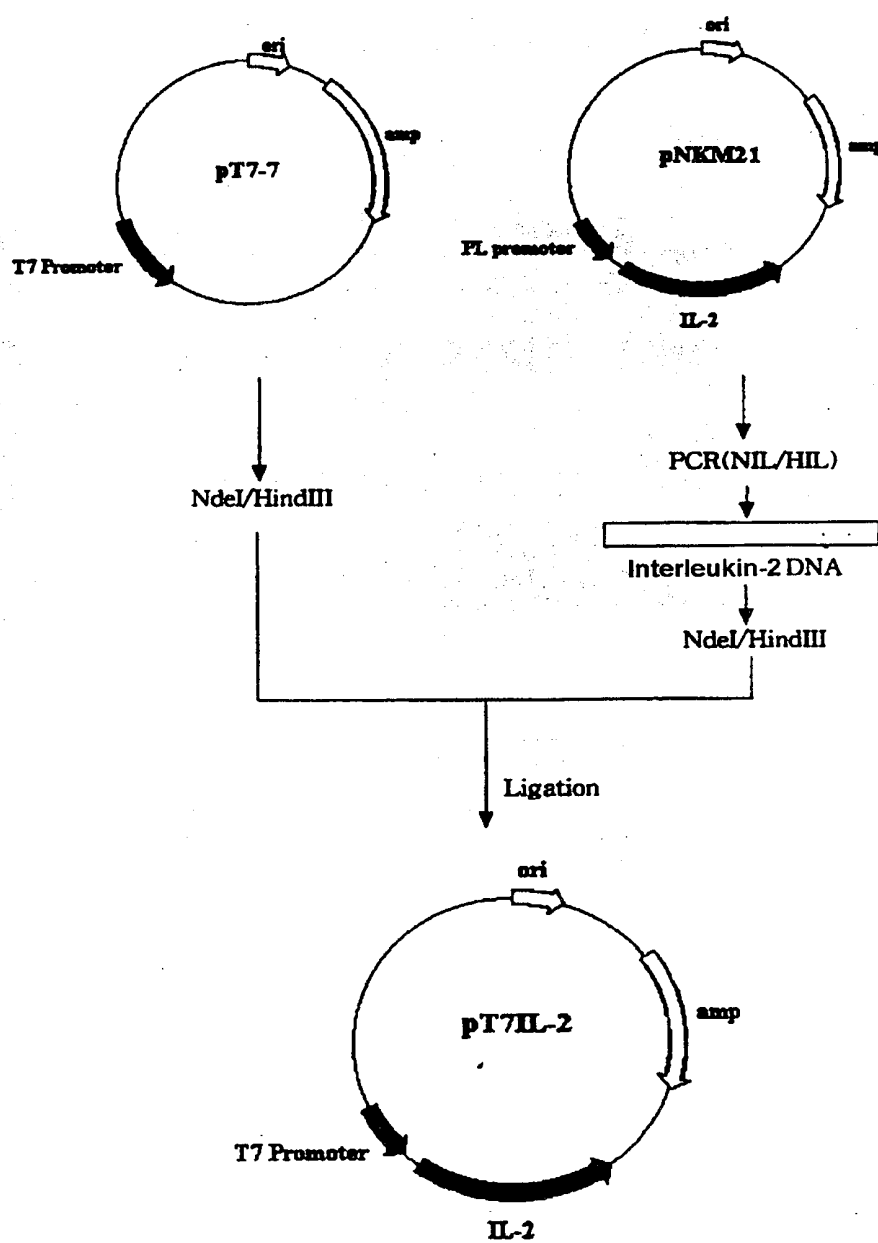


FIG. 2

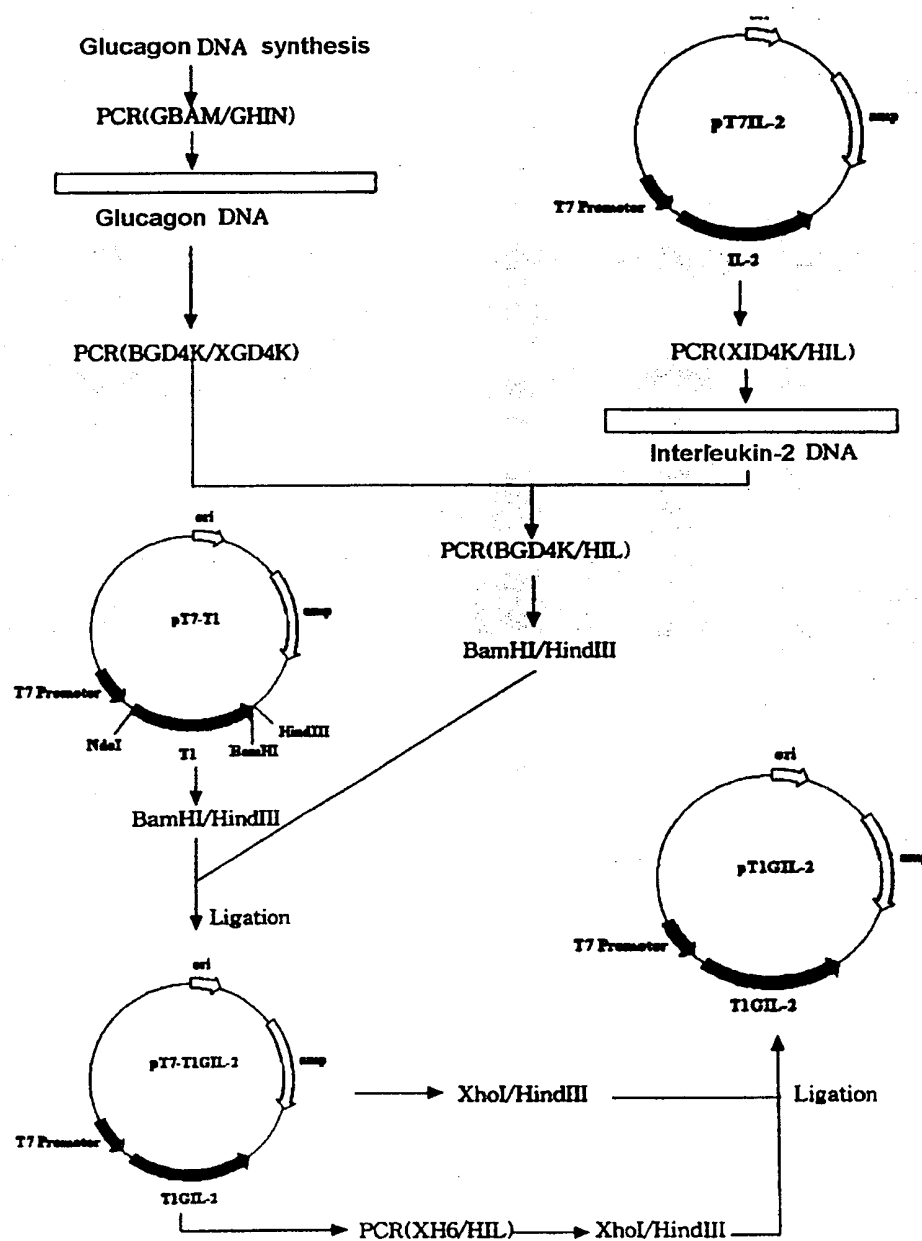


FIG. 3

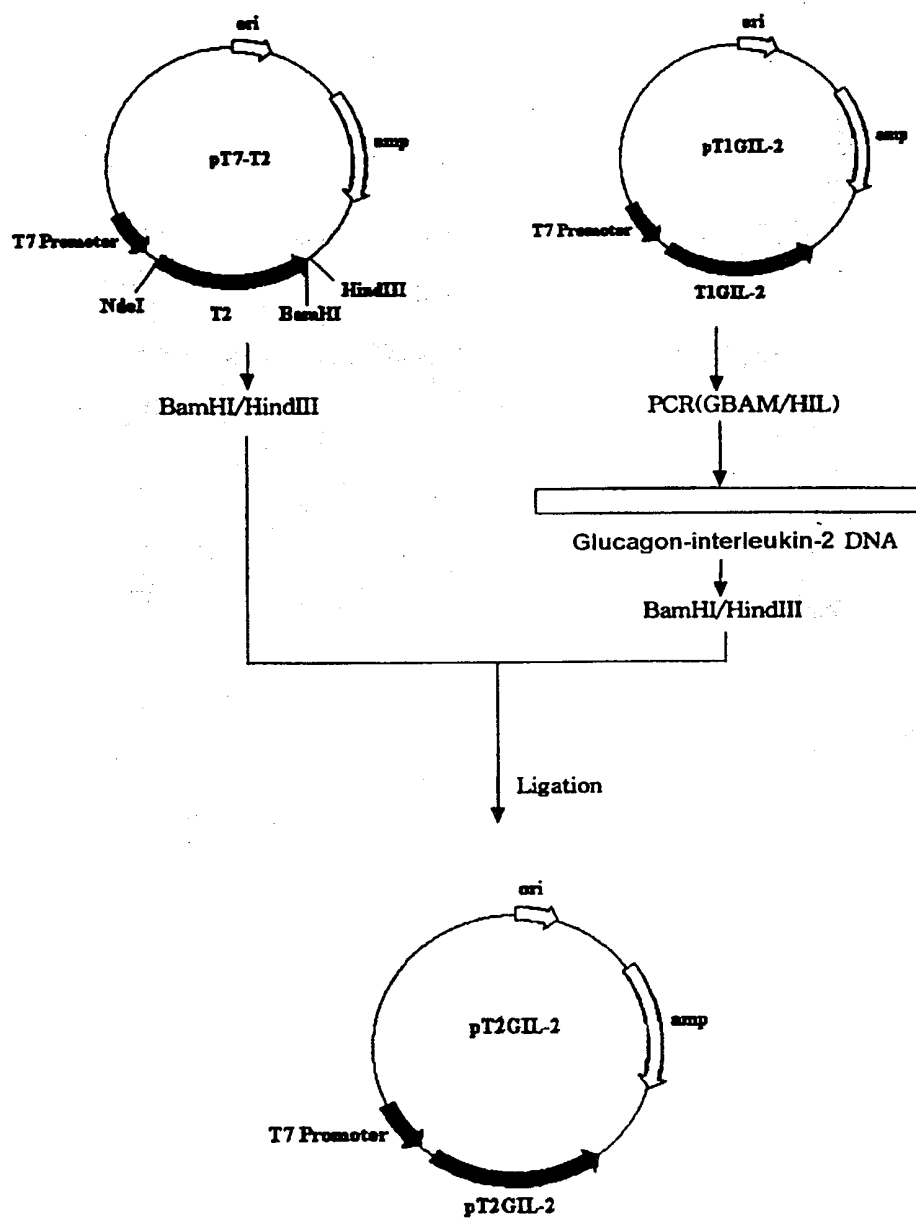


FIG. 4

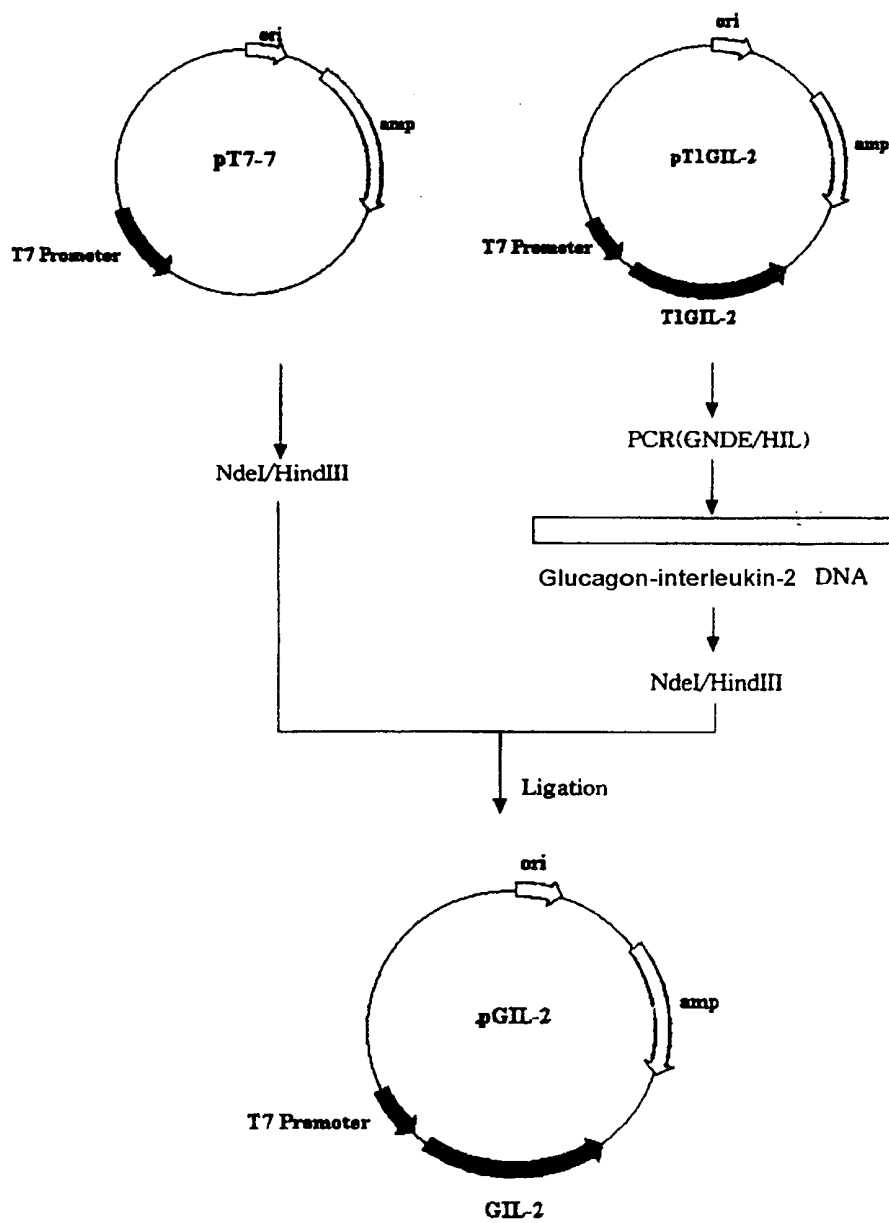


FIG. 5

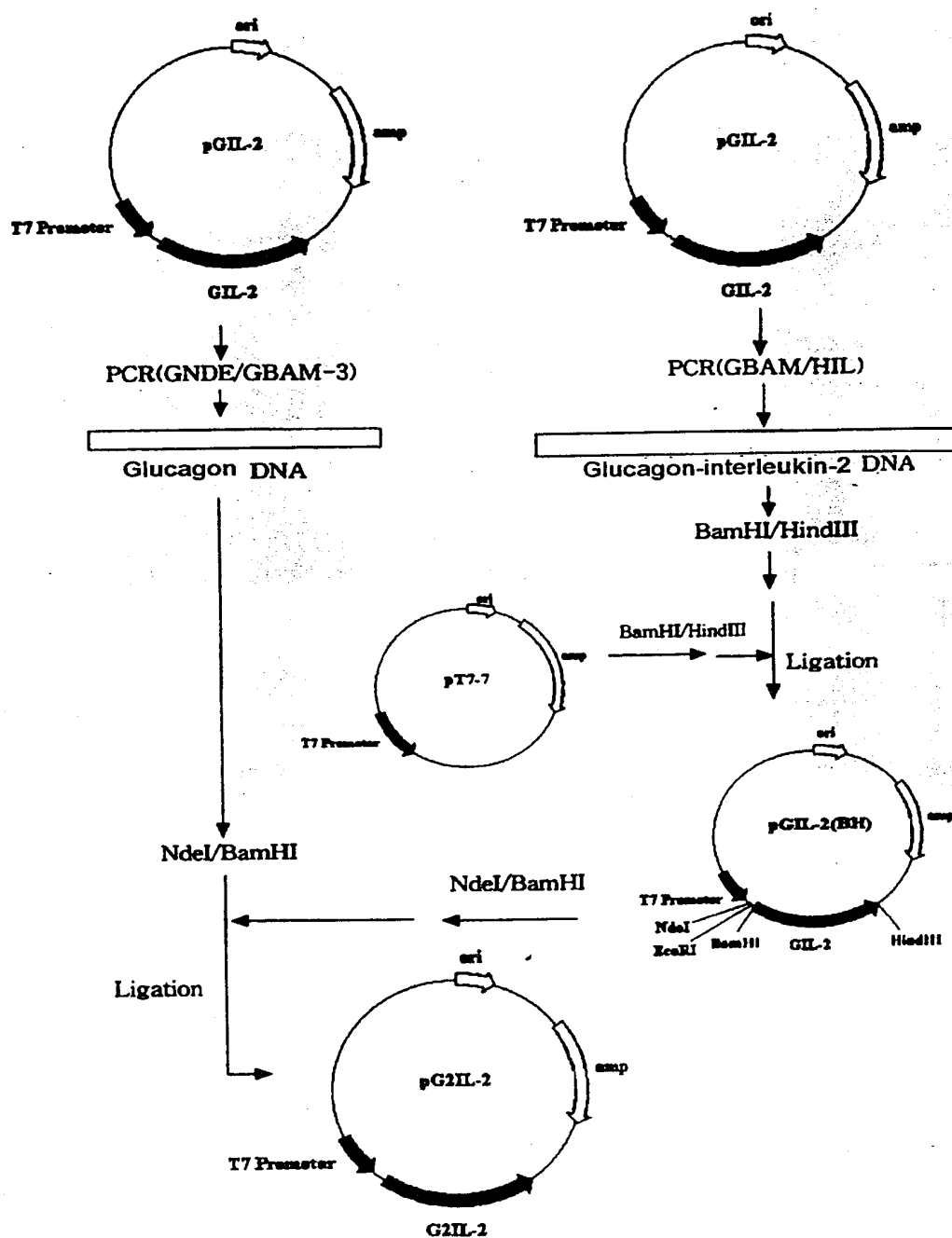


FIG. 6

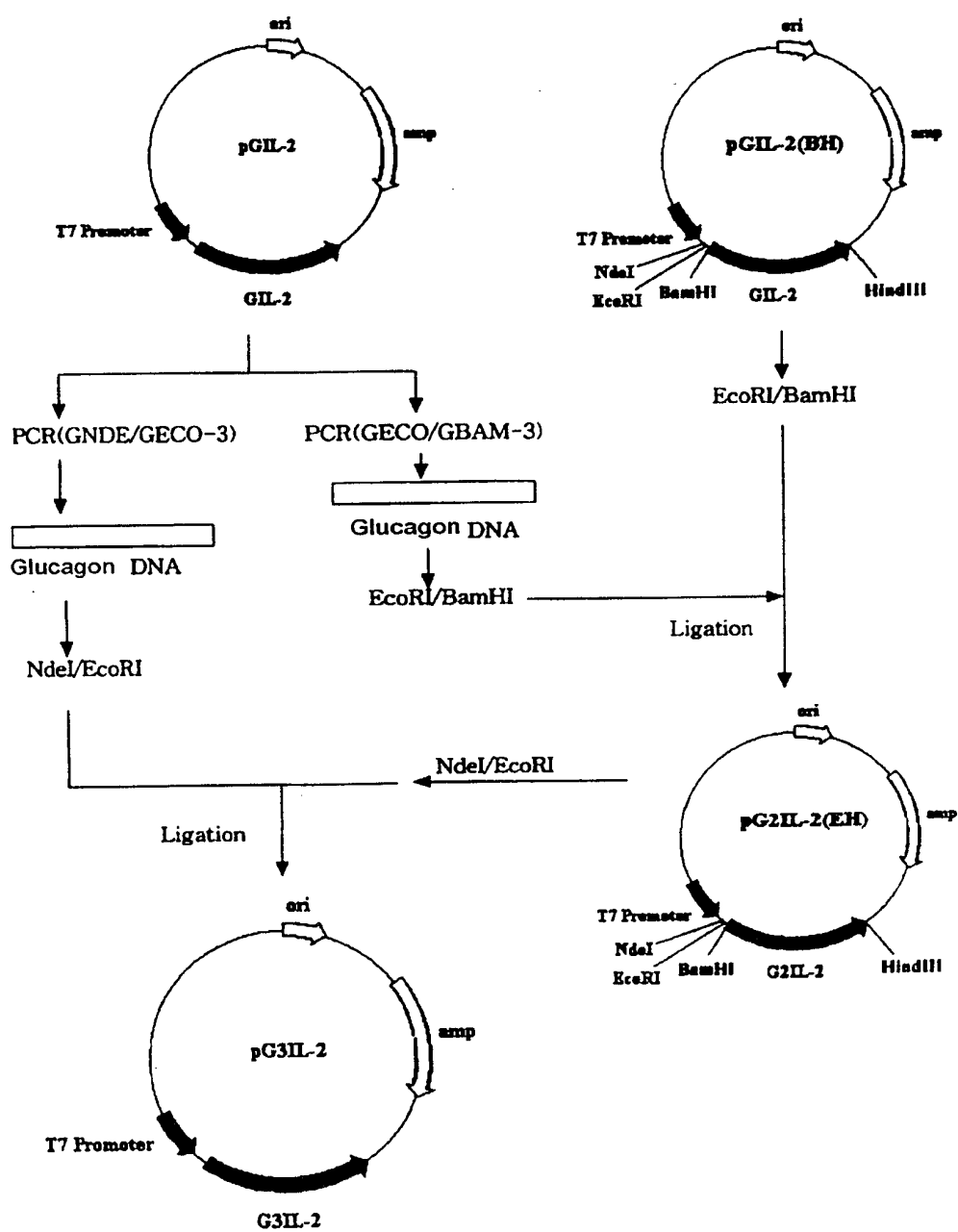


FIG. 7

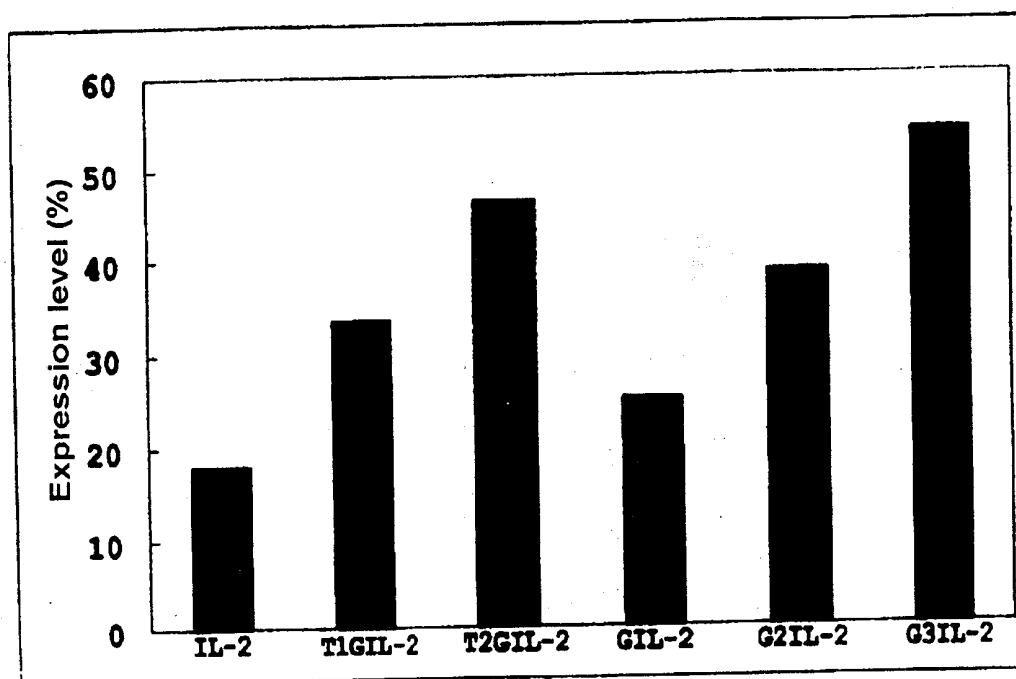


FIG. 8

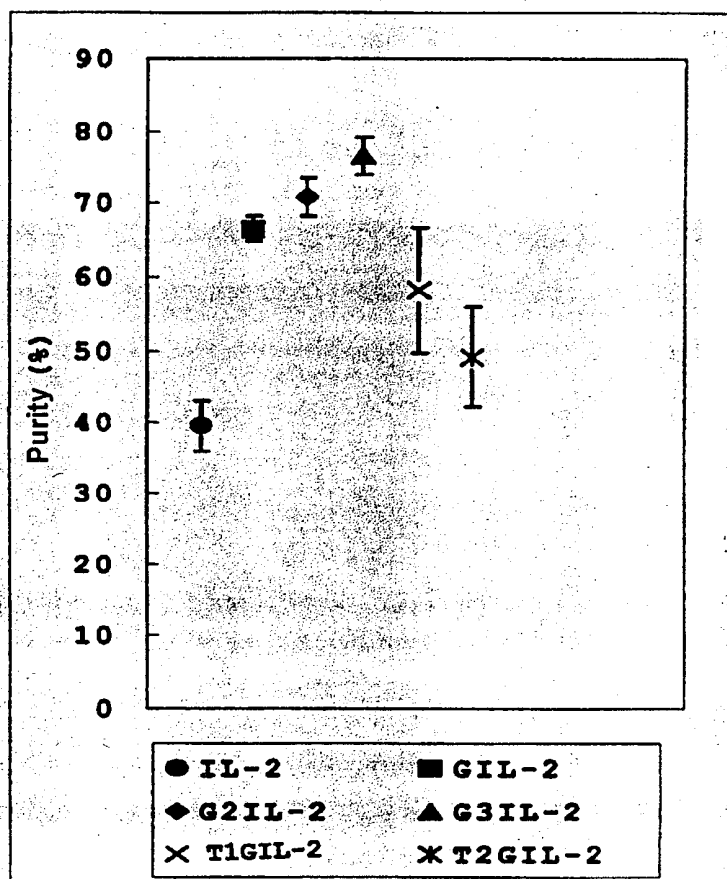
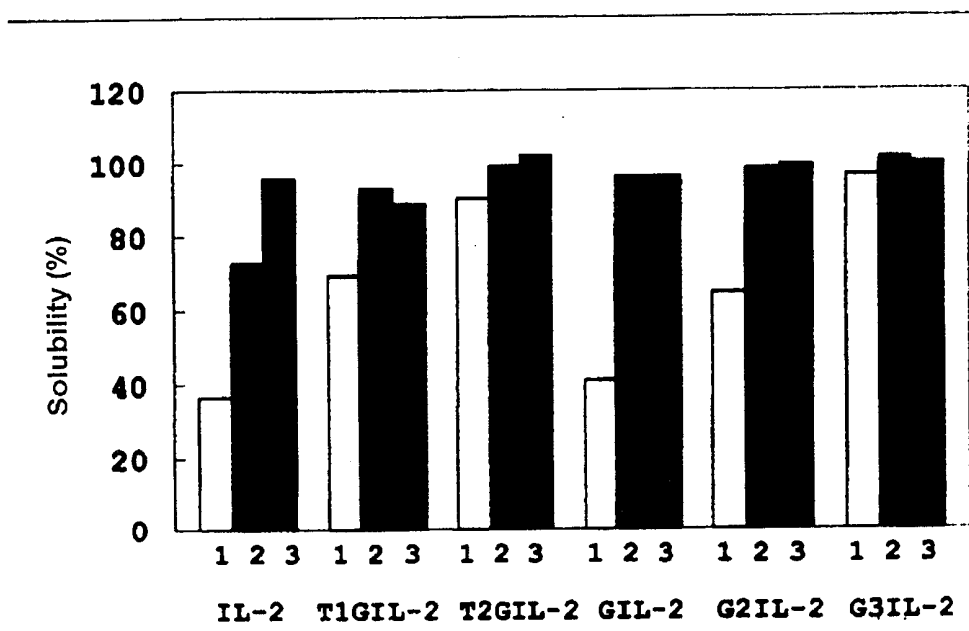


FIG. 9



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FIG. 10

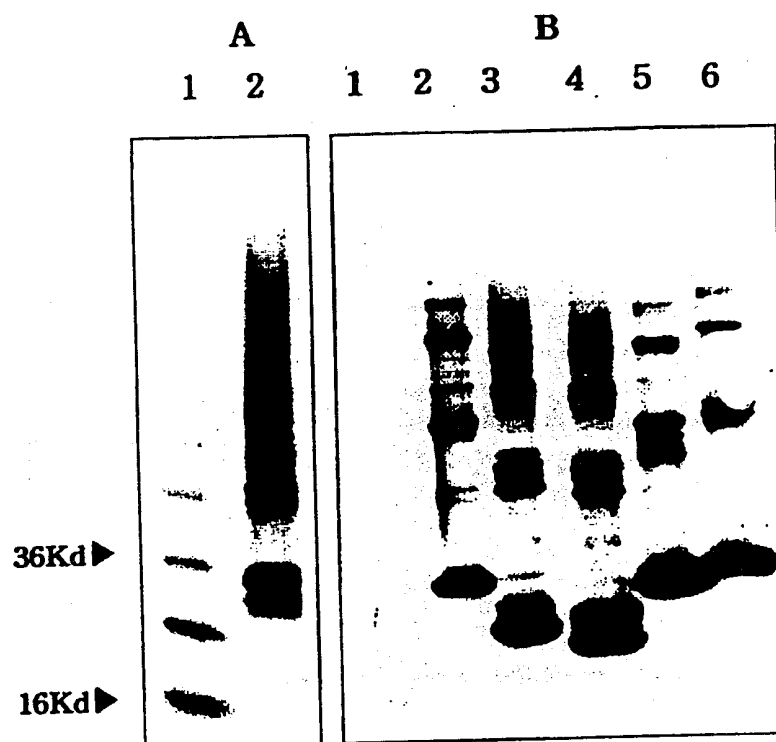
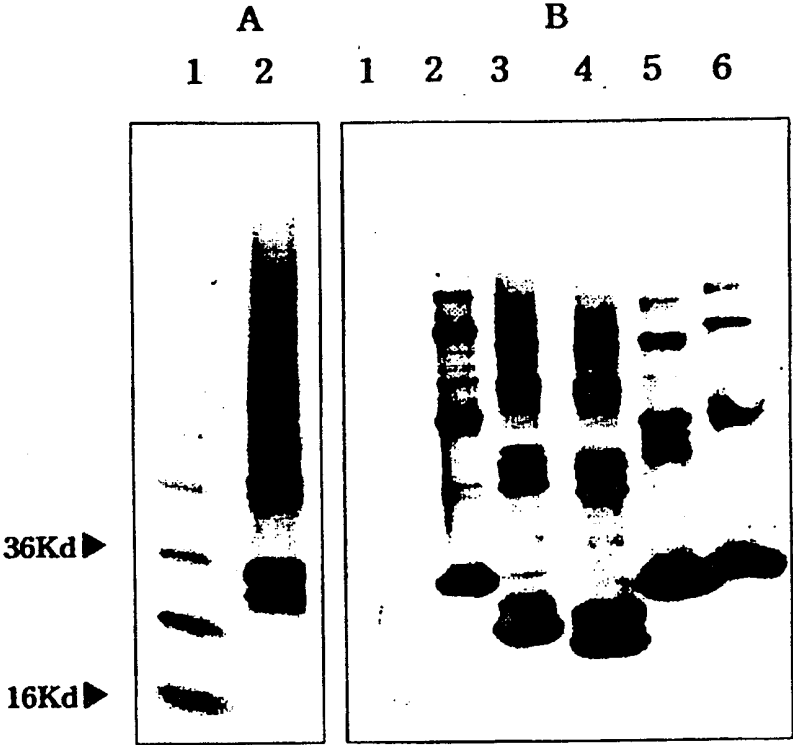


FIG. 11



SEQUENCE LISTING

<110> Korea Research Institute of Bioscience and Biotechnology et al.

<120> Preparation of Peptides by Use of Human Glucagon Sequence as a Fusion Expression Partner

<130> Ofpo-02-06

<160> 3

<170> KOPATIN 1.0

<210> 1

<211> 29

<212> PRT

<213> Homo sapiens

<400> 1

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1 5 10 15

Arg Arg Ala Gln Asp Phe Val Gln Trp Leu Met Asn Thr
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<212> DNA

<213> Artificial Sequence

<220>

<223> modified DNA sequence for human glucagon

<400> 2

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<213> Artificial Sequence

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR00/00187

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/70, C12N 15/62, C12N 1/21

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)

IPC7 C12N 15/70,

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)

"glucagon, fusion-partner, protein, expression, production " NCBI pubmed, USPTO-US, EP, WO patent databases, IBM patent database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6,033,877 A (Research Foundation of State University of New York) 7 March 2000 (7. 03. 2000)	1-9
A	US 5,686,268 A (Pfizer Inc.) 11 Nov. 1997 (11. 11. 97.)	1-9
A	US 5,670,340 A (Suntory Limited) 23 Sept 1997 (23. 09. 97)	1-9
A	US 5,506,120 A (M & D Research Co., Ltd) 09 Apr. 1996 (09. 04. 96)	1-9

☐ 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

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