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(54) PREPARATION METHOD FOR THE PRODUCTION OF ACTIVE AND SOLUBLE PROTEINS IN PROKARYOTES AND POLYCISTRONIC VECTORS THEREFOR

HERSTELLUNGSVERFAHREN ZUR PRODUKTION AKTIVER UND LÖSLICHER PROTEINE IN PROKARYONTEN UND POLYCISTRONISCHE VEKTOREN DAFÜR

PROCEDE PERMETTANT DE PRODUIRE DES PROTEINES ACTIVES ET SOLUBLES DANS DES CELLULES PROCARYOTES ET VECTEURS POLYCISTRONIQUES CORRESPONDANTS

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Description

Technical Field

[0001] The present invention relates to a method of producing a target protein in biologically-active, soluble forms in prokaryotes, and polycistronic vectors therefor.

Background Art

[0002] The production system of heterogeneous proteins using recombinant *E. coli* ensures rapid cellular growth rates and high density cultures using inexpensive substrates and uses relatively well-identified genes in comparison with cases using other organism, thereby making it possible to design various vector systems for facilitating the high expression and purification of heterogeneous proteins (Jeffrey G. T. and Amanda A. et al, (1997), Applied Biochemistry and Biotechnology 66, 197-238).

[0003] However, when *E. coli* is used as a host cell for the production of eukaryotic proteins, *E. coli* cannot perform post-translational modification such as glycosylation because it does not possess intracellular factors required for protein maturation. In addition, when a heterogeneous protein is expressed in high levels, it is often accumulated in the form of inclusion bodies, which are insoluble precipitates.

[0004] Inclusion bodies are typically formed by interaction between hydrophobic surfaces of folding intermediates of a target protein due to imbalance between the production rate and the folding rate of the target protein. In this case, inclusion bodies may be easily isolated, be typically less affected by proteinases and be accumulated in high concentrations in cells, thereby securing high yields and easy isolation of a target protein. Due to these advantages, the strategy of expressing a protein as inclusion bodies is utilized in the production of proteins unfavorable for in vivo folding. However, a target protein expressed as inclusion bodies requires an additional refolding process to recover its biological activity. The refolding of a target protein to an active form is dependent on experience, and is thus always not successful and makes it difficult to scale up the production of recombinant proteins in industrial scales. In addition, high molecular weight antibody proteins, tissue plasminogen activator (tPA) and factor VIII are very difficult to produce in active forms by a refolding process.

[0005] As described above, since inclusion body proteins should be refolded to have their structure and biological activity intact (Andrew D. Guise, Shauna M. West, and Julian B. Chaudhuri (1996), Molecular Biotechnology 6, 53-64), a target protein is expressed as a soluble protein using the so-called "in vivo protein folding technique" to induce its correct three-dimensional structure formation in vivo. Since this technique improves problems caused when a heterogeneous protein is expressed as inclusion bodies, it has an industrial importance in producing heterogeneous proteins in *E. coli.*

[0006] The following three strategies are typically used for in vivo folding of proteins.

[0007] The first strategy involves the control of protein expression sites and culture environments. When a target protein is designed to be expressed in the cytoplasm, although the target protein is harmful to cells, the cells are not damaged, and the protein is mostly expressed in very high levels. Also, this method facilities the preparation of expression vectors. As another method, the secretion of a target protein to the periplasm has advantages of simplifying protein purification and, compared to the method of expressing a protein in the cytoplasm, reducing protein degradation by proteinases and making disulfide bonding possible to some degree due to a relatively oxidative environment. The advantages further include that an authentic protein can be obtained by removing an N-terminal secretory signal. However, a secreted protein may be aggregated, resulting in formation of inclusion bodies, and reduced folding may occur. In a further method, the secretion of a target protein to culture media may solve the problems associated with protein folding and degradation by proteinases. However, *E. coli* rarely secretes proteins to culture media, and, even when proteins are secreted to media, proteins are greatly diluted, thus making purification rather difficult. This method is effective only in particular proteins and is thus not a generalized method to prevent inclusion bodies from being formed. Also, the fermentation control is frequently used to increase a soluble protein, and, in most cases, is the most economical method (Korean Pat. Application No. 1997-50023). The reduction of culture temperature is not applied to all proteins, but is a very effective method in many cases because it typically leads to decrease the production rate of a protein below the folding rate of the protein, resulting in no accumulation of folding intermediates with strong aggregation to each other (Schein, C. H. and M. H. M. Noteborn (1988), Biotechnology 6, 291-294; More, J. T., Uppal, F. Maley and G. F. Maley (1993), Protein. Expr. Purif. 4, 160-163).

[0008] The second strategy involves the co-expression of chaperones and protein foldases. The chaperones refer to proteins that function to help formation of the desired three-dimensional structure of protein and prevent unnecessary intermolecular or intramolecular interactions. Chaperone proteins derived from *E. coli* include GroEL, GroES, DnaK, HtpG, SecB and PapD, which protect folding intermediates and prevent aggregation and precipitation, and all of the *E. coli* chaperone proteins except for PapD (present in the periplasmic membrane) are present in the cytoplasm (Korean

Pat. Application No. 2003-7008657; Hartl, F. U., R. Holdan and T. Langer (1994), Trends Biochem. Sci. 19, 20-25; Bernadea-Clark, E. and G. Georgiou (1994), American Chem. Soc. Symp. Ser. Vol 470, ACS). Foldases refer to an auxiliary protein family that serves to facilitate covalent boding or isomerization during folding. Enzymes stimulating the disulfide bond formation of proteins include DsbA, DsbB, DsbC and DsbD (Creighton, T. E., A. Zapun and N. J. Darby (1995), TIBTECH. 13, 18-27; Gottesman, M. E. and W. A. Hendrickson (2000. Curr. Opin. Microbiol. 3, 197-202).

[0009] The third strategy involves the use of fusion proteins. Many proteins have been developed as fusion proteins, which include glutathione-S-transferase, maltose-binding protein, Protein A, tumor necrosis factor-α and lysyl-tRNA synthetase (Smith, D. B. and Johnson, K. S. (1988), Gene 67, 31-40.; Bedouelle, H. and Duplay, P. (1988), Euro. J. Biochem. 171, 541-549.; Nisson, B. et al. (1987), Prot. Eng. 1, 107-113; Korean Pat. Application No. 1996-44010). Also, as described in U.S. Pat. No. 6,027,888, a soluble eukaryotic protein having disulfide bonds can be produced by being expressed in a fused form with disulfide isomerase. In addition, as described in Korean Pat. Application No. 2002-0040497, an H-chain human ferritin protein can be produced as a soluble fusion protein with a L-chain human ferritin protein that is expressed in an insoluble form in *E. coli.* As described above, various attempts were made to express heterogeneous proteins in soluble fusion protein forms. However, the fusion effect varies according to the type of fusion proteins, as follows: fusion proteins are expressed as inclusion bodies; only a portion of them are expressed as soluble forms; and a protein fused with a target protein functions to aid the folding of the target protein (Savvas C. Makrides (1996), Microbiological Review, 512-538).

[0010] Thus, there is an urgent need for techniques allowing the high level production of biologically-active, soluble recombinant proteins in high efficiency and high concentrations.

Disclosure of the Invention

[0011] Based on the above background, the present inventors intended to develop a novel vector system capable of producing in high levels a heterogeneous protein expressed as a biologically active form (but not inclusion bodies) in prokaryotes instead of finding fusion proteins useful for producing proteins by recombinant DNA technology.

[0012] As a result, the present inventors found that an expression vector system based on the polycistronic expression of a gene encoding a target protein and a beta-lactamase gene highly expresses both the target protein and beta-lactamase in prokaryotes, resulting in the expression of the target protein in a soluble form in a higher percentage, and is thus effective in the mass production of proteins. Using the established protein expression system, the present inventors developed a method of mass-producing a target protein in a biologically active form, thereby leading to the present invention.

[0013] It is therefore an object of the present invention to provide a method of producing a target protein expressed as a biologically active, soluble form instead of inclusion bodies, in prokaryotic cells.

[0014] It is another object of the present invention to provide a polycistronic vector system for producing the above target protein in a biologically active form.

Brief Description of the Drawings

[0015] The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

FIG. 1 is a diagram for a process of preparing an expression vector carrying a human growth hormone gene, pTT191;

FIG. 2 is a photograph showing a result of analysis for human growth hormone expression on a SDS-PAGE gel after *E. coli* BL21 (DE3) was transformed with a pTT191 expression vector (lane 1: protein size marker; lane 2: standard of human growth hormone; lane 3: supernatant obtained by disrupting the IPTG-induced transformant; and lane 4: pellet obtained by disrupting the IPTG-induced transformant);

FIG. 3 is a diagram for a process of preparing pT0191 and pT0-CSF expression vectors respectively carrying a human growth hormone (hGH) gene and a human granulocyte-colony stimulating factor (G-CSF) gene;

FIG. 4 is a photograph showing a result of analysis for human growth hormone expression on a SDS-PAGE gel after *E. coli* BL21 (DE3) was transformed with a pT0191 expression vector (lane 1: protein size marker; lane 2: standard of human growth hormone; lane 3: whole proteins obtained by disrupting the IPTG-induced transformant; lane 4: supernatant obtained by disrupting the IPTG-induced transformant; and lane 5: pellet obtained by disrupting the IPTG-induced transformant);

FIG. 5 is a construct of a pTR0191 prepared by inserting a human growth hormone (hGH) gene into an expression vector carrying a beta-lactamase gene in a reverse-orientation to the hGH gene;

FIG. 6 is a photograph showing a result of analysis for human growth hormone expression on a SDS-PAGE gel after *E. coli* BL21 (DE3) was transformed with a pTR0191 expression vector (lane 1: protein size marker; lane 2: standard of human growth hormone; lanes 3 and 4: pellets obtained by disrupting the IPTG-induced transformant;

and lane 5 and 6: supernatants obtained by disrupting the IPTG-induced transformant);

FIG. 7 is a photograph showing a result of analysis for human G-CSF expression on a SDS-PAGE gel after *E. coli* BL21Star(DE3)pLysS was transformed with a pT0-CSF expression vector (lane 1: protein size marker; lane 2: supernatant obtained by disrupting the transformant before IPTG induction; lanes 3 and 4: supernatants obtained by disrupting the IPTG-induced transformant; and lane 5 and 6: pellets obtained by disrupting the IPTG-induced transformant);

FIG. 8 is a photograph showing a result of analysis for interferon-α2b expression on a SDS-PAGE gel after *E. coli* BL21(DE3) was transformed with a pT0-IFN expression vector (lane 1: protein size marker; lane 2: pellet obtained by disrupting the IPTG-induced transformant; and lane 3: supernatants obtained by disrupting the IPTG-induced transformant);

FIG. 9 is a photograph showing a result of analysis for basic fibroblast growth factor expression on a SDS-PAGE gel after *E. coli* BL21 (DE3) was transformed with a pT0-bFGF expression vector (lane 1: protein size marker; lane 2: pellets obtained by disrupting the IPTG-induced transformant; and lane 3: supernatants obtained by disrupting the IPTG-induced transformant);

FIG. 10 is a photograph showing a result of analysis for insulin-like growth factor-1 expression on a SDS-PAGE gel after *E. coli* BL21(DE3) was transformed with a pTO-IGF1 expression vector (lane 1: protein size marker; lane 2: pellet obtained by disrupting the IPTG-induced transformant; and lane 3: supernatant obtained by disrupting the IPTG-induced transformant);

FIG. 11 is a photograph showing a result of analysis for insulin-like growth factor-2 expression on a SDS-PAGE gel after *E. coli* BL21(DE3) was transformed with a pT0-IGF2 expression vector (lane 1: protein size marker; lane 2: pellet obtained by disrupting the IPTG-induced transformant; and lane 3: supernatant obtained by disrupting the IPTG-induced transformant);

FIG. 12 is a photograph showing a result of analysis for keratinocyte growth factor expression on a SDS- PAGE gel after *E. coli* BL21(DE3) was transformed with a pT0-KGF expression vector (lane 1: protein size marker; lane 2: pellets obtained by disrupting the IPTG-induced transformant; and lane 3: supernatant obtained by disrupting the IPTG-induced transformant); and

FIG. 13 is a photograph showing a result of analysis for keratinocyte growth factor expression on a SDS-PAGE gel after *E. coli* BL21(DE3) was transformed with a pT0N-KGF expression vector (lane 1: protein size marker; lane 2: pellets obtained by disrupting the IPTG-induced transformant; and lane 3: supernatants obtained by disrupting the IPTG-induced transformant).

Best Mode for Carrying Out the Invention

[0016] In one aspect, the present invention relates to a method of producing an active, soluble target protein in a prokaryotic cell, which is based on expressing the target protein and beta-lactamase as a first cistron and a second cistron, respectively, in a polycistron.

[0017] The present inventors found that, when human growth hormone expressed as inclusion bodies in prokaryotic cells and beta-lactamase were polycistronically expressed, both human growth hormone and beta-lactamase were produced in high concentrations, and the expressed human growth hormone was present in an active, soluble form. In contrast, when basic fibroblast growth factor and keratinocyte growth factor were expressed using kanamycin instead of beta-lactamase under same conditions, the target proteins were mostly expressed as inclusion bodies. Based on this finding, the present inventors polycistronically coexpressed various target proteins that are expressed in the form of inclusion bodies in prokaryotic cells but have medical usefulness, with beta-lactamase. As a result, the target proteins in this system were produced in active, soluble forms.

[0018] Thus, in another aspect, the present invention relates to a polycistronic vector to produce a heterogeneous proteins in an active, soluble form.

[0019] In an embodiment, the present invention relates to a polycistronic vector for expressing target proteins in an active, soluble form in a prokaryotic cell, which comprises (i) a promoter operable in the prokaryotic cell, (ii) a first cistron including a DNA sequence encoding the target protein, and (iii) a second cistron including a DNA sequence encoding beta-lactamase.

[0020] The term "polycistron", as used herein, refers to a system where a single mRNA is synthesized from an same promoter, cistrons are separated from each other by a termination codon and an initiation codon, a ribosome binding site is present for each cistron, and proteins corresponding to each cistron are simultaneously expressed from the single mRNA transcribed in a single promoter. Herein, the "cistron" means a nucleotide sequence encoding for a single protein or polypeptide, and includes a 5' initiation codon and a 3' termination codon. In addition, the first and second cistrons do not mean the sequence in a DNA sequence but only indicate an individual cistron.

[0021] In a preferred aspect, in the polycistron of the present invention, the first cistron including a DNA sequence encoding a target protein may be in a 5' to 3' direction operably linked to the second cistron including a DNA sequence

encoding beta-lactamase, or the second cistron including a DNA sequence encoding beta-lactamase may be in a 5' to 3' direction operably linked to the first cistron including a DNA sequence encoding a target protein

[0022] The term "vector", as used herein, refers to a DNA construct that contains a DNA sequence operably linked to a suitable regulatory sequence capable of expressing DNA in a suitable host, and, in detail, may be constructed to contain a promoter sequence, a terminator sequence, a marker gene and other suitable sequences including a suitable regulatory sequence. Such a vector may be a plasmid, a pharge, a cosmid, or the like (Molecular Cloning: Laboratory Mannual second edition, Sambrook et al., Cold Spring Harbor Laboratory Press (1989)). The preparation of such a vector, mutagenesis, sequence analysis, DNA introduction into cells, gene expression and protein analysis are described in detail in Current Protocols in Molecular Biology, edited by Ausubel et al., John Wiley & Sons (1992). When introduced into a suitable host, a vector may be replicated or function independently of the host genome, or, in some cases, may be integrated into the host genome. Plasmids are at present the most common form of vectors, and, in the present invention, the terms "plasmid" and "vector" may be used interchangeably. With respect to the objects of the present invention, a vector is a vector suitable for protein expression in prokaryotic cells, and is a polycistronic vector polycistronically expressing a heterogeneous target protein and beta-lactamase.

[0023] The term "operably linked", as used herein, means that an expression regulatory sequence is linked in such a way of regulating the transcription and translation of a polynucleotide sequence encoding a target protein, and includes maintaining a precise translation frame in such a way that a polypeptide of a target protein encoded by a polynucleotide sequence is produced when the polypeptide sequence is expressed under the control of regulatory sequences (including a promoter).

[0024] The term "promoter", as used herein, means a minimum sequence sufficient for triggering transcription. With respect to the objects of the present invention, a promoter inducible by an external signal or an effector is used. Promoters useful for the expression of a target protein in prokaryotic cells include T7, tac, trc, lac, lpp, phoA, recA, araBAD, proU, cst-1, tetA, cadA, nar, lpp-lac, starvation promoters, cspA, T7-lac operator, T3-lac operator, T5-lac operator, T4 gene 32, and nprM-lac operator. Preferred are T7, tac, lac, T7-lac operator, T3-lac operator, T5-lac operator and T4 gene 32, and more preferred are T7, tac and T7-lac operator. The most preferred promoter is T7 promoter. T7 promoter can be controlled by T7 RNA polymerase and the expression of a T7 RNA polymerase can be controlled by IPTG(isopropyl-β-D-thiogaloactosidase). T7 promoter can induce expression of a target protein in a desired time using IPTG. This is because it is preferable that a prokaryotic host cell, for example, *E. coli* is grown until a cell number is increased while a target protein is not expressed, and, after the *E. coli* cell number is sufficiently increased, the expression of the target protein is induced.

[0025] A ribosome binding site is typically located in about 10 bp upstream of an initiation codon, and functions to precisely and effectively initiate mRNA translation in polycistronic operon systems of phages or prokaryotes.

[0026] A target protein to be expressed using the polycistronic vector of the present invention may include all proteins having medical applications. In particular, proteins having demands for medical purposes but being known to be produced as inclusion bodies upon high expression in host cells by genetic engineering are suitable as the target protein of the present invention. Examples of the target protein include human growth hormone (hGH), granulocyte-colony stimulating factor (G-CSF), interferons (IFN), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), keratinocyte growth factor (KGF), erythropoietin (EPO), thrombopoietin (TPO), human epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), transforming growth factor (TGF), tumor necrosis factor (TNF), angiogenin, angiotensin, interleukin (IL), and tissue plasminogen activator (tPA). More preferred are hGH, G-CSF, IFN-α2b, bFGF, IGF-1, IGF-2, KGF, EPO, IL-7 and TPO. These target proteins may be in a natural or modified form, and may include their variants including deletions, substitutions or additions of the whole sequence or its fragment. In another embodiment of the present invention, hGH, G-CSF, IFN-α2b, bFGF, IGF-1, IGF-2 and KGF were expressed.

[0027] The target protein to be expressed in the present invention may be present itself, or may be present in the form of a fusion protein, such as a fused form with a sequence increasing solubility, to facilitate purification, provide various functions by being fused with an antibody or enzyme, or increase solubility. When the target protein contains a sequence facilitating, for example, purification, the target protein may be expressed in the form of a fusion protein with such a sequence. Such a fused target protein may be present in a sequence of a fusion partner-a peptide linker-a target protein, but may be prepared in various organizations according to the types of target proteins and fusion partners. More preferably, the fusion partner is employed for facilitating the purification of a produced protein, and is exemplified by histidine-tag, glutathione-S-transferase, maltose-binding protein, protein A, protein G, flag peptide, thioredoxin, S-peptide, avidin, streptavidin, galactose binding protein, cellulose-binding domain, chitin-binding domain, polyarginine, polycysteine and polyphenylalanine. In a further embodiment of the present invention, a histidine tag containing ten histidines was used. The peptide linker linking the target protein to such a fusion partner includes a sequence recognizable by a proteinase, and is exemplified by enterokinase, thrombin, factor Xa, urokinase, TEV protease and subtilisin, which have a high sequence specificity.

[0028] The term "active", as used herein, refers to a soluble protein that has biological activity by being stably expressed

in a transformant with a recombinant vector and being folded into a native form without additional denaturation or refolding.

[0029] The term "soluble", as used herein, means the nature with which a protein is not easily precipitated in an aqueous solution and does not easily form inclusion bodies or other aggregates.

[0030] The beta-lactamase (bla) used in the present invention, as a factor for selecting a host cell transformed with an expression vector, is a protein providing a resistance to ampicillin. In the polycistronic vector of the present invention, the arrangement of a cistron encoding a target protein and another cistron encoding beta-lactamase may be changed by a certain purpose, but the cistron encoding beta-lactamase is preferably located in the downstream region of the cistron encoding a target protein.

[0031] pT0 expression vectors used in embodiments of the present invention are vectors in which a fused target protein gene (fusion partner-peptide linker-target protein gene) or a target protein gene itself is operably linked to the downstream region of T7 promoter of pET3a, and which overexpresses the target protein and beta-lactamase when the fused target protein gene(or the target protein gene) and a beta-lactamase gene are transcribed under the control of an same promoter. The pT0 expression vectors prepared in the present invention include pT0191, pT0-CSF pT0-IFN, pT0-bFGF, pT0-IGF1, pT0-IGF2, pT0-KGF and pT0N-KGF, which each carry fused hGH, G-CSF, IFN-α2b, bFGF, IGF-1, IGF-2 and KGF genes, and a non-fused KGF gene. When these vectors were expressed, most of the target proteins were expressed in soluble, active forms in comparison with a control.

[0032] On the other hand, the control expression vector of pTT used in embodiments of the present invention are vectors in which a fused target protein gene (fusion partner-peptide linker-target protein gene) is operably linked to the downstream region of T7 promoter of pET3a, but in which the fused target protein gene and a beta-lactamase gene are expressed under the control of different promoters. *E. coli* transformed with such a vector overexpressed the fused target protein in vivo, but a large quantity of the fusion protein was expressed as inclusion bodies (Example 2). Another control vector, pTR0191, is a plasmid prepared by converting a beta-lactamase gene to a reverse orientation in a pT0191 expression vector and operably linking a fused human growth hormone gene to the downstream region of T7 promoter, and in which the target protein and the beta-lactamase gene are not located under the control of the same promoter. When the pTR0191 was expressed in a host cell, the fusion protein was mostly expressed as inclusion bodies, and beta-lactamase was expressed in low levels (Example 5).

[0033] Thus, in one detailed aspect, to simultaneously overexpress beta-lactamase and a target protein itself or a target fusion protein and express the target protein in an active form in a higher percentage, the present invention provides polycistronic vectors, pT0191, pT0-CSF, pT0-IFN, pT0-bFGF, pT0-IGF1, pT0-IGF2, pT0-KGF and pT0N-KGF, which carry respectively genes encoding hGH (SEQ ID NO. 5), G-CSF (SEQ ID NO. 7), IFN-α2b (SEQ ID NO. 9), bFGF (SEQ ID NO. 11), IGF-1 (SEQ ID NO. 13), IGF-2 (SEQ ID NO. 15) and KGF (SEQ ID NO. 23), which are fused to a pT0 expression vector derived from a pET3a expression vector, and non-fused KGF (SEQ ID NO. 25). Of them, the pT0191 and pT0-IFN are introduced into *E.coli* BL21(DE3), and the pT0-CSF is introduced into *E.coli* BL21 Star(DE3)pLysS. The resulting transformants were deposited at KCTC (Korean Collection for Type Cultures; KRIBB, 52, Oun-dong, Yusong-ku, Taejon, Korea) on March 11, 2004, under accession numbers KCTC-10610BP, KCTC-10612BP and KCTC-10611BP, respectively.

[0034] The polycistronic expression vectors of the present invention may be introduced into a host cell to transform the host cell by certain methods known in the art, including chemical methods using $CaCl_2$ and electroporation.

[0035] The term "transformed", as used herein, refers to introduction into a prokaryotic cell in such a manner as to allow a gene carried by the polycistronic vector to be expressed.

[0036] If a recombinant nucleotide sequence of a fusion protein is suitably transcribed to mRNA in a cell, and the cell is able to express proteins, a certain prokaryotic cell can be used, and a Gram-negative bacterium, *E. coli,* and a Gram-positive bacterium, *Bacillus*, are preferred. More preferred is *E. coli,* and most preferred are *E. coli* BL21 (DE3), *E. coli* BL21 Star (DE3)pLysS, *E. coli* HMS (DE3) and *E. coli* AD494 (DE3). The above host cells possess bacteriophage T_7 RNA polymerase, and the present invention is not limited to the examples. The bacteriophage-derived T7 promoter used in the present invention is more effectively expressed by bacteriophage T_7 RNA polymerase than by *E. coli* RNA polymerase (Studier FW et al. (1990), Method Enzymol. 185, 60-89). Thus, the pT0 expression vectors are preferably expressed by being introduced into *E. coli* BL21 (DE3) or *E. coli* BL21 Star (DE3) pLysS which carries T_7 RNA polymerase gene under control of the lacUV5 promoter. When the pT0-CSF expression vector of the present invention is introduced into *E. coli* BL21 (DE3), BL21 Star (DE3) pLysS, HMS (DE3) or AD494(DE3), a target fusion protein may be expressed in an active form with an efficiency of 70% or higher.

[0037] Thus, in still another aspect, the present invention provides transformants transformed with the above polycistronic expression vectors. In detail, the transformants include *E. coli* transformed with pT0191, pT0-CSF, pT0-IFN, pT0-bFGF, pT0-IGF1, pT0-IGF2, pT0-KGF or pT0N-KGF.

[0038] The transformants transformed with the expression vectors according to the present invention are cultured in suitable media under suitable conditions in a manner of allowing a DNA sequence encoding a target protein to be expressed. A method of expressing a recombinant protein by culturing a transformant is known in the art. For example, a transformant is inoculated in a suitable medium for seed culture, and the seed culture is inoculated in a production

culture medium and grown under suitable conditions, thereby inducing protein expression. In the production culture, microbial growth is performed separately from the induction of recombinant protein expression, thereby increasing recombinant protein yield.

[0039] Thus, in still another aspect, the present invention provides a method of producing an active, soluble protein, comprising culturing the transformant and recovering a soluble target protein from a culture.

[0040] From the culture obtained by culturing a transformant, a target protein is recovered in substantially pure forms, and thus can be used for medical purposes. The recovery of a recombinant protein may be achieved by various isolation and purification methods known in the art. Typically, to remove cell debris, a cell lysate is centrifuged, and the supernatant is subjected to precipitation, dialysis and various column chromatographies. Examples of the column chromatography include ion exchange chromatography, gel-filtration chromatography, HPLC, reverse phased HPLC, preparative SDS-PAGE, and affinity column chromatography.

[0041] The purification of the soluble, active protein according to the present invention may be achieved by typical purification methods such as ultrafiltration and ion exchange chromatography without a refolding process after cell disruption and centrifugation, thereby facilitating the isolation of an active target protein.

[0042] A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as the limit of the present invention.

EXAMPLE 1: Construction of pTT191 expression vector

[0043] A control expression vector pTT191 was prepared to express in high yields a fusion protein containing human growth hormone as inclusion bodies.

[0044] A fusion gene encoding a fusion protein (SEQ ID NO. 5) containing human growth hormone, which is linked to a histidine-tag and an enterokinase recognition sequence, was prepared by a PCR ligation method (PCR ligation method, Willem P.C. Stemmer, and Herbert L. Heyneker (1995) Gene 164, 49-53; Scott W. Altmann, and Robert A. Kastelein (1995) Protein Expression and Purification 6, 722-726; Ana Paula de Mattos Areas, and Paulo Lee Ho (2002) Protein Expression and Purification 25, 481-487). The PCR ligation method was carried out as follows. To a PCR tube, 50 pmole of each of pairs of synthetic oligonucleotides containing twenty complementarily overlapped bases, 2.5 U (1 μl) of Pfu DNA polymerase (Stratagene, USA), 2 μL of 2.5 mM dNTPs (Takara, Japan), and 2 μl of 10x Pfu polymerase buffer were sequentially added, and sterile distilled water was added to a final volume of 20 μl. PCR was carried out using a PCR machine (MJ research, USA). Herein, each of the oligonucleotides served as a template as well as a primer. PCR conditions included denaturation at 94°C for 5 min, and 20 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 30 sec and elongation at 72°C for 30 sec, followed by final elongation at 72°C for 10 min. Two PCR products having a complementary nucleotide sequence (20 bp) at ends were amplified. To a PCR tube, 5 μl of each of the PCR products, 2.5 U (1 μl) of Pfu DNA polymerase (Stratagene, USA), 2 μl of 2.5 mM dNTPs (Takara, Japan), and 2 μl of 10x Pfu polymerase buffer were sequentially added, and sterile distilled water was added to a final volume of 20 μl. PCR was carried out using a PCR machine (MJ research, USA). PCR conditions included denaturation at 94°C for 5 min, and 20 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 30 sec and elongation at 72°C for 30 sec, followed by final elongation at 72°C for 10 min. This procedure was repeated, and, in a final step, PCR was carried with 30 cycles, thus generating a synthetic gene. The synthesized gene was run on a 1% agarose gel and isolated from the gel using QIAQuick gel extraction kit (Qiagen, USA). As a result, a fused human growth hormone (somatotropin) gene (SEQ ID NO. 5) was obtained, which included a histidine-tag (SEQ ID NO. 1) and an enterokinase recognition sequence (SEQ ID NO. 3) and an Nde I recognition sequence at both ends. Thereafter, the pTT191 expression vector was constructed as shown in FIG. 1. The synthesized fusion gene was digested with NdeI, separated on a 1% agarose gel, and isolated from the gel. The linearized fusion gene was ligated to NdeI-digested pET3a (Novagen, USA) that was also pretreated with CIAP (calf intestine alkaline phosphatase; NEB, USA). The CIAP treatment was carried out at 37°C for one hour to prevent self-ligation of the NdeI-digested pET3a. The ligation was carried out at 16°C for 18 hrs using T4 DNA ligase (NEB, USA), thus generating pTT191. Then, *E. coli TOP10* (Invitrogen, USA) was transformed with the pTT191. The plasmid DNA was prepared from the resulting transformant and introduced into *E.coli* BL21 (DE3) (Novagen, USA). The resulting *E.coli* BL21 (DE3) transformant transformed with the pTT191 expression vector was selected on ampicillin-containing LB plates, and designated as "*E.coli* BL21 (DE3)/pTT191". The correct insertion of the fusion gene containing a human growth hormone gene in the pTT191 expression vector was confirmed by digestion with restriction enzymes AlwNI and HindIII and DNA sequencing.

EXAMPLE 2: Expression of the fusion protein with human growth hormone in the *E.coli* BL21 (DE3)/pTT191 transformant

[0045] The expression pattern of the fusion protein with human growth hormone was tested in *E. coli* transformed with the control expression vector pTT191.

[0046] The *E.coli* BL21 (DE3)/pTT191 transformed with the pTT191 expression vector was cultured in LB medium

(Luria-Bertani medium) at 30°C for 12 hrs, and the expression of the fusion protein was then induced with IPTG (Isopropyl-μ-D-Thiogalactopyranoside). After IPTG induction, cells were collected by centrifugation and disrupted. After centrifugation, the supernatant was used to investigate the expression of the fusion protein. As a result, as shown in FIG. 2, the fusion protein containing human growth hormone had the predicted molecular weight of about 24 kDa, but mainly expressed as inclusion bodies.

EXAMPLE 3: Construction of pT0191 expression vector

[0047] A pT0191 expression vector was prepared to express the fusion protein containing human growth hormone in a soluble form in a higher percentage.

[0048] To insert a gene encoding the fusion protein with human growth hormone into a pET3a vector, PCR was carried out using pTT191 as a template, thus providing an NdeI recognition site and a HindIII recognition site to each end of a nucleotide sequence encoding the fusion protein. To a PCR tube, 100 ng of pTT191 plasmid (Example 1) as a template, 2.5 U (1 μl) of Pfu DNA polymerase (Stratagene, USA), 30 pmole of primer A (5'-AAACATATGGGCCATCATCATCATCATCATCATCATCAC-3': SEQ ID NO. 19), 30 pmole of primer B (5'-AAAAAGCTTTTACTAGAAGCCACAGCTGCC-3': SEQ ID NO. 20), 2 μl of 2.5 mM dNTPs (Takara, Japan), and 2 μl of 10x Pfu polymerase buffer were sequentially added, and sterile distilled water was added to a final volume of 20 μl. PCR was carried out using a PCR machine (MJ research, USA). PCR conditions included denaturation at 94°C for 5 min, and 30 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 30 sec and elongation at 72°C for 2 min, followed by final elongation at 72°C for 10 min. The amplified gene was digested with NdeI and HindIII restriction enzymes, separated on a 1% agarose gel and purified from the gel. The pET3a expression vector was digested with NdeI and HindIII and separated on a 1% agarose gel, and a 4119-bp fragment was purified from the gel. The NdeI/HindIII-treated fusion gene and pET3a fragment were ligated to each other at 16°C for 18 hrs using T4 DNA ligase, thus generating pT0191. Then, *E. coli TOP10* (Invitrogen, USA) was transformed with the pT0191 (FIG. 3). The plasmid DNA was prepared from the resulting transformant and introduced into *E.coli* BL21 (DE3). The resulting *E.coli* BL21 (DE3) transformant transformed with the pT0191 expression vector was selected on ampicillin-containing LB plates, and designated as *"E.coli* BL21 (DE3)/pT0191 (KCTC10610BP)". The correct insertion of the fusion gene containing a human growth hormone gene in the pT0191 expression vector was confirmed by digestion with restriction enzymes NdeI and HindIII and DNA sequencing.

EXAMPLE 4: Expression of the fusion protein with human growth hormone in the *E. coli* BL21(DE3)/pT0191 transformant

[0049] The expression pattern of the fusion protein with human growth hormone was tested in *E. coli* transformed with the pT0191 expression vector.

[0050] The *E.coli* BL21 (DE3)/pT0191 transformed with the pT0191 expression vector was cultured in LB medium at 30°C for 12 hrs, and the expression of the fusion protein was then induced with IPTG. Thereafter, the expression of the fusion protein was estimated. As shown in FIG. 4, the fusion protein mainly expressed in an active form and was present in a centrifuged supernatant, and had a molecular weight of about 24 kDa. Unlike the *E.coli* BL21 (DE3)/pTT191 transformant, the *E.coli* BL21 (DE3)/pT0191 transformant was found to overexpress the target fusion protein along with beta-lactamase. The expression of beta-lactamase was confirmed by N-terminal sequencing.

EXAMPLE 5: Construction of pTR0191 expression vector and expression of human growth hormone in *E. coli* BL21 (DE3)/pTR0191 transformant

[0051] The pT0191 plasmid prepared in Example 3 was digested with SphI and HindIII, and a 3812-bp fragment was purified. A gene encoding beta-lactamase contained in the pT0191 plasmid was amplified by PCR using two primers (primer 1: 5'-AAAAAGCTTAAGGAGATGGCGCCCA-3' (SEQ ID NO. 21); primer 2: 5'-AAAGCATGCCTAGAAGCCACAGCTG-3' (SEQ ID NO. 22)), thus generating a 950-bp fragment in which the positions of the SphI and HindIII sites were exchanged with each other. Then, the 950-bp fragment was ligated to the 3812-bp fragment using T4 DNA ligase, thus generating a pTR0191 expression vector in which the human growth hormone gene had a different orientation from the beta-lactamase gene (FIG. 5). *E. coli* By21 (DE3) was transformed with the prepared expression vector, and protein expression was carried out at 30°C. As shown in FIG. 6, the target protein was expressed mainly as inclusion bodies, and the beta-lactamase was expressed in lower levels than the case of using the pT0191 expression vector.

EXAMPLE 6: Construction of pT0-CSF expression vector

[0052] A pT0-CSF expression vector was prepared to express in high yields a fusion protein containing human granulocyte-colony stimulating factor (G-CSF) in a soluble form. A gene (SEQ ID NO. 7) encoding a fusion protein containing

human G-CSF linked to a histidine-tag and an enterokinase recognition sequence was synthesized according to the same PCR ligation method as in Example 1, and the pT0-CSF expression vector was constructed according to the same method as in Example 3 (FIG. 3). The pT0-CSF expression vector was introduced into *E.coli* BL21 Star (DE3) pLysS (Invitrogen, USA), and the resulting transformant was designated as "*E.coli* BL21 Star(DE3)pLysS/pT0-CSF (KCTC10611BP)". The correct insertion of the fusion gene containing a human G-CSF gene into the pT0-CSF expression vector was confirmed by digestion with NdeI and HindIII and DNA sequencing.

EXAMPLE 7: Expression of the fusion protein with human G-CSF in the *E. coli* B21 Star(DE3)pLysS/pT0-CSF transformant

[0053] The expression pattern of the fusion protein with human G-CSF was investigated in *E. coli* transformed with the pT0-CSF expression vector.

[0054] The *E. coli* B21 Star(DE3)pLysS/pT0-CSF transformed with the pT0-CSF expression vector was cultured in LB medium at 30°C for 12 hrs, and the expression of the fusion protein was then induced with IPTG. As shown in FIG. 7, the fusion protein containing human G-CSF was expressed mainly in an active form and present in a centrifugal supernatant, and had a molecular weight of about 20 kDa. Like the *E.coli* BL21 (DE3)/pT0191 transformant, the *E. coli* B21 Star(DE3)pLysS/pT0-CSF transformant transformed with the pT0-CSF expression vector was found to overexpress the target fusion protein containing human G-CSF along with beta-lactamase.

[0055] In addition, the pT0-CSF expression vector was introduced into *E. coli* BL21 (DE3) (Novagen, USA), *E. coli* HMS (DE3) (Novagen, USA) and *E. coli* AD494 (DE3) (Novagen, USA) to investigate the expression pattern of the fusion protein containing human G-CSF (also designated herein as human G-CSF fusion protein) in the *E. coli* strains. The results are given in Table 1, below. As apparent from the data of Table 1, the fusion protein was mainly expressed in an active form.

TABLE 1

Host cell (*E.coli*)	Volume (arbitary unit [a.u.])			Percentage of active form*
	Total	Active form	Inclusion bodies	
BL21 (DE3)	15,043	10,610	4,433	70.5%
BL21 Star (DE3) pLysS	28,169	25,554	2,614	90.7%
HMS (DE3)	13,376	12,361	1,015	92.4%
AD 494 (DE3)	22,843	16,886	5,957	73.9%
*: measured by a densitometer after electorphoresis				

EXAMPLE 8: Construction of pT0-IFN, pT0-bFGF, pT0-IGF1, pT0-IGF2, pT0-KGF and pT0N-KGF expression vectors

[0056] Several expression vectors, pT0-IFN, pT0-bFGF, pT0-IGF1, pT0-IGF2, pT0-KGF and pT0N-KGF, were prepared to express in high yields various target proteins themselves or fusion proteins containing target proteins in soluble forms. Genes coding for an IFN-α2b fusion protein (SEQ ID NO. 9), a bFGF fusion protein (SEQ ID NO. 11), an IGF-1 fusion protein (SEQ ID NO. 13), an IGF-2 fusion protein (SEQ ID NO. 15) and a KGF fusion protein (SEQ ID NO. 23), which each are linked to a histidine-tag and an enterokinase recognition sequence, and a non-fused KGF itself (SEQ ID NO. 25) were prepared according to the same PCR ligation method as in Example 1, wherein primers were designed to provide a NdeI site and a HindIII site at each end of a sequence of each of the coding genes to insert fusion protein into pET3a vector. The amplified genes were digested with NdeI and HindIII, separated on a 1% agarose gel, and purified from the gel. A pET3a expression vector was digested with NdeI and HindIII and separated on a 1% agarose gel, and a 4119-bp fragment was purified from the gel. Each of the NdeI/HindIII-treated fused genes and pET3a fragment was ligated to each other at 16°C for 18 hrs using T4 DNA ligase, thus generating expression vectors, pT0-IFN, pT0-bFGF, pT0-IGF1, pT0-IGF2, pT0-KGF and pT0N-KGF, respectively. Then, *E. coli TOP10* was transformed with the expression vectors. The plasmid DNA was prepared from each of the resulting transformants and introduced into *E.coli* BL21 (DE3). The correct insertion of each gene into the corresponding expression vector was confirmed by digestion with NdeI and HindIII and DNA sequencing.

EXAMPLE 9: Expression of each target protein in *E. coli* BL21 (DE3)/pT0-IFN, pT0-bFGF, pT0-IGF1, pT0-IGF2, pT0-KGF and pT0N-KGF transformants

[0057] The expression pattern of each target protein was investigated in *E. coli* transformed with the expression vectors, pT0-IFN, pT0-bFGF, pT0-IGF1, pT0-IGF2, pT0-KGF and pT0N-KGF.

[0058] The *E.coli* BL21 (DE3) transformants transformed with the above expression vectors were individually cultured in LB medium at 30°C for 12 hrs, and the expression of each target protein was then induced with IPTG. The results are given in FIGS. 8 to 13. As shown in the figures, the expressed target proteins were present in a centrifugal supernatant, thus indicating that the target proteins are expressed in soluble, active forms.

Industrial Applicability

[0059] As described hereinbefore, the present invention provides expression vectors overexpressing a target protein along with beta-lactamase. The expression vectors can produce in high levels a soluble, active form of heterogeneous target proteins in prokaryotic cells where the proteins are mostly expressed as inclusion bodies when other expression vectors are used.

SEQL

[0060]

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<120> Preparation method for the production of active and soluble proteins in prokaryotes and polycistronic vectors therefor

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Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His
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65 70 75 80

Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Ala Trp Asp Glu Thr
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Leu Leu Asp Lys Phe Tyr Thr Glu Leu Tyr Gln Gln Leu Asn Asp Leu
100 105 110

Glu Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met
115 120 125

Lys Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr
130 135 140

Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val
145 150 155 160

Arg Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu
165 170 175

Ser Leu Arg Ser Lys Glu
180

<210> 11
<211> 531
<112> DNA
<213> Artificial Sequence

<220>
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<221> CDS
<222> (7)..(522)

<220>
<221> source
<222> (58)..(522)
<223> Human basic fibroblast growth factor

<400> 11

aaacat atg ggc cat cat cat cat cat cat cat cat cac gac gac 48
Met Gly His His His His His His His His His Asp Asp
1 5 10

gac gac aaa atg gca gcc ggt agc atc acc acg ctg ccg gcc ctg ccg 96
Asp Asp Lys Met Ala Ala Gly Ser Ile Thr Thr Leu Pro Ala Leu Pro
15 20 25 30

gag gat ggc ggc agc ggc gcc ttc ccg ccg ggc cac ttc aag gac ccg 144
Glu Asp Gly Gly Ser Gly Ala Phe Pro Pro Gly His Phe Lys Asp Pro
35 40 45

aag cgt ctg tac tgc aag aac ggt ggc ttc ttc ctg cgc atc cac ccg 192
Lys Arg Leu Tyr Cys Lys Asn Gly Gly Phe Phe Leu Arg Ile His Pro
50 55 60

gac ggc cgt gtt gac ggt gtc cgt gag aag agc gac cct cac atc aag 240
Asp Gly Arg Val Asp Gly Val Arg Glu Lys Ser Asp Pro His Ile Lys
65 70 75

ctg caa ctg caa gca gaa gag cgt ggt gtt gtg tct atc aaa ggt gtg 288
Leu Gln Leu Gln Ala Glu Glu Arg Gly Val Val Ser Ile Lys Gly Val
80 85 90

tgt gct aac cgt tac ctg gct atg aag gaa gat ggt cgt ctg ctg gct 336
Cys Ala Asn Arg Tyr Leu Ala Met Lys Glu Asp Gly Arg Leu Leu Ala
95 100 105 110

tct aaa tgt gtt acg gat gag tgt ttc ttc ttc gaa cgt ctg gaa tct 384
Ser Lys Cys Val Thr Asp Glu Cys Phe Phe Phe Glu Arg Leu Glu Ser
115 120 125

aat aac tac aat act tac cgt tcc cgt aaa tac acc agc tgg tat gtg 432
Asn Asn Tyr Asn Thr Tyr Arg Ser Arg Lys Tyr Thr Ser Trp Tyr Val
130 135 140

gca ctg aaa cgt act ggt cag tat aaa ctg ggt tcc aaa acc ggt cct 480
Ala Leu Lys Arg Thr Gly Gln Tyr Lys Leu Gly Ser Lys Thr Gly Pro
145 150 155

ggt cag aaa gct atc ctg ttt ctg cca atg tct gct aag agc taaaagct 530
Gly Gln Lys Ala Ile Leu Phe Leu Pro Met Ser Ala Lys Ser
160 165 170

t 531

<210> 12
<211> 172
<212> PRT
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<400> 12

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1 5 10 15

Lys Met Ala Ala Gly Ser Ile Thr Thr Leu Pro Ala Leu Pro Glu Asp
20 25 30

Gly Gly Ser Gly Ala Phe Pro Pro Gly His Phe Lys Asp Pro Lys Arg
35 40 45

Leu Tyr Cys Lys Asn Gly Gly Phe Phe Leu Arg Ile His Pro Asp Gly
50 55 60

Arg Val Asp Gly Val Arg Glu Lys Ser Asp Pro His Ile Lys Leu Gln
65 70 75 80

Leu Gln Ala Glu Glu Arg Gly Val Val Ser Ile Lys Gly Val Cys Ala
85 90 95

Asn Arg Tyr Leu Ala Met Lys Glu Asp Gly Arg Leu Leu Ala Ser Lys
100 105 110

Cys Val Thr Asp Glu Cys Phe Phe Phe Glu Arg Leu Glu Ser Asn Asn
115 120 125

Tyr Asn Thr Tyr Arg Ser Arg Lys Tyr Thr Ser Trp Tyr Val Ala Leu
130 135 140

Lys Arg Thr Gly Gln Tyr Lys Leu Gly Ser Lys Thr Gly Pro Gly Gln
145 150 155 160

Lys Ala Ile Leu Phe Leu Pro Met Ser Ala Lys Ser
165 170

<210> 13
<211> 279
<212> DNA
<213> Artificial Sequence

<220>
<223> Human insulin like growth factor-1 fused with histidine tag and enterokinase recognition sequence

<220>
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<222> (7)..(267)

<120>
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<222> (58)..(267)
<223> Human insulin like growth factor-1

<400> 13

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Met Gly His His His His His His His His His Asp Asp
1 5 10

gac gac aaa ggt ccg gaa acc ctg tgc ggc gct gag ctg gtt gac gct 96
Asp Asp Lys Gly Pro Glu Thr Leu Cys Gly Ala Glu Leu Val Asp Ala
15 20 25 30

ctg caa ttc gtt tgc ggt gac cgt ggt ttc tac ttc aac aaa ccg act 144
Leu Gln Phe Val Cys Gly Asp Arg Gly Phe Tyr Phe Asn Lys Pro Thr
35 40 45

ggt tac ggt tcc tct tct cgt cgt gct ccg cag acc ggt atc gtt gac 192
Gly Tyr Gly Ser Ser Ser Arg Arg Ala Pro Gln Thr Gly Ile Val Asp
50 55 60

gaa tgc tgc ttc cgt tct tgc gac ctg cgt cgt ctg gaa atg tac tgc 240
Glu Cys Cys Phe Arg Ser Cys Asp Leu Arg Arg Leu Glu Met Tyr Cys
65 70 75

gct ccg ctg aaa ccg gcg aag tct gct taa tgaaagctt 279
Ala Pro Leu Lys Pro Ala Lys Ser Ala
80 85

<210> 14
<211> 87
<212> PRT
<213> Artificial Sequence

<400> 14

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1 5 10 15

Lys Gly Pro Glu Thr Leu Cys Gly Ala Glu Leu Val Asp Ala Leu Gln
20 25 30

Phe Val Cys Gly Asp Arg Gly Phe Tyr Phe Asn Lys Pro Thr Gly Tyr
35 40 45

Gly Ser Ser Ser Arg Arg Ala Pro Gln Thr Gly Ile Val Asp Glu Cys
50 55 60

Cys Phe Arg Ser Cys Asp Leu Arg Arg Leu Glu Met Tyr Cys Ala Pro
65 70 75 80

Leu Lys Pro Ala Lys Ser Ala
85

<210> 15
<211> 267
<212> DNA
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<220>
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<220>
<221> CDS
<222> (7)..(258)

<220>
<221> source
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<223> Human insulin like growth factor-2

<400> 15

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Met Gly His His His His His His His His His Asp Asp
1 5 10

gac gac aaa gct tac cgc ccg agc gag acc ctg tgc ggc ggt gag ctg 96
Asp Asp Lys Ala Tyr Arg Pro Ser Glu Thr Leu Cys Gly Gly Glu Leu
15 20 25 30

gtg gac acc ctc cag ttc gtc tgt ggt gac cgc ggc ttc tac ttc agc 144
Val Asp Thr Leu Gln Phe Val Cys Gly Asp Arg Gly Phe Tyr Phe Ser
35 40 45

cgt ccg gca agc cgt gtg agc cgt cgc agc cgt ggc atc gtt gag gag 192
Arg Pro Ala Ser Arg Val Ser Arg Arg Ser Arg Gly Ile Val Glu Glu
50 55 60

tgc tgt ttc cgc agc tgt gac ctg gcc ctc ctg gag acg tac tgt gct 240
Cys Cys Phe Arg Ser Cys Asp Leu Ala Leu Leu Glu Thr Tyr Cys Ala
65 70 75

acc ccg gcc aag tcc gag ta aaagctt 267
Thr Pro Ala Lys Ser Glu
80

<210> 16
<211> 84
<212> PRT
<213> Artificial Sequence

<400> 16

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1 5 10 15

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20 25 30

Thr Leu Gln Phe Val Cys Gly Asp Arg Gly Phe Tyr Phe Ser Arg Pro
35 40 45

Ala Ser Arg Val Ser Arg Arg Ser Arg Gly Ile Val Glu Glu Cys Cys
50 55 60

Phe Arg Ser Cys Asp Leu Ala Leu Leu Glu Thr Tyr Cys Ala Thr Pro
65 70 75 80

Ala Lys Ser Glu

<210> 17
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<220>
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<220>

<221> CDS
<222> (1)..(858)

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Met Ser Ile Gln His Phe Arg Val Ala Leu Ile Pro Phe Phe Ala Ala
1 5 10 15

ttt tgc ctt cct gtt ttt gct cac cca gaa acg ctg gtg aaa gta aaa 96
Phe Cys Leu Pro Val Phe Ala His Pro Glu Thr Leu Val Lys Val Lys
20 25 30

gat gct gaa gat cag ttg ggt gca cga gtg ggt tac atc gaa ctg gat 144
Asp Ala Glu Asp Gln Leu Gly Ala Arg Val Gly Tyr Ile Glu Leu Asp
35 40 45

ctc aac agc ggt aag atc ctt gag agt ttt cgc ccc gaa gaa cgt ttt 192
Leu Asn Ser Gly Lys Ile Leu Glu Ser Phe Arg Pro Glu Glu Arg Phe
50 55 60

cca atg atg agc act ttt aaa gtt ctg cta tgt ggc gcg gta tta tcc 240
Pro Met Met Ser Thr Phe Lys Val Leu Leu Cys Gly Ala Val Leu Ser
65 70 75 80

cgt gtt gac gcc ggg caa gag caa ctc ggt cgc cgc ata cac tat tct 288
Arg Val Asp Ala Gly Gln Glu Gln Leu Gly Arg Arg Ile His Tyr Ser
85 90 95

cag aat gac ttg gtt gag tac tca cca gtc aca gaa aag cat ctt acg 336
Gln Asn Asp Leu Val Glu Tyr Ser Pro Val Thr Glu Lys His Leu Thr
100 105 110

gat ggc atg aca gta aga gaa tta tgc agt gct gcc ata acc atg agt 384
Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala Ala Ile Thr Met Ser
115 120 125

gat aac act gcg gcc aac tta ctt ctg aca acg atc gga gga ccg aag 432
Asp Asn Thr Ala Ala Asn Leu Leu Leu Thr Thr Ile Gly Gly Pro Lys
130 135 140

gag cta acc gct ttt ttg cac aac atg ggg gat cat gta act cgc ctt 480
Glu Leu Thr Ala Phe Leu His Asn Met Gly Asp His Val Thr Arg Leu
145 150 155 160

gat cgt tgg gaa ccg gag ctg aat gaa gcc ata cca aac gac gag cgt 528
Asp Arg Trp Glu Pro Glu Leu Asn Glu Ala Ile Pro Asn Asp Glu Arg
165 170 175

gac acc acg atg cct gca gca atg gca aca acg ttg cgc aaa cta tta 576
Asp Thr Thr Met Pro Ala Ala Met Ala Thr Thr Leu Arg Lys Leu Leu
180 185 190

act ggc gaa cta ctt act cta gct tcc cgg caa caa tta ata gac tgg 624
Thr Gly Glu Leu Leu Thr Leu Ala Ser Arg Gln Gln Leu Ile Asp Trp
195 200 205

atg gag gcg gat aaa gtt gca gga cca ctt ctg cgc tcg gcc ctt ccg 672
Met Glu Ala Asp Lys Val Ala Gly Pro Leu Leu Arg Ser Ala Leu Pro
210 215 220

gct ggc tgg ttt att gct gat aaa tct gga gcc ggt gag cgt ggg tct 720
Ala Gly Trp Phe Ile Ala Asp Lys Ser Gly Ala Gly Glu Arg Gly Ser
225 230 235 240

cgc ggt atc att gca gca ctg ggg cca gat ggt aag ccc tcc cgt atc 768
Arg Gly Ile Ile Ala Ala Leu Gly Pro Asp Gly Lys Pro Ser Arg Ile
245 250 255

gta gtt atc tac acg acg ggg agt cag gca act atg gat gaa cga aat 816
Val Val Ile Tyr Thr Thr Gly Ser Gln Ala Thr Met Asp Glu Arg Asn
260 265 270

aga cag atc gct gag ata ggt gcc tca ctg att aag cat tgg ta 860
Arg Gln Ile Ala Glu Ile Gly Ala Ser Leu Ile Lys His Trp
275 280 285

a 861

<210> 18
<211> 286
<212> PRT
<213> Artificial Sequence

<400> 18

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Phe Cys Leu Pro Val Phe Ala His Pro Glu Thr Leu Val Lys Val Lys
20 25 30

Asp Ala Glu Asp Gln Leu Gly Ala Arg Val Gly Tyr Ile Glu Leu Asp
35 40 45

Leu Asn Ser Gly Lys Ile Leu Glu Ser Phe Arg Pro Glu Glu Arg Phe
50 55 60

Pro Met Met Ser Thr Phe Lys Val Leu Leu Cys Gly Ala Val Leu Ser
65 70 75 80

Arg Val Asp Ala Gly Gln Glu Gln Leu Gly Arg Arg Ile His Tyr Ser
85 90 95

Gln Asn Asp Leu Val Glu Tyr Ser Pro Val Thr Glu Lys His Leu Thr
100 105 110

Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala Ala Ile Thr Met Ser
115 120 125

Asp Asn Thr Ala Ala Asn Leu Leu Leu Thr Thr Ile Gly Gly Pro Lys
130 135 140

Glu Leu Thr Ala Phe Leu His Asn Met Gly Asp His Val Thr Arg Leu
145 150 155 160

Asp Arg Trp Glu Pro Glu Leu Asn Glu Ala Ile Pro Asn Asp Glu Arg
165 170 175

Asp Thr Thr Met Pro Ala Ala Met Ala Thr Thr Leu Arg Lys Leu Leu
180 185 190

Thr Gly Glu Leu Leu Thr Leu Ala Ser Arg Gln Gln Leu Ile Asp Trp
195 200 205

Met Glu Ala Asp Lys Val Ala Gly Pro Leu Leu Arg Ser Ala Leu Pro
210 215 220

Ala Gly Trp Phe Ile Ala Asp Lys Ser Gly Ala Gly Glu Arg Gly Ser
225 230 235 240

Arg Gly Ile Ile Ala Ala Leu Gly Pro Asp Gly Lys Pro Ser Arg Ile
245 250 255

Val Val Ile Tyr Thr Thr Gly Ser Gln Ala Thr Met Asp Glu Arg Asn
260 265 270

Arg Gln Ile Ala Glu Ile Gly Ala Ser Leu Ile Lys His Trp
275 280 285

<210> 19
<211> 42
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<220>
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<400> 19
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<210> 20
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<212> DNA
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<220>
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<400> 20

aaaaagcttt tactagaagc cacagctgcc 30

<210> 21
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer 1 for preparmg pTR0191

<400> 21
aaaaagctta aggagatggc gccca 25

<210> 22
<211> 25
<212> DNA
<113> Artificial Sequence

<220>
<223> Primer 2 for preparing p1R0191

<400> 22
aaagcatgcc tagaagccac agctg 25

<210> 23
<211> 555
<212> DNA
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<220>
<223> Human keratinocyte growth factor fused with histidine tag and enterokinase recognition sequence

<220>
<221> CDS
<222> (1)..(546)

<220>
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<222> (58)..(546)
<223> Human keratinocyte growth factor

<400> 23

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Lys His Met Gly His His His His His His His His His Asp Asp
1 5 10 15

gac gac aaa tgc aat gac atg act cca gag caa atg gct acc aat gtg 96
Asp Asp Lys Cys Asn Asp Met Thr Pro Glu Gln Met Ala Thr Asn Val
20 25 30

aac tgt tcc agc cct gag cgt cac acc cgt agc tat gat tac atg gaa 144
Asn Cys Ser Ser Pro Glu Arg His Thr Arg Ser Tyr Asp Tyr Met Glu
35 40 45

ggt ggt gat atc cgt gtg cgt cgt ctc ttc tgt cgt acc cag tgg tat 192
Gly Gly Asp Ile Arg Val Arg Arg Leu Phe Cys Arg Thr Gln Trp Tyr
50 55 60

ctg cgt atc gat aaa cgt ggc aaa gta aaa ggc acc caa gag atg aag 240

Leu Arg Ile Asp Lys Arg Gly Lys Val Lys Gly Thr Gln Glu Met Lys
65 70 75 80

aat aat tac aat atc atg gaa atc cgt acc gtg gca gtt ggt att gtg 288
Asn Asn Tyr Asn Ile Met Glu Ile Arg Thr Val Ala Val Gly Ile Val
85 90 95

gca atc aaa ggt gtg gaa agc gag ttc tat ctg gca atg aac aag gaa 336
Ala Ile Lys Gly Val Glu Ser Glu Phe Tyr Leu Ala Met Asn Lys Glu
100 105 110

ggt aaa ctc tat gca aag aaa gaa tgc aat gaa gat tgt aac ttc aaa 384
Gly Lys Leu Tyr Ala Lys Lys Glu Cys Asn Glu Asp Cys Asn Phe Lys
115 120 125

gaa ctg att ctg gaa aac cat tac aac acc tat gca tct gct aaa tgg 432
Glu Leu Ile Leu Glu Asn His Tyr Asn Thr Tyr Ala Ser Ala Lys Trp
130 135 140

acc cac aac ggt ggt gaa atg ttt gtt gcc ctg aat caa aag ggt att 480
Thr His Asn Gly Gly Glu Met Phe Val Ala Leu Asn Gln Lys Gly Ile
145 150 155 160

cct gta cgt ggt aag aag acg aag aaa gaa cag aaa acc gcc cac ttt 528
Pro Val Arg Gly Lys Lys Thr Lys Lys Glu Gln Lys Thr Ala His Phe
165 170 175

ctg cct atg gca atc act taaa agctt 555
Leu Pro Met Ala Ile Thr
180

<210> 24
<211> 182
<212> PRAT
<213> Artificial Sequence

<400> 24

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1 5 10 15

Asp Asp Lys Cys Asn Asp Met Thr Pro Glu Gln Met Ala Thr Asn Val
20 25 30

Asn Cys Ser Ser Pro Glu Arg His Thr Arg Ser Tyr Asp Tyr Met Glu
35 40 45

Gly Gly Asp Ile Arg Val Arg Arg Leu Phe Cys Arg Thr Gln Trp Tyr
50 55 60

Leu Arg Ile Asp Lys Arg Gly Lys Val Lys Gly Thr Gln Glu Met Lys
65 70 75 80

Asn Asn Tyr Asn Ile Met Glu Ile Arg Thr Val Ala Val Gly Ile Val
85 90 95

Ala Ile Lys Gly Val Glu Ser Glu Phe Tyr Leu Ala Met Asn Lys Glu
100 105 110

Gly Lys Leu Tyr Ala Lys Lys Glu Cys Asn Glu Asp Cys Asn Phe Lys
115 120 125

Glu Leu Ile Leu Glu Asn His Tyr Asn Thr Tyr Ala Ser Ala Lys Trp
130 135 140

Thr His Asn Gly Gly Glu Met Phe Val Ala Leu Asn Gln Lys Gly Ile
145 150 155 160

Pro Val Arg Gly Lys Lys Thr Lys Lys Glu Gln Lys Thr Ala His Phe
165 170 175

Leu Pro Met Ala Ile Thr
180

<210> 25
<111> 507
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(498)
<223> Human keratinocyte growth factor

<400> 25

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Lys His Met Cys Asn Asp Met Thr Pro Glu Gln Met Ala Thr Asn Val
1 5 10 15

aac tgt tcc agc cct gag cgt cac acc cgt agc tat gat tac atg gaa 96
Asn Cys Ser Ser Pro Glu Arg His Thr Arg Ser Tyr Asp Tyr Met Glu
20 25 30

ggt ggt gat atc cgt gtg cgt cgt ctc ttc tgt cgt acc cag tgg tat 144
Gly Gly Asp Ile Arg Val Arg Arg Leu Phe Cys Arg Thr Gln Trp Tyr
35 40 45

ctg cgt atc gat aaa cgt ggc aaa gta aaa ggc acc caa gag atg aag 192
Leu Arg Ile Asp Lys Arg Gly Lys Val Lys Gly Thr Gln Glu Met Lys
50 55 60

aat aat tac aat atc atg gaa atc cgt acc gtg gca gtt ggt att gtg 240
Asn Asn Tyr Asn Ile Met Glu Ile Arg Thr Val Ala Val Gly Ile Val
65 70 75 80

gca atc aaa ggt gtg gaa agc gag ttc tat ctg gca atg aac aag gaa 288
Ala Ile Lys Gly Val Glu Ser Glu Phe Tyr Leu Ala Met Asn Lys Glu
85 90 95

ggt aaa ctc tat gca aag aaa gaa tgc aat gaa gat tgt aac ttc aaa 336
Gly Lys Leu Tyr Ala Lys Lys Glu Cys Asn Glu Asp Cys Asn Phe Lys
100 105 110

gaa ctg att ctg gaa aac cat tac aac acc tat gca tct gct aaa tgg 384
Glu Leu Ile Leu Glu Asn His Tyr Asn Thr Tyr Ala Ser Ala Lys Trp
115 120 125

acc cac aac ggt ggt gaa atg ttt gtt gcc ctg aat caa aag ggt att 432

Thr His Asn Gly Gly Glu Met Phe Val Ala Leu Asn Gln Lys Gly Ile
130 135 140

cct gta cgt ggt aag aag acg aag aaa gaa cag aaa acc gcc cac ttt 480
Pro Val Arg Gly Lys Lys Thr Lys Lys Glu Gln Lys Thr Ala His Phe
145 150 155 160

ctg cct atg gca atc act ta aaagctt 507
Leu Pro Met Ala Ile Thr
165

<210> 26
<211> 166
<212> PRT
<213> Homo sapiens

<400> 26

Lys His Met Cys Asn Asp Met Thr Pro Glu Gln Met Ala Thr Asn Val
1 5 10 15

Asn Cys Ser Ser Pro Glu Arg His Thr Arg Ser Tyr Asp Tyr Met Glu
20 25 30

Gly Gly Asp Ile Arg Val Arg Arg Leu Phe Cys Arg Thr Gln Trp Tyr
35 40 45

Leu Arg Ile Asp Lys Arg Gly Lys Val Lys Gly Thr Gln Glu Met Lys
50 55 60

Asn Asn Tyr Asn Ile Met Glu Ile Arg Thr Val Ala Val Gly Ile Val
65 70 75 80

Ala Ile Lys Gly Val Glu Ser Glu Phe Tyr Leu Ala Met Asn Lys Glu
85 90 95

Gly Lys Leu Tyr Ala Lys Lys Glu Cys Asn Glu Asp Cys Asn Phe Lys
100 105 110

Glu Leu Ile Leu Glu Asn His Tyr Asn Thr Tyr Ala Ser Ala Lys T가

115 120 125

Thr His Asn Gly Gly Glu Met Phe Val Ala Leu Asn Gln Lys Gly Ile
130 135 140

Pro Val Arg Gly Lys Lys Thr Lys Lys Glu Gln Lys Thr Ala His Phe
145 150 155 160

Leu Pro Met Ala Ile Thr
165

Claims

1. A method of producing an active, soluble target protein in a prokaryotic cell, which is **characterized by** expressing the target protein and beta-lactamase as a first cistron and a second cistron, respectively, in a polycistron.

2. The method according to claim 1, wherein the polycistron is derived from a recombinant vector comprising (i) a promoter oparable in the prokaryotic cell, (ii) a first cistron including a DNA sequence encoding the target protein and (iii) a second cistron including a DNA sequence encoding beta-lactamase which are operable linked in a 5' to 3' direction.

3. The method according to claim 1, wherein the target protein is selected from the group consisting of human growth hormone, granulocyte-colony stimulating factor, interferons, basic fibroblast growth factor, insulin-like growth factors, keratinocyte growth factor, erythropoietin, thrombopoietin, human epidermal growth factor, platelet-derived growth factor, vascular endothelial growth factor, nerve growth factor, transforming growth factor, tumor necrosis factor, angiogenin, angiotensin and interleukins.

4. The method according to claim 3, wherein the target protein is selected from the group consisting of human growth hormone, granulocyte-colony stimulating factor, interferon-α2b, basic fibroblast growth factor, insulin-like growth factor-1, insulin-like growth factor-2 and keratinocyte growth factor.

5. The method according to claim 1, wherein the prokaryotic cell is Escherichia coli (E.coli).

6. The method according to claim 5, wherein the E.coli is selected from the group consisting of E.coli BL21 (DE3), E.coli BL21 Star (DE3) pLys S, E.coli HMS (DE3) and E.coli AD494 (DE3).

7. A polycistronic vector for expressing a target protein in an active, soluble form in a prokaryotic cell, comprising:

   (i) a promoter operable in the prokaryotic cell;
   (ii) a first cistron including a DNA sequence encoding the target protein; and
   (iii) a second cistron including a DNA sequence encoding beta-lactamase.

8. The polycistronic vector according to claim 7, wherein the vector comprises (i) a promoter operable in the prokaryotic cell, (ii) a first cistron including a DNA sequence encoding the target protein and (iii) a second cistron including a DNA sequence encoding beta-lactamase which are operably linked in a 5' to 3' direction.

9. The polycistronic vector according to claim 7, wherein the vector comprises (i) a promoter operable in the prokaryotic cell, (ii) a second cistron including a DNA sequence encoding beta-lactamase and (iii) a first cistron including a DNA sequence encoding the target protein which are operably linked in a 5' to 3' direction.

10. The polycistronic vector according to claim 7, wherein the target protein is selected from the group consisting of human growth hormone, granulocyte-colony stimulating factor, interferons, basic fibroblast growth factor, insulin-

like growth factors, keratinocyte growth factor, erythropoietin, thrombopoietin, human epidermal growth factor, platelet-derived growth factor, vascular endothelial growth factor, nerve growth factor, transforming growth factor, tumor necrosis factor, angiogenin, antiotensin and interleukins.

11. The polycistronic vector according to claim 10, wherein the target protein is selected from the group consisting of human growth hormone, granulocyte-colony stimulating factor, interferon-α2b, basic fibroblast growth factor, insulin-like growth factor-1, insulin-like growth factor-2 and keratinocyte growth factor.

12. The polycistronic vector according to claim 7, wherein the promoter is selected from the group consisting of T7, tac, trc, lac, lpp, phoA, recA, araBAD, proU, cst-1 tetA, cadA, nar, lpp-lac, starvation promoters, cspA, T7-lac operator, T3-lac operator, T5-lac operator, T4 gene 32 and nprM-lac operator.

13. The polycistronic vector according to claim 12, wherein the promoter is T7 promoter.

14. The polycistronic vector according to claim 7, wherein the vector is selected from the group consisting of pT0191 (pET3a-T7 promoter-human growth hormone-β-lactamase), pT0-CSF (pET3a-T7 promoter-CSF-β-lactamase), pT0-IFN (pET3a-T7 promoter-IFN-β-lactamase), pT0-bFGF (pET3a-T7 promoter-bFGF-β-lactamase), pT0-IGF1 (pET3a-T7 promoter-IGF1-β-lactamase), pT0-IGF2 (pET3a-T7 promoter-IGF2-β-lactamase), pT0-KGF (pET3a-T7 promoter-KGF-β-lactamase) and pTON-KGF (pET3a-T7 promoter-N-KGF-β-lactamase).

15. A transformant transformed with the expression vector of claim 7.

16. The transformant according to claim 15, wherein the transformant is Escherichia coli (E.coli).

17. A method of producing an active, soluble target protein, which is **characterized by** culturing the transformant of claim 16 and recovering an expressed target protein from a culture.

Patentansprüche

1. Ein Verfahren zum Herstellen eines aktiven löslichen Targetproteins in einer prokaryontischen Zelle, **dadurch** charakterisiert, dass das Targetprotein und β-Lactamase als ein erstes Cistron bzw. ein zweites Cistron in einem Polycistron exprimiert werden.

2. Das Verfahren gemäß Anspruch 1, wobei das Polycistron von einem rekombinanten Vector erhalten wird, der (i) einen Promotor, der in der prokaryontischen Zelle wirksam ist, (ii) ein erstes Cistron, das eine DNA Sequenz, die für das Targetprotein kodiert, einschließt, und (iii) ein zweites Cistron, das eine DNA Sequenz, die β-Lactamase kodiert, einschließt, welche operativ in einer 5' → 3'-Richtung verknüpft sind, umfasst.

3. Das Verfahren gemäß Anspruch 1, wobei das Targetprotein ausgewählt ist aus der Gruppe bestehend aus menschlichem Wachstumshormon, Granulocyten-Kolonie-Stimulierungsfaktor, Interferonen, dem basischen Fibroblast-Wachstumsfaktor, insulinähnlichen Wachstumsfaktoren, dem Keratinocyten-Wachstumsfaktor, Erythropoietin, Thrombopoietin, menschlichem epidermalem Wachstumsfaktor, von Blutplättchen freigesetztem Wachstumsfaktor, vascularem endothelialem Wachstumsfaktor, Nervenwachstumsfaktor, transformierendem Wachstumsfaktor, Tumornecrosefaktor, Angiogenin, Angiotensin und Interleukinen.

4. Das Verfahren gemäß Anspruch 3, wobei das Targetprotein ausgewählt ist aus der Gruppe bestehend aus menschlichem Wachstumshormon, Granulocyten-Kolonie-Stimulierungsfaktor, Interferon-a2b, basischem Fibroblasten-Wachstumsfaktor, insulinähnlichem Wachstumsfaktor-1, insulinähnlichem Wachstumsfaktor-2 und Keratinocyten-Wachstumsfaktor.

5. Das Verfahren gemäß Anspruch 1, wobei die prokaryontische Zelle Escherichia coli (E.coli) ist.

6. Das Verfahren gemäß Anspruch 5, wobei das E.coli ausgewählt ist aus der Gruppe bestehend aus E.coli BL21 (DE3), E.coli BL21 Star (DE3) pLys S, E.coli HMS (DE3) und E.coli AD494 (DE3).

7. Ein polycistronischer Vector zur Expression eines Targetproteins in einer aktiven löslichen Form in einer prokaryontischen Zelle, umfassend:

(i) einen Promotor, der in der prokaryontischen Zelle wirksam ist;
(ii) ein erstes Cistron, das eine DNA Sequenz, die das Targetprotein kodiert, einschließt und
(iii) ein zweites Cistron, das eine DNA Sequenz, die für β-Lactamase kodiert, einschließt.

8. Der polycistronische Vector gemäß Anspruch 7, wobei der Vector (i) einen Promotor, der in der prokaryontischen Zelle wirksam ist, (ii) ein erstes Cistron, das eine DNA Sequenz, die das Targetprotein kodiert, einschließt, und (iii) ein zweites Cistron, das eine DNA Sequenz, für β-Lactamase kodiert, einschließt, welche operativ in einer 5' → 3'-Richtung verknüpft sind, umfasst.

9. Der polycistronische Vector gemäß Anspruch 7, wobei der Vector (i) einen Promotor, der in der prokaryontischen Zelle wirksam ist, (ii) ein zweites Cistron, das eine DNA Sequenz, die β-Lactamase kodiert, einschließt und (iii) ein erstes Cistron, das eine DNA Sequenz, die das Targetprotein kodiert, einschließt, welche operativ in einer 5' → 3'-Richtung verknüpft sind, umfasst.

10. Der polycistronische Vector gemäß Anspruch 7, wobei das Targetprotein ausgewählt ist aus der Gruppe bestehend aus menschlichem Wachstumshormon, Granulocyten-Kolonie-Stimulierungsfaktor, Interferonen, dem basischen Fibroblast-Wachstumsfaktor, insulinähnlichen Wachstumsfaktoren, dem Keratinocyten-Wachstumsfaktor, Erythropoietin, Thrombopoietin, menschlichem epidermalem Wachstumsfaktor, von Blutplättchen freigesetztem Wachstumsfaktor, vascularem endothelialem Wachstumsfaktor, Nervenwachstumsfaktor, transformierendem Wachstumsfaktor, Tumornecrosefaktor, Angiogenin, Angiotensin und Interleukinen.

11. Der polycistronische Vector gemäß Anspruch 10, wobei das Targetprotein ausgewählt ist aus der Gruppe bestehend aus menschlichem Wachstumshormon, Granulocyten-Kolonie-Stimulierungsfaktor, Interferon-α2b, basischem Fibroblasten-Wachstumsfaktor, insulinähnlichem Wachstumsfaktor-1, insulinähnlichem Wachstumsfaktor-2 und Keratinocyten-Wachstumsfaktor.

12. Der polycistronische Vector gemäß Anspruch 7, wobei der Promotor ausgewählt ist aus der Gruppe bestehend aus T7, tac, trc, lac, lpp, phoA, recA, araBAD, proU, cst-1 tetA, cadA, nar, lpp-lac, Unterernährungspromotoren, cspA, T7-lac Operator, T3-lac Operator, T5-lac Operator, T4 Gen 32 und nprm-lac Operator.

13. Der polycistronische Vector gemäß Anspruch 12, wobei der Promotor der T7 Promotor ist.

14. Der polycistronische Vector gemäß Anspruch 7, wobei der Vector ausgewählt ist aus der Gruppe bestehend aus pT0191 (pET3a-T7 Promotor-menschliches Wachstumshormon-β-Lactamase), pT0-CSF (pET3a-T7 Promotor-CSF-β-Lactamase), pT0-IFN (pET3a-T7 Promotor-IFN-β-Lactamase), pT0-bFGF (pET3a-T7 Promotor-bFGF-β-Lactamase), pTO-IGF1 (pET3a-T7 Promotor-IGF1-β-Lactamase), pTO-IGF2 (pET3a-T7 Promotor-IGF2-β-Lactamase), pT0-KGF (pET3a-T7 Promotor-KGF-β-Lactamase) und pTON-KGF (pET3a-T7 Promotor-N-KGF-β-Lactamase).

15. Eine transformierte Zelle, transformiert mit dem Expressionsvector gemäß Anspruch 7.

16. Die transformierte Zelle gemäß Anspruch 15, wobei die transformierte Zelle Escherichia coli (E.coli) ist.

17. Ein Verfahren zur Herstellung eines aktiven löslichen Targetproteins, charakterisiert durch das Kultivieren der transformierte Zelle gemäß Anspruch 16, und Gewinnen eines exprimierten Targetproteins aus einer Kultur.

Revendications

1. Procédé de production d'une protéine cible soluble active dans une cellule procaryote, qui est **caractérisé par** l'expression de la protéine cible et de bêta-lactamase comme premier cistron et d'un deuxième cistron, respectivement, dans un polycistron.

2. Procédé selon la revendication 1, dans lequel le polycistron est dérivé d'un vecteur recombinant comprenant (i) un promoteur opérationnel dans la cellule procaryote, (ii) un premier cistron comprenant une séquence d'ADN codant pour la protéine cible et (iii) un deuxième cistron comprenant une séquence d'ADN codant pour la bêta-lactamase qui sont liés de manière opérationnelle dans le sens 5' à 3'.

3. Procédé selon la revendication 1, dans lequel la protéine cible est choisie dans le groupe constitué par l'hormone de croissance humaine, le facteur de croissance hématopoïétique G-CSF, les interférons, le facteur de croissance basique des fibroblastes, les facteurs de croissance insulinomimétiques, le facteur de croissance de kératinocytes, l'érythropoïétine, la thrombopoïétine, le facteur de croissance de l'épiderme humain, le facteur de croissance dérivé des plaquettes, le facteur de croissance endothélial vasculaire, le facteur de croissance du tissu, nerveux, le facteur de croissance transformant, le facteur de nécrose tumorale, le facteur angiogénique, l'angiotensine et les interleukines.

4. Procédé selon la revendication 3, dans lequel la protéine cible est choisie dans le groupe constitué par l'hormone de croissance humaine, le facteur de croissance hématopoïétique G-CSF, l'interféron α2b, le facteur de croissance basique des fibroblastes, le facteur de croissance insulinomimétique de type 1, le facteur de croissance insulinomimétique de type 2 et le facteur de croissance de kératinocytes.

5. Procédé selon la revendication 1, dans lequel la cellule procaryote est Escherichia coli (E. coli).

6. Procédé selon la revendication 5, dans lequel la cellule E. coli est choisie dans le groupe constitué par E. coli BL21 (DE3), E. coli BL21 Star (DE3) pLys S, E. coli HMS (DE3) et E. coli A494 (DE3).

7. Vecteur polycistronique pour l'expression d'une protéine cible dans une forme soluble active dans une cellule procaryote, comprenant :

(i) un promoteur opérationnel dans la cellule procaryote ;
(ii) un premier cistron comprenant une séquence d'ADN codant pour la protéine cible ; et
(iii) un deuxième cistron comprenant une séquence d'ADN codant pour la bêta-lactamase.

8. Vecteur polycistronique selon la revendication 7, dans lequel le vecteur comprend (i) un promoteur opérationnel dans la cellule procaryote, (ii) un premier cistron comprenant une séquence d'ADN codant pour la protéine cible et (iii) un deuxième cistron comprenant une séquence d'ADN codant pour la bêta-lactamase qui sont liés de manière opérationnelle dans le sens 5' à 3'.

9. Vecteur polycistronique selon la revendication 7, dans lequel le vecteur comprend (i) un promoteur opérationnel dans la cellule procaryote, (ii) un deuxième cistron comprenant une séquence d'ADN codant pour la bêta-lactamase et (iii) un premier cistron comprenant une séquence d'ADN codant pour la protéine cible qui sont liés de manière opérationnelle dans le sens 5' à 3'.

10. Vecteur polycistronique selon la revendication 7, dans lequel la protéine cible est choisie dans le groupe constitué par l'hormone de croissance humaine, le facteur de croissance hématopoïétique G-CSF, les interférons, le facteur de croissance basique des fibroblastes, les facteurs de croissance insulinomimétiques, le facteur de croissance de kératinocytes, l'érythropoïétine, la thrombopoïétine, le facteur de croissance de l'épiderme humain, le facteur de croissance dérivé des plaquettes, le facteur de croissance endothélial vasculaire, le facteur de croissance du tissu nerveux, le facteur de croissance transformant, le facteur de nécrose tumorale, le facteur angiogénique, l'angiotensine et les interleukines.

11. Vecteur polycistronique selon la revendication 10, dans lequel la protéine cible est choisie dans le groupe constitué par l'hormone de croissance humaine, le facteur de croissance hématopoïétique G-CSF, l'interféron α2b, le facteur de croissance basique des fibroblastes, le facteur de croissance insulinomimétique de type 1, le facteur de croissance insulinomimétique de type 2 et le facteur de croissance de kératinocytes.

12. Vecteur polycistronique selon la revendication 7, dans lequel le promoteur est choisi dans le groupe constitué par T7, tac, trc, lac, lpp, phoA, recA, araBAD, proU, cst-1 tetA, cadA, nar, lpp-lac, les promoteurs d'inanition, cspA, T7-opérateur lac, T3-opérateur lac, T5-opérateur lac, le gène 32 de T4 et nprM-opérateur lac.

13. Vecteur polycistronique selon la revendication 12, dans lequel le promoteur est le promoteur T7.

14. Vecteur polycistronique selon la revendication 7, dans lequel le vecteur est choisi dans le groupe constitué par pT0191 (pET3a-promoteur T7-hormone de croissance humaine-β-lactamase), pT0-CSF (pET3a-promoteur T7-CSF-F-lactamase), pT0-IFN (pET3a-promoteur T7-IFN-β-lactamase), pT0-bFGF (pET3a-promoteur T7-bFGF-β-lactamase), pT0-IGF1 (pET3a-promoteur T7-IGF1-β-lactamase), pT0-IGF2 (pET3a-promoteur T7-IGF2-β-lactama-

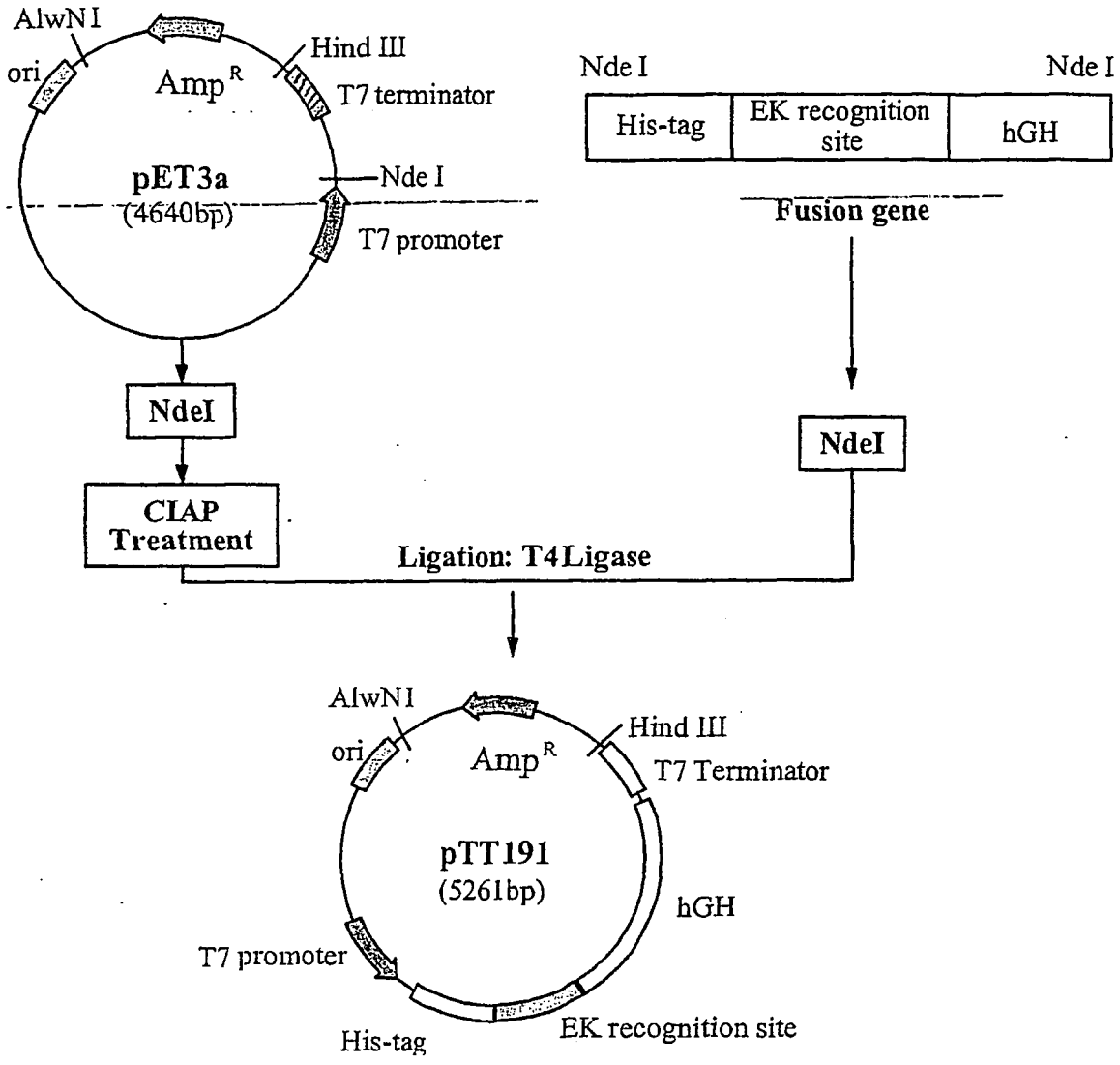
se), pT0-KGF (pET3a-promoteur T7-KGF-β-lactamase) et pT0N-KGF (pET3a-promoteur T7-N-KGF-β-lactamase).

15. Transformant transformé avec le vecteur d'expression selon la revendication 7.

16. Transformant selon la revendication 15, dans lequel le transformant est Escherichia coli (E. coli).

17. Procédé de production d'une protéine cible soluble active, qui est **caractérisé par** la culture du transformant selon la revendication 16 et la récupération d'une protéine cible exprimée à partir d'une culture.

FIG. 1


AlwN I
ori
Amp R
Hind III
T7 terminator
pET3a
(4640bp)
Nde I
T7 promoter
Nde I
Nde I
His-tag
EK recognition site
hGH
Fusion gene
NdeI
NdeI
CIAP Treatment
Ligation: T4Ligase
AlwN I
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T7 Terminator
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(5261bp)
hGH
T7 promoter
His-tag
EK recognition site

FIG. 2

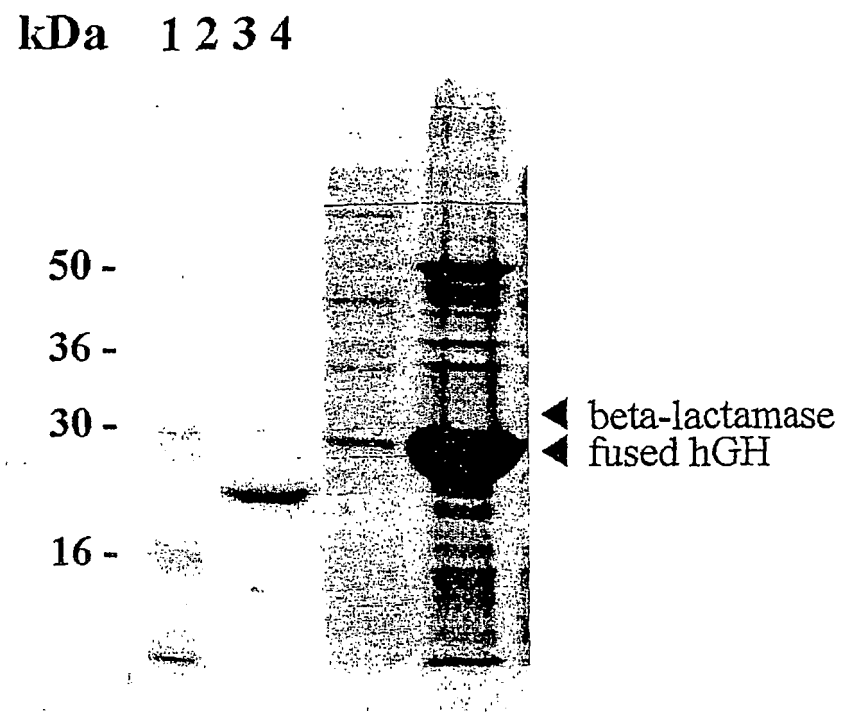


FIG. 3

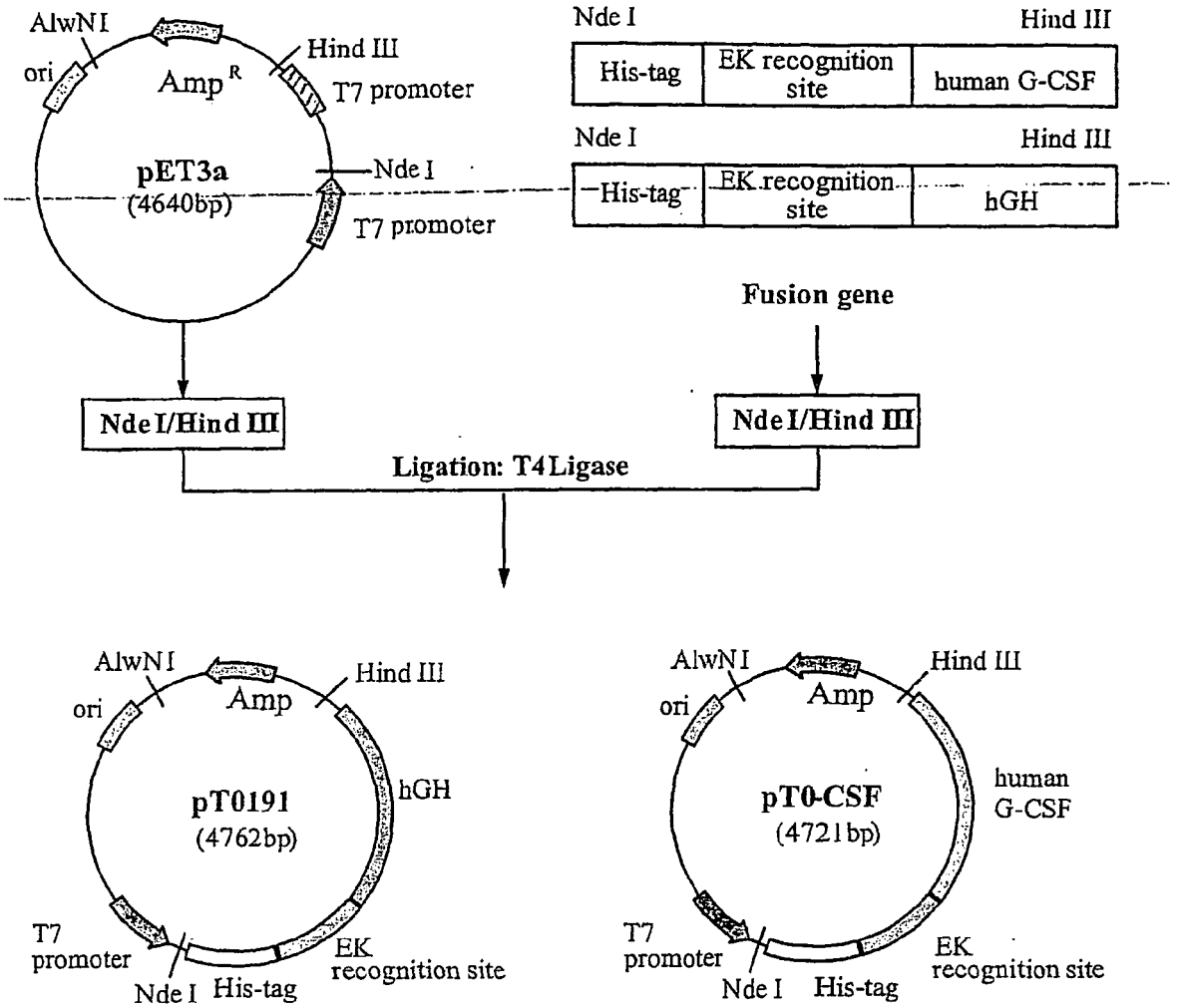

AlwNI
ori
Amp R
Hind III
T7 promoter
pET3a
(4640bp)
Nde I
T7 promoter
Nde I
Hind III
His-tag
EK recognition site
human G-CSF
Nde I
Hind III
His-tag
EK recognition site
hGH
Fusion gene
Nde I/Hind III
Nde I/Hind III
Ligation: T4 Ligase
AlwNI
ori
Amp
Hind III
pT0191
(4762bp)
hGH
T7 promoter
Nde I
His-tag
EK recognition site
AlwNI
ori
Amp
Hind III
pT0-CSF
(4721bp)
human G-CSF
T7 promoter
Nde I
His-tag
EK recognition site

FIG. 4

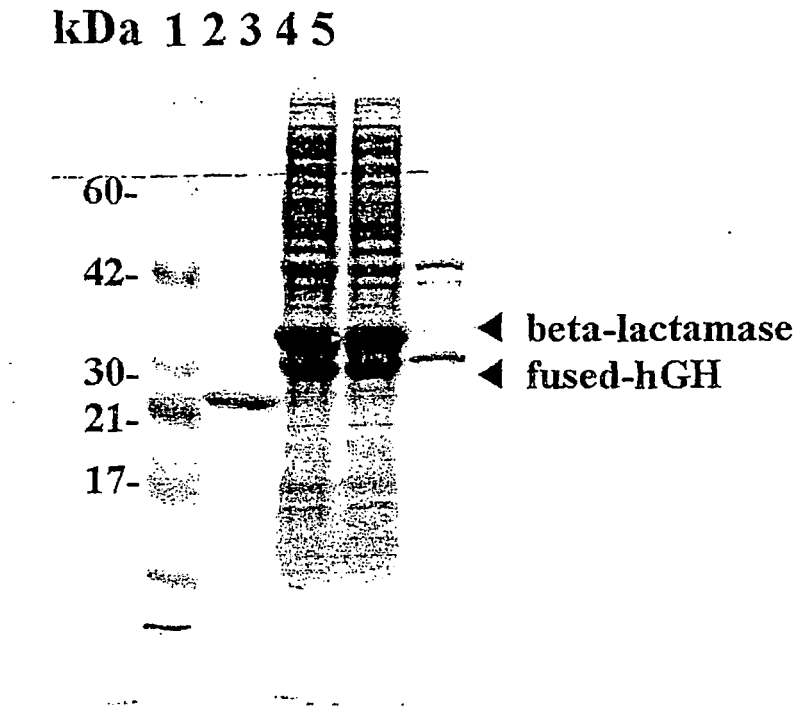


FIG. 5

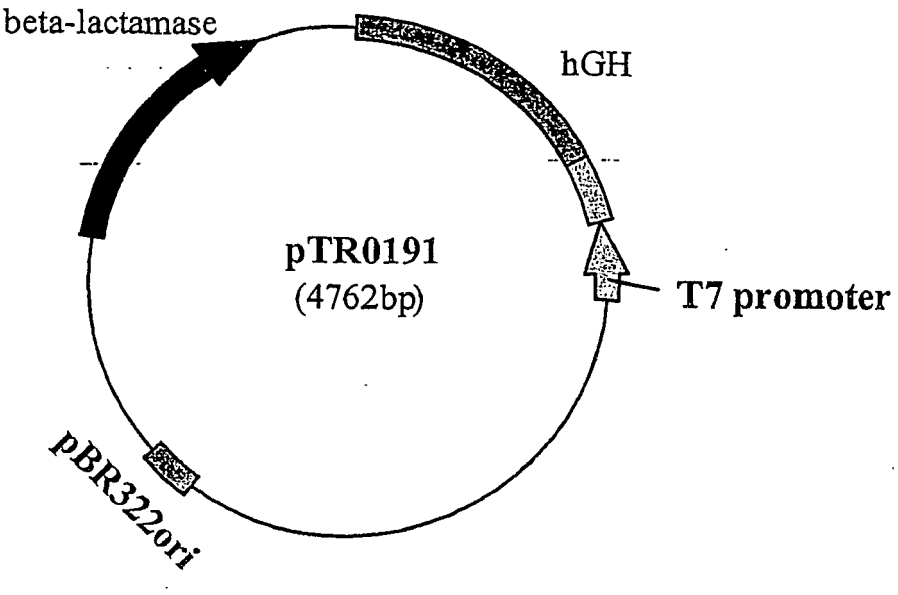


FIG. 6

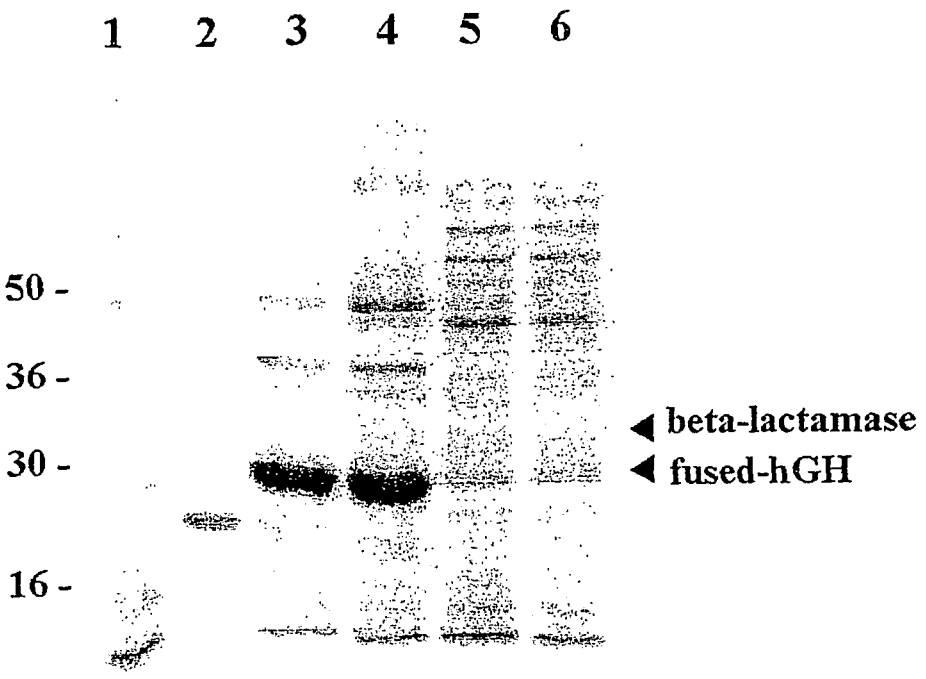


FIG. 7

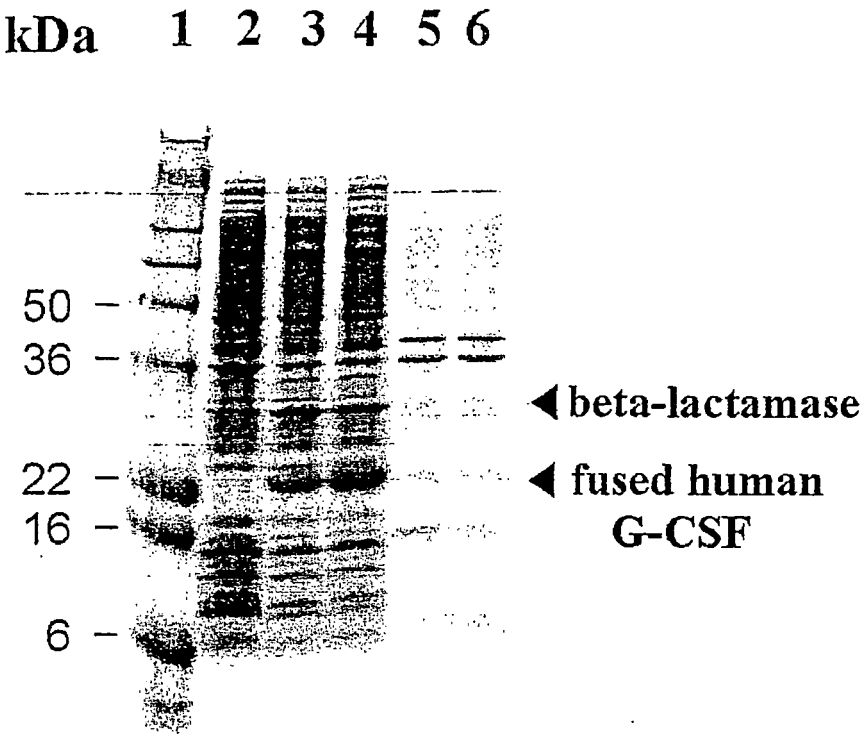


FIG. 8

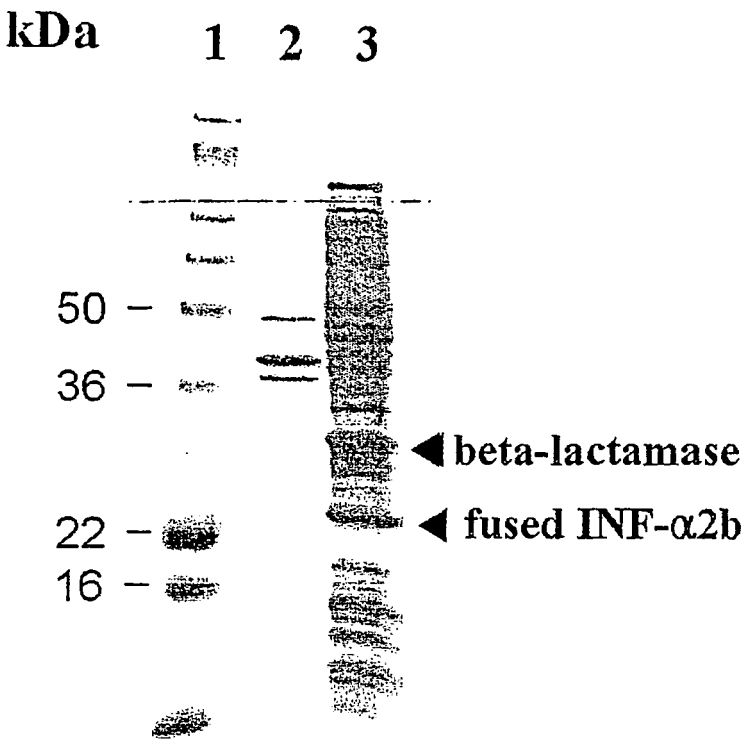


FIG. 9

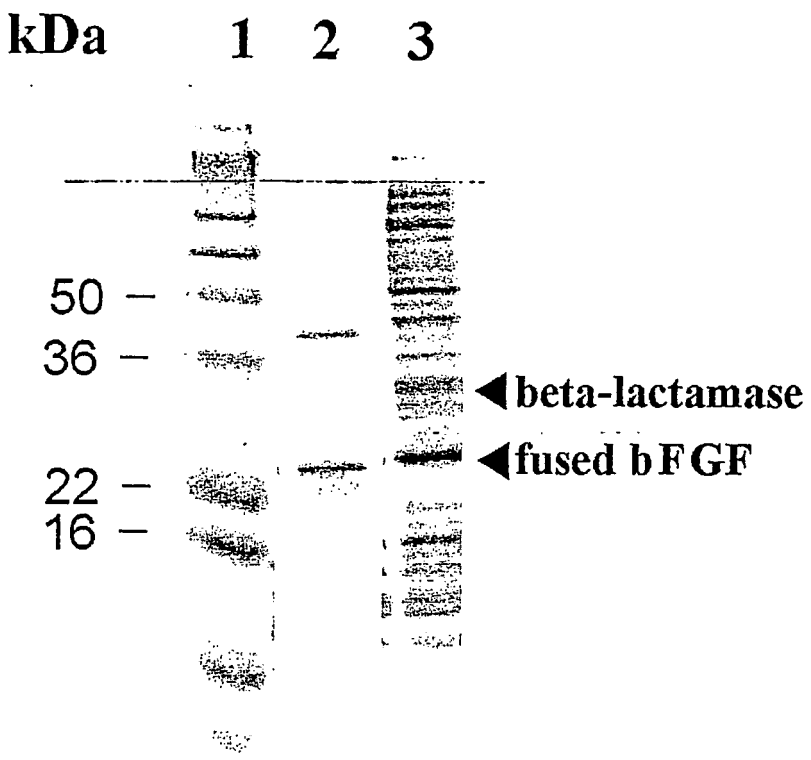


FIG. 10

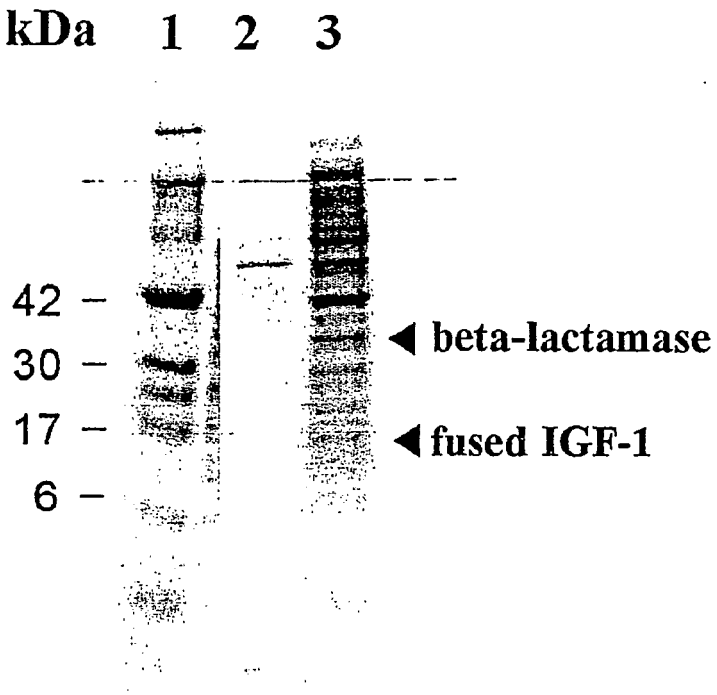


FIG. 11

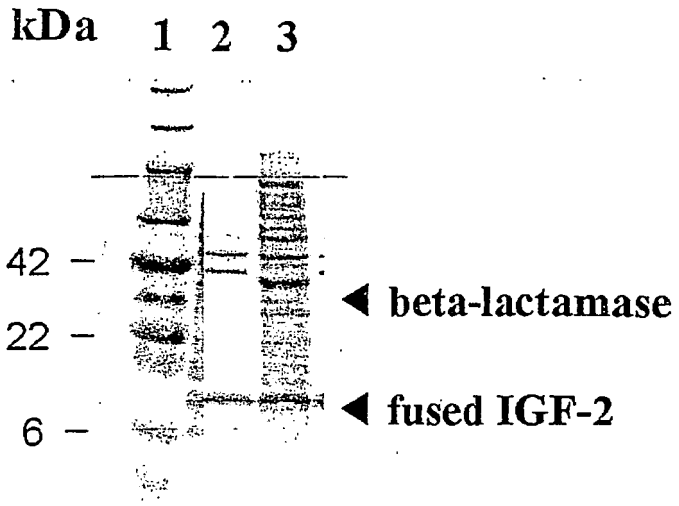


FIG. 12

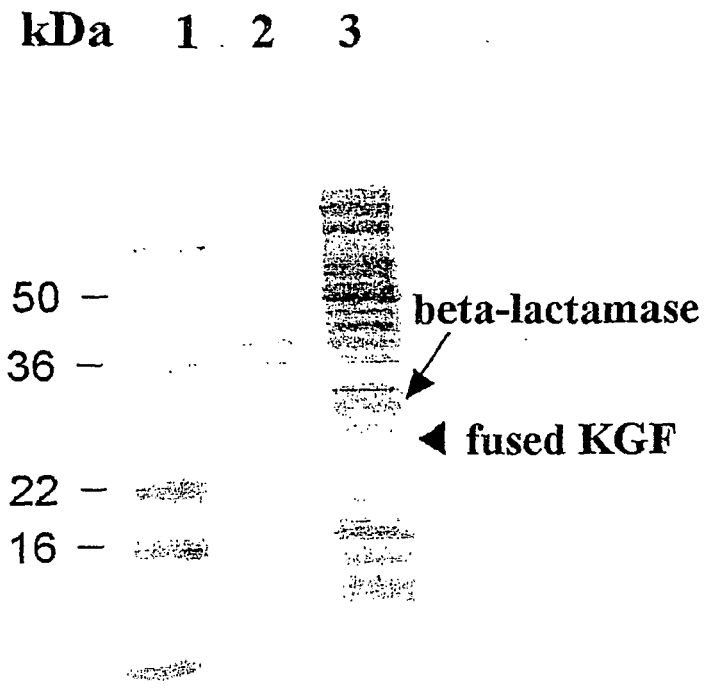
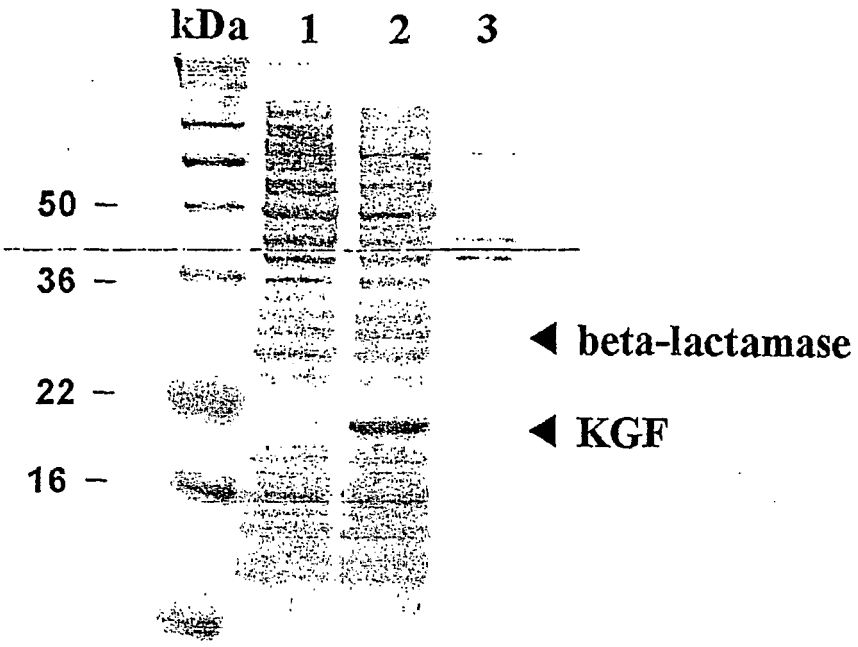


FIG. 13



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