A METHOD FOR THE MASS PRODUCTION OF IMMUNOGLOBULIN Fc REGION DELETED INITIAL METHIONINE RESIDUES

Technical Field

The present invention relates to a method for producing a monomeric or dimeric immunoglobulin Fc region free of initial methionine residues on a mass scale by taking advantage of a recombinant expression vector comprising a nucleotide sequence coding for a recombinant immunoglobulin Fc region including an immunoglobulin hinge region.

Background Art

With advances in genetic engineering, a large number of protein drugs have been developed and utilized. Susceptible to denaturation or proteolytic degradation in the body, protein drugs, however, are difficult to keep at *in vivo* concentrations and titers for a long period of time. An improvement in protein stability *in vivo*, which can lead to the maintenance of *in vivo* concentrations of protein drugs at suitable levels is important not only in promoting the efficacy of therapy, but also in helping patients who need to take frequent injections of their protein drugs, in terms of convenience and cost.

Many attempts have been made to enhance the *in vivo* stability of protein drugs for a long time, exemplified by changing the protein formulation, fusing a protein to another protein, or chemically or biologically attaching a suitable polymer to the surface of a protein.

One of such technique is making a fusion protein with the immunoglobulin Fc fragment.

The Fc fragment mediates effector functions such as complement-dependent cytotoxicity (CDC) or antibody-dependent cell-mediated cytotoxicity (ADCC), as well as antigen binding capacity, which is the unique function of immunoglobulins. Also, the Fc fragment contains a sequence participating in the binding to the neonatal Fc receptor (FcRn), which plays a role in regulating serum IgG levels by increasing the transport of maternal IgG to neonates and the half-life of the IgG (Ghetie and Ward, *Immunology Today* 18: 592-598, 1997), and the sequence regulates the interaction between protein A and protein G. Through the fusion of this Fc fragment with a therapeutic protein, many studies have been performed to enhance the stability of the therapeutic protein.

For example, Korean Pat. No. 249572 discloses a fusion protein which is prepared by linking an IgG1 heavy chain Fc region (Fc) at an amino terminal end thereof to a carboxyl terminal end of a protein, such as an IL4 receptor, an IL7 receptor, a G-CSF receptor or an EPO receptor, and producing

the resulting fusion protein in mammalian cells. U. S. Pat. No. 5,605,690 describes a fusion protein comprising a tumor necrosis factor receptor fused at its carboxyl terminal end to a human IgG1 Fc derivative, the fusion protein being produced in animal cells. Also, Tanox Inc. reported, in U.S. Pat. Nos. 5,723,125 and 5,908,626, a hybrid molecule comprising human interferon alpha or beta that is linked at its carboxyl terminal end to native human IgG4 Fc through a peptide linker, and produced in animal cells. Lexigen Inc., as described in International PCT Application Publication No. WO 00/69913, prepared a native IgG1 Fc linked at its carboxyl terminal end to the amino terminal end of human interferon by genetic recombination without the use of a linker and produced the fusion protein in animal cells. U. S. Pat. Publication No. 20030082679 discloses a fusion protein with an extended serum half-life, which comprises human G-CSF linked at its carboxyl terminal end to the amino terminal end of IgG1 Fc and is produced in animal cells. U. S. Pat. Publication No. 20010053539, U. S. Pat. No. 6,030,613, International PCT Application Publication Nos. WO 99/02709 and WO 01/03737 and European Pat. No. 0464533B1 disclose an Fc fusion protein with a longer serum half-life than a native protein, which comprises an IgG1 Fc or Fc derivative linked at its amino terminal end through a peptide linker or without a peptide linker to the carboxyl terminal end of human EPO, TPO, human growth hormone or human interferon beta, the Fc

fusion protein being produced in animal cells.

These Fc fusion proteins, as described above, increase the serum half-life of target proteins, but entail problems related to the mediation of effector functions by the Fc fragment (U. S. Pat. No. 5,349,053). Through the effector functions of the Fc fragment, they fix complements or bind to cells expressing FcγRs, leading to lysis of specific cells, and induce the production and secretion of several cytokines inducing inflammation, leading to unwanted inflammation. Also, the fusion creates a new amino acid sequence at a connection region between the Fc fragment and the protein partner, which could potentially induce immune responses if administered for a long time.

In this regard, many efforts have been made to prepare an immunoglobulin or immunoglobulin fragment that has a long serum half-life but is deficient in effector functions. Cole et al. reported that, when amino acid residues of the CH2 region at positions 234, 235 and 237, known to play an important role in binding to Fc receptors, are replaced with alanine to thus produce an Fc derivative having a reduced binding affinity to Fc receptors, the ADCC activity is inhibited (Cole et al., *J. Immunol.* 159: 3613-3621, 1997). However, in all of these variants, Fc may have increased immunogenicity or antigenicity compared to the native human Fc fragment due to the presence of unsuitable amino acids, and may lose desirable Fc functions.

Among methods of deleting or reducing undesirable effector functions while maintaining high serum concentrations of an immunoglobulin, one is based on removing sugar moieties from the immunoglobulin. As described in U. S. Pat. No. 5,585,097, an aglycosylated antibody derivative as an anti-CD3 antibody can be prepared by replacing a glycosylated residue of antibodies, the asparagine residue at position 297 of the CH2 domain, with another amino acid. This aglycosylated antibody derivative exhibits reduced effector functions, but still retains its binding affinity to FcRn receptor, with no change in serum half-life. However, this derivative is also problematic in terms of being potentially recognized as a foreign material and rejected by the immune system due to the production of a novel recombinant construct having an abnormal sequence. U.S. Pat. Publication No. 20030073164 discloses a method of producing an Fc derivative using *E. coli* devoid of glycosylation ability so as to prepare a therapeutic antibody deficient in effector functions.

The American company Amgen Inc. described, in U.S. Pat. No. 6,660,843 and U.S. Pat. Publication Nos. 20040044188 and 20040053845, a human IgG1 Fc derivative having amino acid deletions at the first five amino acid residues of the hinge region, which is fused to the amino or carboxyl terminal end of a therapeutic protein or a therapeutic protein mimicked by a peptide, and the production thereof using an *E. coli* host.

However, this fusion protein not having a signal sequence is expressed as inclusion bodies, and thus must be subjected to an additional refolding process. This protein refolding process reduces yields, and, especially in a protein present as a homodimer or a heterodimer, remarkably reduces dimer production. Also, when a protein not having a signal sequence is expressed in *E. coli*, a methionine residue is added to the N-terminus of the expression product due to the feature of the protein expression system of *E. coli*. The aforementioned expression products of Amgen Inc. have an N-terminal methionine residue, which may induce immune responses upon repeated or excessive administration to the body. Also, since these fusion molecules are expressed in a fusion protein form in *E. coli* by linking a gene encoding a therapeutic protein to an Fc gene, they are difficult to express in *E. coli*, or a therapeutic protein is difficult to produce in *E. coli* if its expression in *E. coli* in a fused form results in a significant decrease or loss of activity. Further, since the fusion of two molecules creates a non-naturally occurring abnormal amino acid sequence at the connection region between two proteins, the fusion protein could potentially be recognized as "non-self" by the immune system, and thus induce immune responses.

To solve these problems, the present inventors previously prepared an Fc fragment and a protein drug as separate polypeptides, not using a fusion method based on

genetic recombination but using the best expression systems, and covalently linking the two polypeptides together to use the Fc fragment as a drug carrier. In this case, it is possible to prepare a conjugate of a glycosylated polypeptide drug and an aglycosylated Fc, which does not induce undesirable immune responses but has satisfactory properties of physiological drug activity, *in vivo* duration and stability.

In the above case, since it is preferable that the Fc is in an aglycosylated form, a prokaryotic expression system such as *E. coli* is used. Protein production methods using an *E. coli* expression system have several advantages over conventional methods using animal cells, as follows. An *E. coli* expression vector can be easily constructed, thus allowing rapid evaluation of protein expression. Due to its rapid growth rate, *E. coli* allows mass production of a protein of interest at low cost. Also, a relatively simple expression process can be used. Thus, *E. coli* is more useful for commercial production than other host cells.

Most Fc regions are present as inclusion bodies upon overexpression in E. coli. For this reason, industry demands that Fc regions be expressed in water-soluble form in E. coli. European Pat. No. 0227110 discloses the production of the immunoglobulin G1 Fc region using only the product (the cell lysate) which is expressed in water soluble form upon the overexpression of the immunoglobulin G1 Fc region.

However, only the immunoglobulin expressed in water-soluble form is as low as 15 mg/L in yield, which has no value in terms of industrial usefulness. Korean Pat. Appl'n No. 0092783, overcoming the problem encountered in the prior art, introduces a novel technique of expressing an immunoglobulin Fc region not as inclusion bodies but in a water-soluble form in E. coli through the fusion of the nucleotide sequence corresponding to the Fc region to an E. coli signal sequence. Upon expression in E. coli, the protein of interest is present a soluble form devoid of the signal peptide with the production yield thereof increased to as much as 600 mg/L.

Leading to the present invention, intensive and thorough research into a method of producing active aglycosylated immunoglobulin Fc regions free of immune response, conducted by the present inventors, aiming to increase the production yield to a level suitable for industrialization, resulted in the finding that when a nucleotide sequence encoding an immunoglobulin Fc region is expressed in a form fused at the N terminus to a specific hinge region, the immunoglobulin Fc region is expressed as inclusion bodies which are finally a dimer or a monomer of immbunoglobulin Fc region devoid of initial methionine residues through solubilization and refolding processes.

Disclosure of the Invention

It is therefore an object of the present invention to provide a method of mass-producing an immunoglobulin Fc region free of initial methionine residue, comprising constructing a vector including a nucleotide sequence coding for a recombinant immunoglobulin Fc region containing an immunoglobulin hinge region; transforming a prokaryotic cell with the vector; culturing a resulting transformant; and isolating and purifying the immunoglobulin Fc region expressed in an inclusion body from the transformant.

It is another object of the present invention to provide a dimer or a monomer of an immunoglobulin Fc region prepared by the above method.

Brief Description of the Drawings

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 an electrophoresis gel photograph showing the formation of monomeric and dimeric Fc region fragments from inclusion bodies expressed using an expression vector having a nucleotide encoding a human immunoglobulin IgG4 Fc region;

FIG. 2 shows the results of ELISA for the C1q binding

capacity of human immunoglobulin IgG4 Fc region;

FIG. 3 shows the results of ELISA for the FcγRI binding capacity of human immunoglobulin IgG Fc region;

FIG. 4 shows the results of ELISA for the FcγRIII binding capacity of human immunoglobulin IgG Fc region;

FIG. 5 shows the results of ELISA for the FcRn$\alpha\beta_2$ binding capacity of human immunoglobulin IgG Fc region;

FIG. 6 shows the results of serum half lives of an EPO-PEG-Fc conjugate prepared using a human immunoglobulin IgG Fc region as a carrier;

FIG. 7 is a photograph of a 15% SDS-PAGE gel on which after being mixed with equal volumes of a 2x protein sample buffer, parts of the fermented solutions obtained by growing microbial transformants of Example 2 in fermentors under an expression condition are run;

FIG. 8 is a photograph of an SDS-PAGE gel on which the proteins refolded from the inclusion bodies expressed by the transformants of Example 2 are separated and visualized as bands;

FIG. 9 is a photograph of a 15% SDS-PAGE gel on which after being mixed with equal volumes of a 2x protein sample buffer, parts of the fermented solutions obtained by growing microbial transformants of Example 3 in fermentors under an expression condition are run; and

FIG. 10 is a photograph of a 15% SDS-PAGE gel on which after being mixed with a protein sample buffer free

of a reducing agent such as DTT or beta-mercaptoethanol, the respective products expressed and purified in Example 3 are run.

Best Mode for Carrying Out the Invention

In one aspect, the present invention relates to a method of mass-producing an immunoglobulin Fc region, comprising constructing a vector including a nucleotide sequence coding for a recombinant immunoglobulin Fc region containing an immunoglobulin hinge region; transforming a prokaryotic cell with the vector; culturing the resulting transformant; and isolating and purifying the immunoglobulin Fc region, expressed in an inclusion body form, from the transformant.

The present invention pertains to a method of mass-producing an immunoglobulin Fc region useful as a carrier for protein drugs. When an immunoglobulin Fc region is fused at the N terminus to a hinge region, the resulting recombinant immunoglobulin Fc region is found to be expressed as an inclusion body and then be solubilized and refolded into a dimer or monomer in an active form devoid of the initial methionine residue encoded by the initiation codon. The present invention is of great significance in terms of the finding that, when fused to an immunoglobulin Fc region, a hinge region plays a critical role in

processing and refolding the recombinant Fc region into a native sequence form devoid of the initial methionine residue encoded by the initiation codon.

The hinge region capable of allowing an immunoglobulin Fc region to be mass produced in a recombinant form therewith may be a derivative from IgG, IgA, IgM, IgE or IgD of humans and other animals, including goats, swine, mice, rabbits, hamsters, rats and guinea pigs, with preference for a derivative of IgG, e.g., IgG1, IgG2, IgG3, or IgG4 (SEQ. ID. NOS. 14 to 17). The hinge region useful in the present invention may be a full-length hinge region or a fragment thereof. Preferable is a hinge region fragment having two or more consecutive amino acid sequences, which are more preferable when containing at least one cystein residue therein. Of practical use in the present invention are fragments of the hinge region derived from IgG4 of SEQ. ID. NO. 17, which are represented by SEQ. ID. NOS. 18, 19, 20 and 21. When hinge regions of SEQ. ID. NOS. 18, 19 and 20 are employed, the immunoglobulin Fc region can be prepared in a dimer or monomer form. The hinge region of SEQ. ID. NO. 21 effectively affords the preparation of a monomer of the immunoglobulin Fc region. In other implementations of the present invention, fragments, represented by SEQ. ID. NOS. 48 to 55, of the hinge region derived from IgG1 of SEQ. ID. NO. 14 and, represented by SEQ. ID. NOS. 56 to 60, of the hinge region

derived from IgG2 of SEQ. ID. NO. 15 were used to produce a dimer of the immunoglobulin Fc region.

The immunoglobulin Fc region capable of being produced by the present invention may be a native form isolated from humans and other animals including goats, swine, mice, rabbits, hamsters, rats and guinea pigs, or may be a recombinant or a derivative thereof, obtained from transformed animal cells or microorganisms. Preferred may be an Fc region of IgG, IgA, IgM, IgE and IgD from humans, or a combination or hybrid thereof. The term "combination", as used herein, means that polypeptides encoding single-chain immunoglobulin Fc fragments of the same origin are linked to a single-chain polypeptide of a different origin to form a dimer or multimer. The term "hybrid", as used herein, means that sequences encoding two or more immunoglobulin Fc fragments of different origins are present in a single-chain immunoglobulin Fc fragment. The immunoglobulin may preferably be an Fc region of IgG1, IgG2, IgG3 and IgG4, or a combination or hybrid thereof. Nucleotide sequences encoding human immunoglobulin Fc regions and amino acid sequences limited to the same, useful in the present invention, may be those encoded by nucleotide sequences from the GenBank and/or EMBL databases.

The immunoglobulin Fc region of the present invention includes an amino acid sequence derivative. The term "amino

acid sequence derivative" means a sequence in which one or more amino acid residues differ from a wild-type amino acid sequence, and may naturally occur or be artificially generated. The immunoglobulin Fc region includes derivatives resulting from a deletion, an insertion, a non-conservative or conservative substitution or combinations thereof. An insertion is typically made by the addition of a consecutive amino acid sequence of about 1 to 20 amino acids, or may be made with a longer sequence. A deletion is typically in the range of about 1 to 30 amino acid residues. Amino acid exchanges in proteins and peptides, which do not generally alter the activity of the proteins or peptides, are known in the art (H. Neurath, R. L. Hill, The Proteins, Academic Press, New York, 1979). The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Thy/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu and Asp/Gly, in both directions. Such derivatives may be prepared through a chemical peptide synthesis method or a DNA sequence-based recombination method, which are known in the art (Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory Press, New York, USA, 2d Ed., 1989).

In addition, the immunoglobulin Fc region, if desired, may be modified through phosphorylation, sulfation, acrylation, glycosylation, methylation,

farnesylation, acetylation, amidation, and the like.

The immunoglobulin derivative of the present invention is preferably a functional equivalent to its natural form, thus having a similar biological activity, or, if desired, could be made by altering the property of the natural form. Preferably, the derivatives of the immunoglobulin Fc region are proteins that have increased structural stability against heat, pH, etc., or solubility, or that have improved characteristics in terms of disulfide bond formation, compatibility with an expression host, complement binding, Fc receptor binding and antibody-dependent cell-mediated cytotoxicity (ADCC), so long as the derivatives produced are not so different from natural forms of humans that they induce unwanted immune responses in humans and animals. Preferred derivatives are IgG1 Fc regions which are altered in such a specific residue as to have reduced affinity to Fc receptors mediating antibody-dependent cell-mediated cytotoxicity (ADCC). A derivative produced may contain a deletion or a substitution with another amino acid in the leucine residue at position 234 of an IgG1 CH2 sequence (see the sequence from the Kobat database for the numbering of the amino acid residues). Most preferably, Leu234 is replaced by phenylalanine, an amino acid residue at a corresponding position in IgG4.

In accordance with the present invention, a nucleotide sequence coding for a recombinant immunoglobulin

Fc region in which an immunoglobulin Fc region is fused to an immunoglobulin hinge region is prepared. As used herein, the term "recombinant immunoglobulin Fc region" means an immunoglobulin Fc region linked at the N terminus to a hinge region via a peptide bond.

Depending on the immunoglobulin Fc region, the hinge region to be fused may be chosen. Preferable is a hinge region which is the same in origin as the immunoglobulin Fc region. In the actual practice of the present invention, a nucleotide sequence coding for a recombinant immunoglobulin Fc region consisting of an amino acid sequence set forth as SEQ. ID. NOS. 7, 9, 11 or 13, in which an IgG4-derived Fc region is fused to a hinge region consisting of an amino acid sequence set forth as SEQ. ID. NOS. 18, 19, 20 or 21, was prepared. The nucleotide sequences coding for the recombinant immunoglobulin Fc regions are represented by SEQ. ID. NOS. 6, 8, 10 and 12, respectively.

In another implementation, prepared was a nucleotide sequence coding for a recombinant immunoglobulin Fc region consisting of an amino acid sequence set forth as in SEQ. ID. NO. 23, 25, 27, 29, 31, 33, 35 or 37, in which an IgG1-derived Fc region is fused to a hinge region consisting of an amino acid sequence set forth as one of SEQ. ID. NOS. 48 to 55. The resulting nucleotide sequences encoding the recombinant immunoglobulin Fc regions are represented by SEQ. ID. NOS. 22, 24, 26, 28, 30, 32, 34 and 36.

In a further implementation, prepared was a nucleotide sequence coding for a recombinant immunoglobulin Fc region consisting of an amino acid sequence set forth as in SEQ. ID. NO. 39, 41, 43, 45 or 47, in which an IgG2-derived Fc region is fused to a hinge region consisting of an amino acid sequence set forth as one of SEQ. ID. NOS. 56 to 60. The resulting nucleotide sequences encoding the recombinant immunoglobulin Fc regions are represented by SEQ. ID. NOS. 38, 40, 42, 44 and 46.

In accordance with the present invention, recombinant expression vectors to which nucleotide sequences encoding the recombinants immunoglobulin Fc regions are operably linked are provided.

The term "recombinant expression vector", as used herein, which describes a vector capable of expressing a target protein in a suitable host cell, refers to a genetic construct that comprises essential regulatory elements to which a gene insert is operably linked in such a manner as to be expressed in a host cell.

The term "operably linked", as used herein, refers to a functional linkage between a nucleic acid expression control sequence and a second nucleic acid sequence coding for a target protein in such a manner as to allow general functions. The operable linkage to a recombinant vector may be prepared using a genetic recombinant technique well known in the art, and site-specific DNA cleavage and

ligation may be carried out using enzymes generally known in the art. A suitable expression vector includes expression regulatory elements, such as a promoter, an initiation codon, a stop codon, a polyadenylation signal and an enhancer. The initiation and stop codons are necessary for functionality in an individual to whom a genetic construct has been administered, and must be in frame with the coding sequence. The promoter of the vector may be constitutive or inducible. In addition, expression vectors include a selectable marker that allows selection of host cells containing the vector, and replicable expression vectors include a replication origin. In the detailed practice of the present invention, the following recombinant expression vectors are prepared: pmSCPFc, pmPSCFc, pmCPSFc, pmCPFc, pMEPKFC1, pMSCKFc1, pMDKTFc1, pMCPAFc1, pMPKSFc1, pMCPPFc1, pMPPCFc, pMPCPFc, pmPPCG2Fc, pmPCPG2Fc, pmCPG2Fc, pmCCVG2Fc and pmCVE2Fc.

The recombinant expression vectors expressing the proteins are transformed into host cells.

With respect to the object of the present invention, the host cells are prokaryotic cells in which glycosylation does not occur. Examples of these prokaryotic cells include *Escherichia coli*, *Bacillus subtilis*, *Streptomyces*, *Pseudomonas*, *Proteus mirabilis* and *Staphylococcus*, with preference for *E. coli*. Illustrating, non-limiting examples of *E. coli* strains include BL21 (DE3), JM109, DH series,

TOP10 and HB101. More preferable is the BL21 (DE3) strain. Because it lacks a system for protein glycosylation, *E. coli* can be used as a host cell in which an immunoglobulin Fc region is produced in the form of being devoid of sugar moieties that are present in a CH2 domain of a native immunoglobulin. Sugar moieties of the immunoglobulin CH2 domain do not affect the structural stability of immunoglobulins, but cause immunoglobulins to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) upon association with cells expressing Fc receptors and immune cells, in order to secrete cytokines to induce inflammation. Also, the sugar moieties bind to the C1q part of the first complement component C1, leading to complement fixation. Thus, when an immunoglobulin Fc region is produced in an aglycosylated form and linked to a therapeutic protein, the therapeutic protein is present in the serum for a prolonged period of time without the undesirable effector functions of immunoglobulins.

The transformation of the recombinant expression vectors into prokaryotic cells can be achieved by any method that allows nucleic acids to be introduced into cells and, as known in the art, may be performed by selecting suitable standard techniques according to host cells. These methods include, but are not limited to, electroporation, protoplast fusion, calcium phosphate ($CaPO_4$) precipitation, calcium chloride ($CaCl_2$)

precipitation, agitation with silicon carbide fiber, and PEG-, dextran sulfate- and lipofectamine-mediated transformation.

In the detailed practice of the present invention, the recombinant expression vectors are individually introduced into *E. coli*, thus generating the following transformants: BL21/pmSCPFc(HM11200), BL21/pmPSCFc(HM11201), BL21/pmCPSFc(HM11204), BL21/pmCPAFc(HM11205), BL21/pMEPKFc1(HM11206), BL21/pMSCDFc1(HM11207), BL21/pMDKTFc1(HM11208), BL21/pMCPAFc1(HM11209), BL21/pMPKSFc1(HM11210), BL21/pMCPPFc1(HM11211), BL21/pMPPCFc1(HM11212), BL21/pMPCPFc1(HM11213), BL21/pmPPCPG2Fc(HM11214), BL21/pmPCPG2Fc(HM11215), BL21/pmCPG2Fc(HM11216) and BL21/pmCCVG2Fc(HM11217), BL21/pmCVEG2Fc(HM11218).

The transformants anchoring the recombinant expression vectors thereat are cultured through a general method.

Culture conditions may be easily adjusted according to bacterial strain by those skilled in the art. Typically, the medium used for the culture should contain all nutrients essential for the growth and survival of cells. The medium should contain a variety of carbon sources, nitrogen sources and trace elements. Examples of available carbon sources include glucose, sucrose, lactose, fructose, maltose, starch, carbohydrates such as cellulose, fats such

as soybean oil, sunflower oil, castor oil and coconut oil, fatty acids such as palmitic acid, stearic acid and linoleic acid, alcohols such as glycerol and ethanol, and organic acids such as acetic acid. These carbon sources may be used singly or in combinations of two or more. Examples of available nitrogen sources include organic nitrogen sources, such as peptone, yeast extract, meat extract, malt extract, corn steep liquor (CSL) and soybean whey, and inorganic nitrogen sources, such as urea, ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. These nitrogen sources may be used singly or in combinations of two or more. A phosphorus source may be contained in the medium, which includes potassium dihydrogen phosphate, dipotassium hydrogen phosphate and corresponding sodium-containing salts. In addition, the medium may contain a metal salt, such as magnesium sulfate or iron sulfate. The medium may further include amino acids, vitamins, suitable precursors, and the like. The pH of the culture may be adjusted by adding a compound, such as ammonium hydroxide, potassium hydroxide, ammonia, phosphoric acid and sulfuric acid, to the culture using a suitable method. Also, during the culture, antifoaming agents, such as polyglycol fatty acid esters, may be used to prevent bubble formation. To maintain the culture in a desirable state, oxygen or an oxygen-containing gas (e.g., air) is introduced into the culture.

The temperature of the culture is generally 20°C to 45°C, and preferably 25°C to 45°C. Also, a fermentor can be used for protein production on a large scale. Protein production using a fermentor should be carried out taking into consideration several factors, including the growth rate of host cells and protein expression levels. Protein expression may be induced through adding, for example, IPTG to the medium under suitable culture conditions.

An immunoglobulin Fc region overexpressed as inclusion bodies may be purified through a general technique. The immunoglobulin Fc regions produced in the transformants may be obtained by disrupting cells using a French press, an ultrasonicator, etc., collecting only water-insoluble inclusion bodies containing the immunoglobulin Fc region through centrifugation, solubilizing and denaturing the collected fraction with refolding agents, such as urea, guanidine, arginine, cystein, beta-mercaptoethanol, etc. to the refolding thereof, and purifying the refolded fusion protein through dialysis, various chromatographies, such as gel filtration, ion exchange and reverse phase column chromatography, and ultrafiltration, alone or in combination. Generally, this refolding process is very complicated and is known to produce a very low refolding yield and assure the refolded protein only of lower activity than that of the water-soluble protein.

However, the method of the present invention can overcome the above-mentioned problems and produce an active immunoglobulin Fc region devoid of the initial methionine residue on a mass scale. On the whole, when expressed and produced in E. coli, an exogenous protein has an initial methionine residue encoded by the initiation codon. Repetitive or excessive administration of the protein product having the initial methionine to human bodies may cause an immune response sufficient to reduce the therapeutic effect thereof or to be toxic. However, when the recombinant immunoglobulin Fc region of the present invention is expressed in E. coli, the initial methionine residue is found to be cleaved by aminopeptidase, an intrinsic cytoplasmic enzyme, as measured by N-terminal sequencing analysis (Adams et al., J. Mol. Biol. 33:571-589, 1968, Takeda, Proc. Natl. Acad. Sci. USA 60:1487-1494, 1968). The activity of such aminopeptidases is known to depend on the sequence and structure of the protein of interest (Moerschell et al., J. Biol. Chem. 265:19638-19643, 1990, James et al., Protein Expression and Purification 41:45-52, 2005). A hinge region, when fused to an immunoglobulin Fc region, is affected by aminopeptidase so that the initial methionine is processed to an extent that depends on the amino acid sequence thereof.

Because properties of the hinge region determine the post-translational modification of proteases, the ratio of

dimers to monomers can be effectively controlled by selecting proper hinge regions. In addition, when inclusion bodies are refolded, the formation of accurate dimers is hindered by the mismatching of cysteins in disulfide bonds. However, the method according to the present invention ensures the formation of accurate disulfide bonds, thereby leading to the formation of active dimers.

In addition, the present invention can produce immunoglobulin Fc regions on a larger scale than can conventional methods. For example, an immunoglobulin Fc region is produced at a yield of 15 mg/L according to the method of European Pat. No. EP0227110, in which a G1 Fc region is overexpressed and purified only from a cell lysate containing the water-soluble form thereof, and at a yield of 50 to 600 mg/L according to the method of Korean Pat. Appl'n No. 0092783, in which an immunoglobulin Fc region fused to an E. coli signal sequence is expressed in a water-soluble form, but not as an inclusion body. However, the present invention can produce an immunoglobulin Fc region at a yield of as high as 3 to 6 g/L by purifying an inclusion body of a recombinant immunoglobulin Fc region containing a hinge region. Thus, the method of the present invention ensures a highly useful system for producing immunoglobulin Fc regions on an industrial scale at much higher yield than to conventional methods.

In another aspect, the present invention relates to an immunoglobulin Fc region prepared according to the above method.

The immunoglobulin Fc region produced in prokaryotic cells such as *E. coli* according to the present method does not have specifically limited industrial applications. One exemplary application is use as a carrier for the formation of a conjugate with a certain drug. Construction of the conjugate comprising the immunoglobulin Fc region linked to a drug is not specifically limited. For example, the immunoglobulin Fc region and the drug may be linked together at various ratios, and the linkage may be mediated, for example, through a linker.

The drug includes polypeptides, compounds, extracts and nucleic acids. Preferred is a polypeptide drug (used to have a meaning identical to the word "protein"). Examples of the linker useful in the present invention include peptide and non-peptide linkers, with preference for a non-peptide linker and higher preference for a non-peptide polymer. A preferred example of the immunoglobulin heavy chain is Fc.

If the serum half-life needs to be enhanced, any physiologically active polypeptide may be used without specific limitation as a protein partner of the immunoglobulin Fc region prepared according to the present method to form a conjugate. Such physiologically active

polypeptides include those used for treating or preventing human diseases, which include cytokines, interleukins, interleukin binding protein, enzymes, antibodies, growth factors, transcription regulatory factors, coagulation factors, vaccines, structural proteins, ligand proteins or receptors, cell surface antigens, receptor antagonists, and derivatives and analogues thereof.

In detail, non-limiting examples of the physiologically active polypeptide include human growth hormone, growth hormone releasing hormone, growth hormone releasing peptide, interferons and interferon receptors (e.g., interferon-α, -β and -γ, water-soluble type I interferon receptor, etc.), colony stimulating factors, interleukins (e.g., interleukin-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -12, -13, -14, -15, -16, -17, -18, -19, -20, -21, -22, -23, -24, -25, -26, -27, -28, -29, -30, etc.) and interleukin receptors (e.g., IL-1 receptor, IL-4 receptor, etc.), enzymes (e.g., glucocerebrosidase, iduronate-2-sulfatase, alpha-galactosidase-A, agalsidase alpha and beta, alpha-L-iduronidase, butyrylcholinesterase, chitinase, glutamate decarboxylase, imiglucerase, lipase, uricase, platelet-activating factor acetylhydrolase, neutral endopeptidase, myeloperoxidase, etc.), interleukin and cytokine binding proteins (e.g., IL-18bp, TNF-binding protein, etc.), macrophage activating factor, macrophage peptide, B cell factor, T cell factor, protein A, allergy

inhibitor, cell necrosis glycoproteins, immunotoxin, lymphotoxin, tumor necrosis factor, tumor suppressors, metastasis growth factor, alpha-1 antitrypsin, albumin, alpha-lactalbumin, apolipoprotein-E, erythropoietin, highly glycosylated erythropoietin, angiopoietins, hemoglobin, thrombin, thrombin receptor activating peptide, thrombomodulin, factor VII, factor VIIa, factor VIII, factor IX, and factor XIII, plasminogen activating factor, fibrin-binding peptide, urokinase, streptokinase, hirudin, protein C, C-reactive protein, renin inhibitor, collagenase inhibitor, superoxide dismutase, leptin, platelet-derived growth factor, epithelial growth factor, epidermal growth factor, angiostatin, angiotensin, bone growth factor, bone stimulating protein, calcitonin, insulin, atriopeptin, cartilage inducing factor, elcatonin, connective tissue activating factor, tissue factor pathway inhibitor, follicle stimulating hormone, luteinizing hormone, luteinizing hormone releasing hormone, nerve growth factors (e.g., nerve growth factor, ciliary neurotrophic factor, axogenesis factor-1, brain-natriuretic peptide, glial derived neurotrophic factor, netrin, neurophil inhibitor factor, neurotrophic factor, neuturin, etc.), parathyroid hormone, relaxin, secretin, somatomedin, insulin-like growth factor, adrenocortical hormone, glucagon, cholecystokinin, pancreatic polypeptide, gastrin releasing peptide, corticotropin releasing factor, thyroid

stimulating hormone, autotaxin, lactoferrin, myostatin, receptors (e.g., TNFR(P75), TNFR(P55), IL-1 receptor, VEGF receptor, B cell activating factor receptor, etc.), receptor antagonists (e.g., IL1-Ra etc.), cell surface antigens (e.g., CD 2, 3, 4, 5, 7, 11a, 11b, 18, 19, 20, 23, 25, 33, 38, 40, 45, 69, etc.), monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., scFv, Fab, Fab', F(ab')2 and Fd), and virus derived vaccine antigens. The physiologically active polypeptide useful in the present invention may be a native form, may be produced by genetic recombination using prokaryotic cells, such as *E. coli,* or eukaryotic cells, such as yeast cells, insect cells and animal cells, or may be a derivative having one or more amino acid mutations but showing biological activity identical to that of the native form.

In a preferred embodiment of the present invention, an immunoglobulin Fc region fragment produced using the HM11201 transformant was linked to human erythropoietin (EPO) using polyethylene glycol, thus providing an EPO-PEG-immunoglobulin Fc region protein conjugate. This protein conjugate was found to exhibit extended serum half-life compared not only to the native EPO but also to Aranesp (Amgen), known as a second generation EPO having improved serum half-life. Thus, the immunoglobulin Fc region devoid of the initial methionine residue, obtained from inclusion bodies using a hinge region in accordance with the present

invention, can be used to enhance the serum half-life and physiological activity of the physiologically active polypeptide linked thereto, with no risk of immune response induction.

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 Human Immunoglobulin IgG4 Fc Region Expression Vector, Expression and Purification of IgG4 Fc Region, and N-Terminal Sequence Analysis

<1-1> Construction of IgG4 Fc region expression vector

To clone a heavy chain Fc region including the hinge region of human immunoglobulin IgG4, RT-PCR was carried out with RNA from human blood cells serving as a template, as follows. First, total RNA was isolated from about 6 ml of blood using a Qiamp RNA blood kit (Qiagen), and gene amplification was performed using the total RNA as a template with the aid of a One-Step RT-PCR kit (Qiagen). To amplify genes having different N-terminal sequences, pairs of primers represented by SEQ ID NOS. 1 and 2, 3 and 2, 4 and 2, and 5 and 2 were used. To facilitate a subsequent gene cloning procedure, an Nde I recognition site and the initiation codon ATG, necessary for protein expression,

were introduced into 5′ primers of SEQ ID NOS. 1, 3, 4 and 5, and a BamHI recognition site containing a stop codon into 3′ primers of SEQ ID NO. 2. The amplified Fc region products were digested with Nde I and Hind III, and inserted into a pET22b (Novagen) treated with the same restriction enzyme, thus giving respective recombinant plasmids. These plasmids were designed to have parts of the total amino acid sequence Glu-Ser-Lys-Tyr-Gly-Pro-Pro-Cys-Pro-Ser-Cys-Pro of the IgG4 hinge region, as follows.

The plasmid which contained a gene amplified with SEQ ID NOS. 1 and 2 was named pmSCPFc and anchored thereto a DNA sequence coding for an N-terminal amino acid sequence starting with Met-Ser-Cys-Pro, which was determined through base sequencing to have SEQ ID NO. 6, corresponding to the amino acid sequence of SEQ ID NO. 7. The plasmid which contained a gene amplified with SEQ ID NOS. 3 and 2 was named pmPSCFc and anchored thereto a DNA sequence coding for an N-terminal amino acid sequence starting with Met-Pro-Ser-Cys-Pro, which was determined through base sequencing to have SEQ ID NO. 8, corresponding to the amino acid sequence of SEQ ID NO. 9. A plasmid which contained a gene amplified with SEQ ID NOS. 4 and 2 was named pmCPSFc and anchored thereto a DNA sequence coding for an N-terminal amino acid sequence starting with Met-Cys-Pro-Ser-Cys-Pro, which was determined through base sequencing to have SEQ ID NO. 10, corresponding to the amino acid

sequence of SEQ ID NO. 11. A plasmid which contained a gene amplified with SEQ ID NOS. 5 and 2 was named pmCPFc and anchored thereto a DNA sequence coding for an N-terminal amino acid sequence starting with Met-Cys-Pro, which was determined through base sequencing to have SEQ ID NO. 12 corresponding to SEQ ID NO. 13.

The expression vectors were transformed into *E. coli* BL21 (DE3) to prepare transformants respectively designated BL21/pmSCPFc(HM11200), BL21/pmPSCFc(HM11201), BL21/pmCPSFc(HM11204) and BL21/pmCPFc(HM11205). The transformants BL21/pmSCPFc(HM11200) and BL21/pmPSCFc(HM11201) were deposited at the Korean Culture Center of Microorganisms (KCCM) on June 20, 2005 with Accession Nos. KCCM-10659P and KCCM-10660P, respectively, and the transformants BL21/pmCPSFc(HM11204) and BL21/pmCPFc(HM11205) at KCCM on July 28, 2005 with Accession Nos. KCCM-10665P and KCCM-10666P, respectively.

<1-2> Expression and purification of IgG4 Fc

The bacterial transformants prepared in Example <1-2> were inoculated in respective fermentors (Marubishi Company) and allowed to grow, followed by determining whether they expressed immunoglobulin Fc region fragments.

First, each transformant was grown in 100 ml of LB medium with agitation overnight, and inoculated in a fermentor for large-scale culture. The fermentor was

maintained at 28°C or 35°C. To prevent conversion from an aerobic to an anaerobic environment, the cultures were aerated with 20-vvm air and stirred at 500 rpm. To compensate for the insufficient nutrients for bacterial growth during fermentation, the cultures were supplemented with glucose and yeast extracts according to the fermentation states of bacteria. When the cultures reached an OD_{600} value of 80, an inducer, IPTG, was added to the cultures to induce protein expression. The cultures were further cultured for 40 to 45 hrs to increase the OD value at 600 nm to 100 to 120.

The expression of immunoglobulin Fc, the formation of inclusion bodies, and the dimer formation of the expressed Ig Fc in the *E. coli* transformants were examined as follows. To investigate the overall intracellular expression of the immunoglobulin Fc regions, parts of the fermented solutions were mixed with equal volumes of 2x protein sample buffer and electrophoresed on a 15% SDS-PAGE gel (Criterion Gel, Bio-Rad). As a result, immunoglobulin Fc was observed to be overexpressed in all of the transformants produced. Then, cells were disrupted using an ultrasonicator (Misonix Company). The cell lysate thus obtained was centrifuged to separate water-soluble substances from water-insoluble substances. Most of the overexpressed substances were found to exist as inclusion bodies, as measured by electrophoresis on 15% SDS-PAGE. The

inclusion bodies were subjected to the following refolding process in order to examine to what degree Fc was refolded and whether and to what degree dimeric Fc regions were formed. 10 g of the fermented solution was subjected to ultrasonication in 100 mL of a lysis buffer (10mM Tris, pH9.0, 1mM EDTA, 0.5% Triton X-100, 0.2M NaCl) to disrupt the cells. Centrifugation at 10,000 rpm for 20 min divided the cell lysate into a water-soluble fraction and a water-insoluble fraction as an inclusion body. 2 g of this inclusion body was dissolved in a mixture of 20 mL of 1M Tris (pH 9.0) and 20 mL of a solubilization buffer (6M Guanidine, 50mM Tris) and allowed to react while being gently agitated at 4°C for 30 min. Following completion of the reaction, the inclusion body solution was mixed overnight with 10 volumes of a refolding buffer (2 M urea, 50 mM Tris, 0.25 M Arginine, 3 mM cysteine, pH 9.0) with gentle agitation. To this mixture was added a protein sample buffer free from any reducing agent, such as DTT or beta-mercaptoethanol, followed by electrophoresis on 15% SDS-PAGE (Criterion Gel, Bio-Rad). The protein bands were visualized with a dye such as Coomassie Brilliant. FIG. 1 is a photograph taken of a gel on which proteins refolded from the inclusion bodies expressed by the reformant HM11201 at 32°C (lane 1) and 28°C (lane 2), by HM11200 at 28°C (lane 3) and 32°C (lane 4), by HM11204 at 28°C (lane 5) and 32°C (lane 6), and by HM11205 at 32°C (lane 7) and

28°C (lane 8) were run in the presence of an electric field, along with an Fc protein, as a control, purified from E. coli according to a conventional method (lane C). As seen in FIG. 1, a significant portion of the total proteins is attributed to the Fc protein, much of which exists in a dimeric form after being refolded. However, the Fc proteins differ in the ratio of dimers to monomers from one transformant to another, that is, according to the N-terminal amino acid sequence expressed by the transformant. For example, a significant portion of the Fc proteins of HM11201, which starts with Met-Pro-Ser-Cys-Pro-CH2-CH3, exists in a dimeric form. Almost all of the Fc proteins of HM11205, which start with Met-Cys-Pro-CH2-CH3, exist as monomers, but neither exist in dimeric forms. This is believed to be attributed to the fact that the processing specificity of aminopeptidase in E. coli host cells varies depending on the Fc N-terminal sequence.

<1-3> N-terminal sequence analysis

The dimeric Fc region fragments refolded from the inclusion bodies are different in amino acid sequence from the wild type because of the presence of the initial methionine residue. In order to determine whether the methionine residue is processed by E. coli proteases, N-terminal amino acid sequences of the proteins were analyzed by the Basic Science Research Institute, Seoul, Korea. The

samples used in the N-terminal amino acid sequence analysis were prepared as follows.

First, a PVDF membrane (Bio-Rad) was immersed in methanol for about 2-3 sec to activate it, and was sufficiently wet with a blocking buffer (170 mM glycine, 25 mM Tris-HCl (pH 8.0), 20% methanol). The protein samples separated on a non-reduced SDS-PAGE gel, prepared in Example <1-2>, were blotted onto a PVDF membrane for about one hour using a blotting kit (Hoefer Semi-Dry Transfer unit, Amersham). Proteins transferred onto the PVDF membrane were stained with a protein dye, Coomassie Blue R-250 (Amnesco), for a moment (3-4 sec), and washed with a destaining solution (water: acetic acid: methanol = 5: 1: 4). Then, membrane fragments containing proteins were cut out with scissors and subjected to N-terminal sequence analysis.

As a result, the IgG4 Fc proteins including a hinge region were found to have an N-terminal sequence of Glu-Ser-Lys-Tyr-Gly-Pro-Pro-Cys Pro-Ser-Cys-Pro-CH2-CH3. Amino acid sequences and N-terminal sequences of the proteins expressed in the transformants are given in the following Table 1.

TABLE 1

Trans-formants	N-terminal sequences	Results of sequence analysis	
		dimer	Monomer
HM11200	Met-Ser-Cys-Pro-CH2-	Ser-Cys-Pro-CH2	Pro-CH2

HM11201	Met-Pro-Ser-Cys-Pro-CH2-	Pro-Ser-Cys-Pro-CH2	Pro-Ser-Cys-Pro-CH2
HM11204	Met-Cys-Pro-Ser-Cys-Pro-CH2	Pro-Ser-Cys-Pro-CH2	mixed
HM11205	Met-Cys-Pro-CH2-CH3	-	Pro-CH2

Data from the amino acid sequencing analysis revealed that the Fc fragments refolded from the inclusion bodies produced by the *E. coli* transformants of the present invention were processed to have an accurate N-terminal sequence devoid of the initial methionine residue. The protein product which remains in a monomeric form even after refolding is deprived of cystein residues, and thus it cannot form dimers. In addition, as apparent from FIG. 1, the portion of monomer in the refolded Fc fragments differs from one transformant to another, and no dimers exist in HM11205. These results indicate that the amino acid sequence of the N terminal site has a great influence on the processing of the N terminus, so that a protein having a desired N-terminal sequence can be obtained by modulating the N-terminal sequence. Proteins, even if they have the same amino acid sequence, can be differently processed depending on the culture conditions of E. coli host cells, especially culture temperature, as revealed through the following tests. HM11200, when grown at low temperatures (28°C~32°C), expressed the Fc fusion protein in a solubilized form in the same amount as in the

inclusion body form. The solubilized form of the Fc fusion protein existed as a monomer devoid of the N-terminal amino acid sequence Met-Ser-Cys. Thus, the present inventors recognized that a controlled proportion of monomeric and dimeric immunoglobulin Fc fragments can be obtained by modulating the N-terminal amino acid sequence of the fusion Fc protein and the culture condition of host cells.

To quantitatively determine the expression of immunoglobulin Fc regions in the *E. coli* transformants, immunoglobulin Fc regions of the refolding solution were purified using a protein-A affinity column known to have strong affinity to immunoglobulins, as follows.

Inclusion bodies collected by centrifugation were refolded, and then purified through column chromatography. After 5 ml of a protein-A affinity column (Pharmacia) was equilibrated with PBS, the cell lysates were loaded onto the column at a flow rate of 5 ml/min. Unbound proteins were washed out with PBS, and bound proteins were eluted with 100 mM citrate (pH 3.0). The collected fractions were desalted using a HiPrep 26/10 desalting column (Pharmacia) with 10 mM Tris buffer (pH 8.0). Then, secondary anion exchange column chromatography was carried out using 50 ml of a Q HP 26/10 column (Pharmacia). The primary purified recombinant immunoglobulin Fc regions were loaded onto the Q-Sepharose HP 26/10 column (pharmacia), and the column was eluted with a linear gradient (0-0.2 M NaCl) in 10 mM Tris

buffer (pH 8.0), thus providing highly pure fractions. After being partially purified using the protein-A affinity column, expression levels of the recombinant Ig Fc regions were determined, and the results are given in Table 2, below.

TABLE 2

Plasmids	Transformants	Expression Yields After Protein-A Purification
pmSCPFc	HM11200	5-6 g/L
pmPSCFc	HM11201	4-5 g/L
pmCPSFc	HM11204	4-5 g/L
pmCPFc	HM11205	3-4 g/L

EXAMPLE 2: Construction of Human Immunoglobulin IgG1 Fc Region Expression Vector, Expression and Purification of IgG1 Fc Region, and N-Terminal Sequence Analysis

<2-1> Construction of IgG1 Fc region expression vector

To clone a heavy chain Fc region including the hinge region of human immunoglobulin IgG1, RT-PCR was carried out in the same manner as in Example <1-1>. To amplify genes having different N-terminal sequences, the following primers were used.

TABLE 3

Sequence of 5' Primers Used

	5' Primer Sequence
MEPK	5'GGA ATT CCA TAT GGA GCC CAA ATC TTG TGA CAA AAC TCA C 3'

MSCD	5'GGA ATT CCA TAT GTC TTG TGA CAA AAC TCA CAC ATG CCC 3'
MDKT	5'GGA ATT CCA TAT GGA CAA AAC TCA CAC ATG CCC ACC GTG C 3'
MCPA	5'GGG AAT TCC ATA TGT GCC CAG CAC CTG AAC TCC TGG GG
MPKS	5'GGG AAT TCC ATA TGC CCA AAT CTT GTG ACA AAA CTC AC
MCPP	5'GGG AAT TCC ATA TGT GCC CAC CGT GCC CAG CAC CTG AAC TCC
MPPC	5'GGA ATT CCA TAT GCC ACC GTG CCC AGC ACC TGA ACT CCT G 3'
MPCP	5'GGA ATT CCA TAT GCC GTG CCC AGC ACC TGA ACT CCT GGG G 3'

As for 3' primer, it had the sequence of 5'-CGC GGA TCC TCA TTT ACC CGG AGA CAG GGA GAG GCT CTT C-3' and was used for the amplification of all of the genes having different N-terminal sequences. To facilitate a subsequent gene cloning procedure, an Nde I recognition site was introduced into each of the 5′ primers, and a BamHI recognition site into the 3′ primer. The Fc region products amplified with pairs of the primers were inserted into a vector, thus giving respective recombinant plasmids designed to have parts of the total amino acid sequence Glu-Pro-Lys-Ser-Cys-Asp-Lys-Thr-His-Thr-Cys-Pro-Pro-Cys-Pro of the IgG1 hinge region as follows. The plasmid which contained a gene amplified with the MEPK primer was named pMEPKFc1, and anchored thereto a DNA sequence coding for the CH2 and CH3 of IgG1, starting with Met-Glu-Pro-Lys, which was analyzed through base sequencing to have SEQ ID NO. 22 corresponding to the amino acid sequence of SEQ ID

NO. 23. The plasmid, which contained a gene amplified with the MSCD primer, was named pMSCKFc1 and anchored thereto a DNA sequence coding for the CH2 and CH3 of IgG1, starting with Met-Ser-Cys-Asp, which was analyzed through base sequencing to have SEQ ID NO. 24, corresponding to the amino acid sequence of SEQ ID NO. 25. A plasmid which contained a gene amplified with the MDKT primer was named pMDKTFc1 and anchored thereto a DNA sequence coding for the CH2 and CH3 of IgG1, starting with Met-Asp-Lys-Thr, which was analyzed through base sequencing to have SEQ ID NO. 26 corresponding to the amino acid sequence of SEQ ID NO. 27. A plasmid which contained a gene amplified with the MCPA primer was named pMCPAFc1 and anchored thereto a DNA sequence coding for the CH2 and CH3 of IgG1, starting with Met-Cys-Pro, which was analyzed through base sequencing to have SEQ ID NO. 28, which corresponds to SEQ ID NO. 29. A plasmid which contained a gene amplified with the MPKS primer was named pMPKSFc1, and anchored thereto a DNA sequence coding for the CH2 and CH3 of IgG1, starting with Met-Pro-Lys-Ser, which was analyzed through base sequencing to have SEQ ID NO. 30, which corresponds to SEQ ID NO. 31. A plasmid which contained a gene amplified with the MCPP primer was named pMCPPFc1, and anchored thereto a DNA sequence coding for the CH2 and CH3 of IgG1, starting with Met-Cys-Pro-Pro, which was analyzed through base sequencing to have SEQ ID NO. 32, which corresponds to SEQ ID NO. 33.

A plasmid which contained a gene amplified with the MPPC primer was named pMPPCFc, and anchored thereto a DNA sequence coding for the CH2 and CH3 of IgG1, starting with Met-Pro-Pro-Cys, which was analyzed through base sequencing to have SEQ ID NO. 34, which corresponds to SEQ ID NO. 35. A plasmid which contained a gene amplified with the MPCP primer was named pMPCPFc, and anchored thereto a DNA sequence coding for the CH2 and CH3 of IgG1, starting with Met-Pro-Cys-Pro, which was analyzed through base sequencing to have SEQ ID NO. 36, corresponding to SEQ ID NO. 37. The expression vectors were transformed into *E. coli* BL21 (DE3) to prepare transformants respectively designated as BL21/pMEPKFc1(HM11206), BL21/pMSCDFc1(HM11207), BL21/pMDKTFc1(HM11208), BL21/pMCPAFc1(HM11209) BL21/pMPKSFc1(HM11210), BL21/pMCPPFc1(HM11211), BL21/pMPPCFc1(HM11212) and BL21/pMPCPFc1(HM11213).

<2-2> Expression and purification of IgG1 Fc

As in the case of IgG4, bacterial transformants prepared in Example <2-1> were inoculated in respective fermentors (Marubishi Company) and allowed to grow, followed by determining whether they expressed immunoglobulin Fc region fragments.

First, each transformant was grown in 100 ml of LB medium with agitation overnight and inoculated in the fermentor for large-scale culture. The fermentor was

maintained at 28°C or 35°C. To prevent conversion from an aerobic to an anaerobic environment, the cultures were aerated with 20-vvm air and stirred at 500 rpm. To compensate for the insufficient nutrients for bacterial growth during fermentation, the cultures were supplemented with glucose and yeast extracts according to the fermentation states of bacteria. When the cultures reached an OD_{600} value of 80, an inducer, IPTG, was added to the cultures to induce protein expression. The cultures were further cultured for 40 to 45 hrs to increase the OD value at 600 nm to 100 to 120.

The expression of immunoglobulin Fc, the formation of inclusion bodies, and the dimer formation of the expressed Ig Fc in the *E. coli* transformants were examined as follows. To investigate overall intracellular expression of the immunoglobulin Fc regions, the fermented solutions were aliquoted before and after the induction.

Parts of the fermented solutions were mixed with equal volumes of 2x protein sample buffer and electrophoresed on a 15% SDS-PAGE gel (Criterion Gel, Bio-Rad) under the following reducing conditions. The electrophoresis results are given in FIG. 7. A control of IgG4 Fc was run in lane 1, while the expression levels of the HM11208 transformant according to time are shown in lanes 2 to 4 and the expression levels of the HM11206 transformant according to time in lanes 5 to 7. Expression

levels in HM11207, HM11212, HM11209, HM11210, HM11213 and HM11211 transformants are shown in lanes 8 to 13, respectively. As seen in FIG. 7, a single 30 kDa-band (Fc region), which was not observed before the IPTG induction, very clearly appeared in all of the samples subjected to IPTG induction, indicating that the recombinant IgG1 Fc regions were expressed by contrast with the G4Fc control. Also, the Fc regions were overexpressed, amounting to at least about 30% of the total amount of proteins expressed.

To quantitatively determine the expression of immunoglobulin Fc regions in the *E. coli* transformants, immunoglobulin Fc regions of the refolding solution were purified using a protein-A affinity column known to have strong affinity to immunoglobulin in the same manner as that used for IgG4 Fc.

Of the transformants, the pMSCDFc plasmid transformant was measured to have the highest expression rate, amounting to as much as 340 mg per 10 g of inclusion body, while the pMDKTFc, pMEPKFc, pMPPCFc and pMPCPFc transformants showed expression rates of 133.3 mg, 159 mg, 110 mg and 120 mg, respectively.

Contents of dimeric IgG1 Fc in the expressed products were measured in the same manner as that used for the content of dimeric IgF4 Fc. Cells of the fermentation solutions were disrupted using an ultrasonicator (Misonix Company). The cell lysate thus obtained was centrifuged to

separate water-soluble substances from water-insoluble substances. Most of the overexpressed substances were found to exist as inclusion bodies as measured by electrophoresis on 15% SDS-PAGE. The inclusion bodies were refolded in order to examine to what degree Fc was refolded and whether and to what degree dimeric Fc regions were formed. The refolded Fc proteins were purified using a protein-A affinity column and mixed with a protein sample buffer free of a reducing agent, such as DTT or beta-mercaptoethanol, followed by electrophoresis on 15% SDS-PAGE (Criterion Gel, Bio-Rad). The protein bands were visualized with a dye such as Coomassie Brilliant.

FIG. 8 is a photograph taken of a gel on which protein-A column isolates of the proteins refolded from the inclusion bodies expressed by the reformant HM11208 (lane 1), by the reformant HM11206 (lane 2), by the reformant HM11207 (lane 4), by the reformant HM11212 (lane 5) and by the reformant HM11213 (lane 7) were run in the presence of an electric field under a non-reducing condition, along with an IgG4 Fc protein used as a control (lanes 3, 6, and 8). As shown in FIG. 8, all of the IgG1 Fc fragments used in the test were found to form dimers, although the amount thereof differed to some degree.

<2-3> N-terminal sequence analysis

As recognized in the case of IgG4 Fc, the N-terminal

amino acid sequence determined the post-translational processing as to whether the initial methionine residue remained or whether the initial methionine residue was accurately processed, or along with other amino acid residues to give amino acid sequences different from the desired one. In order to examine whether the methionine residue was processed by E. coli proteases, different N-terminal amino acid sequences of the IgG1 Fc regions were analyzed by the Basic Science Research Institute, Seoul, Korea. The analysis results are summarized in Table 4, below.

TABLE 4

Transformants	N-Terminal Sequencing Results (Dimers)
HM11208	Met
HM11206	Met
HM11207	Ser
HM11212	Pro
HM11213	Pro

As seen in Table 4, the initial methionine residue remains unprocessed in the transformants of HM11208 and HM11206, in which IgG1 Fc regions were overexpressed in dimeric forms while the fermented products of HM11207, HM11212 and HM11213 have no initial methionine residues as a result of the accurate post-translational processing.

Taken together, data obtained through the above-mentioned experiments indicate that when an IgG1 Fc region

is expressed in E. coli, the N-terminal sequence thereof determines the expression, expression level, dimer proportion and N-terminal processing thereof, and that Fc regions devoid of initial methionine residues can be produced on a mass scale by taking advantage of the N-terminal sequence. The IgG1 Fc regions obtained according to the present invention can be used to enhance the serum half-life and physiological activity of the physiologically active polypeptide linked thereto without immune response induction due to the addition of exogenous amino acid residues.

EXAMPLE 3: Construction of Human Immunoglobulin IgG2 Fc Region Expression Vector

<3-1> Construction of IgG2 Fc region expression vector

To clone a heavy chain Fc region including the hinge region of IgG2, RT-PCR was carried out in the same manner as that used for IgG4 Fc region. To amplify genes having different N-terminal sequences, the following primers were used.

TABLE 5

	5' Primer Sequences
G2MPPCSS	5' GGG AAT TCC ATA TGC CAC CGT GCC CAG CAC CAC CTG TGG CAG G 3'
G2MPCPSS	5' GGG AAT TCC ATA TGC CGT GCC CAG CAC CAC CTG TGG CAG GAC 3'
G2MCPSS	5' GGG AAT TCC ATA TGT GCC CAG CAC CAC CTG TGG CAG

	GAC 3'
G2MCCVSS	5' GGG AAT TCC ATA TGT GTT GTG TCG AGT GCC CAC CGT GCC CAG C 3'
G2MCVESS	5' GGG AAT TCC ATA TGT GTG TCG AGT GCC CAC CGT GCC CAG CAC C 3'

The 3' primer had the sequence of 5'-CGC GGA TCC TCA TTT ACC CGG AGA CAG GGA GAG GCT CTT C-3' and was applied for the amplification of all of the genes having different N-terminal sequences. To facilitate a subsequent gene cloning procedure, an Nde I recognition site was introduced into each of the 5′ primers, and a BamHI recognition site into the 3′ primer. The Fc region products amplified with pairs of the primers were inserted into a vector, thus giving respective recombinant plasmids designed to have parts of the total amino acid sequence Glu-Arg-Lys-Cys-Cys-Val-Glu-Cys-Pro-Pro-Cys-Pro of the IgG1 hinge region, as follows. The plasmid which contained a gene amplified with the G2MPPCSS primer was named pmPPCG2Fc and anchored thereto a DNA sequence coding for the CH2 and CH3 of IgG2, starting with Met-Pro-Pro-Cys, which was analyzed through base sequencing to have SEQ ID NO. 38, corresponding to the amino acid sequence of SEQ ID NO. 39. The plasmid which contained a gene amplified with the G2MPCPSS primer was named pmPCPG2Fc and anchored thereto a DNA sequence coding for the CH2 and CH3 of IgG2, starting with Met-Pro-Cys-Pro, which was analyzed through base sequencing to have SEQ ID NO. 40, corresponding to the amino acid sequence of SEQ ID

NO. 41. A plasmid which contained a gene amplified with the G2MCPSS primer was named pmCPG2Fc and anchored thereto a DNA sequence coding for the CH2 and CH3 of IgG2, starting with Met-Cys-Pro, which was analyzed through base sequencing to have SEQ ID NO. 42, corresponding to the amino acid sequence of SEQ ID NO. 43. A plasmid which contained a gene amplified with the G2MCCVSS primer was named pmCCVG2Fc, and anchored thereto a DNA sequence coding for the CH2 and CH3 of IgG2, starting with Met-Cys-Cys-Val-Glu-Cys-Pro-Pro-Cys-Pro, which was analyzed through base sequencing to have SEQ ID NO. 44, which corresponded to SEQ ID NO. 45. A plasmid which contained a gene amplified with the G2MCVESS primer was named pmCVEG2Fc and anchored thereto a DNA sequence coding for the CH2 and CH3 of IgG2, starting with Met-Cys-Val-Glu-Cys-Pro-Pro-Cys-Pro, which was analyzed through base sequencing to have SEQ ID NO. 46, which corresponds to SEQ ID NO. 47. The expression vectors were transformed into *E. coli* BL21 (DE3) to prepare transformants respectively designated BL21/pmPPCPG2Fc (HM11206), BL21/pmPCPG2Fc (HM11207), BL21/pmCPG2Fc (HM11216), BL21/pmCCVG2Fc (HM11217) and BL21/pmCVEG2Fc (HM11218).

<3-2> Expression, Purification and N-terminal Sequence Analysis of IgG2 Fc

As in the case of IgG4, the bacterial transformants

prepared in Example <3-1> were inoculated in respective fermentors (Marubishi Company) and allowed to grow, followed by determining whether they expressed immunoglobulin Fc region fragments. The culture conditions were not significantly different from those set forth for IgG4 Fc. The IgG2 Fc region fragments were found to be overexpressed under various conditions, including temperature, medium composition, inducer concentration, etc. as measured by SDS-PAGE in a reducing condition. FIG. 9 shows the result of a 15% SDS-PAGE of the fermentation solutions mixed with equal volumes of 2x protein sample buffer. An IgG4 Fc fragment was used as a control in lane 1 while the fragments expressed by the HM11214, HM11215, HM11216, HM11217 and HM11218 were run in lanes 2 to 6, respectively. As seen in FIG. 9, all of the five transformants used in the experiment overexpressed the Fc fragments.

The content of dimeric IgG4 Fc in the expressed products was measured in the same manner as described above. Cells of the fermentation solutions were disrupted and the water-insoluble substances of the cell lysate were refolded, after which only Fc region fragments were purified using a protein-A affinity column. The purified expression products were mixed with a protein sample buffer free of a reducing agent, such as DTT or beta-mercaptoethanol, and separated on 15% SDS-PAGE (Criterion

Gel, Bio-Rad). The protein bands were visualized with a dye such as Coomassie Brilliant. FIG. 10 shows the result of electrophoresis. An IgG4 Fc fragment was used as a control in lanes 1 and 7 while dimers of the fragment from the HM11214, HM11215, HM11216, HM11217, and HM11218 were observed in lanes 2 to 6. As is understood from data of FIG. 10, the expression products of the transformants, although different from one another with respect to N-terminal sequence or expression condition, can all form dimers.

In order to examine whether the methionine residue is processed by E. coli proteases, different N-terminal amino acid sequences of the dimeric IgG4 Fc regions were analyzed by the Basic Science Research Institute in Seoul, Korea. The initial methionine residue was removed from the products from the HM11214 and HM11215 transformants, both of which have a proline residue at position 2.

As apparent from these experiments, IgG2 Fc regions can be expressed on a large scale in E. coli. In addition, data obtained in the above-mentioned experiments indicate that the N-terminal sequence of an IgG1 Fc region determines the expression, expression level, dimer proportion and N-terminal processing thereof, and that Fc regions devoid of initial methionine residues can be produced on a mass scale by taking advantage of the N-terminal sequence. The IgG1 Fc regions obtained according

to the present invention can be used to enhance the serum half-life and physiological activity of the physiologically active polypeptide linked thereto without immune response induction due to the addition of exogenous amino acid residues.

EXAMPLE 4: C1q Binding Assay-Using ELISA

To determine whether the derivatives prepared in Example <1-2> and proteins corresponding to the Fc regions of immunoglobulins, expressed in the *E. coli* transformants and purified, bind to human C1q, an enzyme linked immunosorbent assay (ELISA) was carried out as follows. As test groups, immunoglobulin Fc regions produced by the HM11200 and HM11201 transformants prepared in the above Examples were used. As standards, a glycosylated immunoglobulin (IVIGG-globulin S, Green Cross PBM) was used. The test and standard samples were prepared in 10 mM carbonate buffer (pH 9.6) at a concentration of 1 μg/ml. The samples were aliquotted into a 96-well plate (Nunc) in an amount of 200 ng per well, and the plate was coated overnight at 4°C. Then, each well was washed with PBS-T (137 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.05% Tween 20) three times, blocked with 250 μl of a blocking buffer (1% bovine serum albumin in PBS-T) at room temperature for 1 hr, and washed again with the same PBS-T

three times. The standard and test samples were diluted in PBS-T to a predetermined concentration and added to antibody-coated wells, and the plate was incubated at room temperature for 1 hr and washed with PBS-T three times. Thereafter, 2 μg/ml C1q (R&D Systems) was added to the plate and reacted at room temperature for 2 hrs, and the plate was washed with PBS-T six times. 200 μl of a 1:1000 dilution of a human anti-human C1q antibody-peroxidase conjugate (Biogenesis, USA) in the blocking buffer was added to each well and reacted at room temperature for 1 hr. After each well was washed with PBS-T three times, equal volumes of color reagents A and B (Color A: stabilized peroxide and Color B: stabilized chromogen; DY 999, R&D Systems) were mixed, and 200 μl of the mixture was added to each well, followed by incubation for 30 min. Then, 50 μl of a reaction termination solution, 2 M sulphuric acid, was added to each well. The plate was read using a microplate reader (Molecular Device). The absorbance of standard and test samples was measured at 450 nm, and the results are given in FIG. 2.

As shown in FIG. 2, the immunoglobulin Fc region proteins produced in *E. coli* according to the present invention exhibited markedly reduced binding affinity to C1q. These results indicate that the immunoglobulin Fc region proteins of the present invention rarely have the risk of inducing immune responses such as cytotoxicity and

inflammation in the body when used as a carrier for physiologically active polypeptides in a conjugate form.

EXAMPLE 5: Assay for Binding to FcγRI, FcγRIII and FcRnαβ$_2$ Using ELISA

Immunoglobulin Fc is known to bind to the hematocyte receptors to FcγRI and FcγRIII to mediate effector functions such as antibody-dependent cytotoxicity. To determine whether the immunoglobulin Fc produced in E. coli mediates such effector functions, each of the receptors was obtained and assayed for binding ability through ELISA. Also, the immunoglobulin Fc was assayed for binding to the receptor FcRn, which is known to have influence on the *in vivo* metabolism of immunoglobulin, in the same manner.

<5-1> Construction of Human FcγRI, FcγRIII and FcRnαβ$_2$ Expression Strains

Total RNA was isolated from human peripheral blood mononuclear cells using a kit (Qiagen, Cat. No. ???), and was used to fish for genes encoding extracellular ligand binding domains of human FcγRI, FcγRIII and FcRnαβ$_2$ through RT-PCR and PCT. The genes were fused to a GST (Glutathione S-transferase) gene and cloned in respective mammal cell expression vectors anchoring thereto a dehydrofolate reductase gene. The recombinant pHM000 plasma thus prepared was transfected into CHO cells. In this regard, CHO cells

were inoculated at a count of $1x10^6$ cells per 6-cm culture dish, incubated at 37°C or 24 hours in a 5% CO2 incubator, and washed twice with Opti-MEM (Gibco., Cat. no. 31985-070). 1 ml of the Opti-MEM containing 10 μg of pHM000 was mixed with 1 ml of Lipofectamine™ Reagent (Invitrogen, Cat. no. 18324-020). After being allowed to stand for 20 min, the resulting mixture was added to the prepared CHO cells. These cells were incubated at 37°C for 18 hours in a 5% CO_2 incubator and refreshed with DMEM/F12, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, before incubation for an additional 48 hours. In order to select transformed strains, the cells were treated with 0.5% trypsin (Gibco., Cat. no. 15400-054) in the selection medium α-MEM (Welgene, Cat. no. LM008-02) which included 10% dialyzed fetal bovine serum, 1% penicillin-streptomycin, and 800ug/ml of geneticin (Mediatech, Cat. No. 61-234RG), followed by centrifugation. The cells thus transformed were transferred to a T25 culture dish (Nunc) and cultured at 37°C in a 5% CO_2 incubator to 90% or higher confluency. In order to determine the expression levels of FcγRI, FcγRIII and FcRnαβ_2, the selected strains were incubated at 37°C in a 5% CO_2 incubator with increasing concentrations of MTX (Sigma, Cat. No. M-8407) from 20 nM by an increment of 20 nM every two weeks.

<5-2> Production and Purification of Human FcγRI, FcγRIII

and FcRn$\alpha\beta_2$

FcγRI, FcγRIII and FcRn$\alpha\beta_2$ were purified as follows. The selected cell strains were inoculated in Cell Factory (Nunc, Cat. no. 170009) at a count of 3.5×10^8 cells per factory and grown at 37°C for 48 hours in a 5% CO_2 incubator, and then washed twice with 1 liter of PBS per factory. The cells were supplemented with 1 liter of the production medium CHO-A-SFM containing 0.3 mM sodium butyrate (Sigma, Cat. no. B-5887) and cultured at 33°C in a 5% CO_2 incubator, during which the expression supernatant was recovered every other day 7 times in total. The collected supernatant was centrifuged, filtered through a 0.22 μm filtering system (Corning), concentrated using a concentration system (PALL, Cat. no. PN OS010C70), and loaded on a chelating sepharose FF resin (Amersharm pharmacia, Cat. no. 17-0575-02) charged with 0.1M nickel sulfide (Sigma, Cat. no. N4887), so that the GST of FcγRI, FcγRIII, and FcRn$\alpha\beta_2$ were bound to the nickel. Bound FcγRI, FcγRIII, and FcRn$\alpha\beta_2$ were separated and purified from the column using 50mM NaPi(pH 8.0), 300mM NaCl, and 250mM imidazole.

<5-3> Assay for binding to FcγRI

The FcγRI purified in Example <5-2> was diluted to a concentration of 0.75 μg/ml in PBS (pH 7.4), aliquoted onto a 96-well plate (Nunc, Maxisorp) at an amount of 100 μl per

well, and incubated for 18 hours at 4°C so that the receptor was attached to the bottom of the 96-well plate. Each well of the 96-well plate was washed three times with 300 μl of a washing buffer PBS (pH 7.4) containing 0.05% Tween-20 (Amresco, Cat. no. 0777). Then, 300 μl of PBS (pH 7.4) containing 0.1% Tween-20 and 3% BSA (bovine serum albumin, Amresco, Cat. no. 0332) was added to each well so as to prevent the undesirable attachment of other substances to the bottom of the well and incubated at 37°C for 1 hour, after which the reaction solution was completely removed therefrom. With human serum IgG and the Fc separated by the treatment of human serum IgG with papain serving as controls, HM11200 and the HM11201 product purified in Example 2 were diluted to a concentration of 9 μg/ml in respective assay buffers, followed by repeating a 1:3 serial dilution with the assay buffer seven times. 100 μl of the dilution was placed in each well of a 96-well plate and allowed to react at 25°C for 2 hours with shaking at a constant rate, and the wells were washed six times with a washing buffer. In order to examine whether the HM11200, the HM11201 product and the controls, all of which were anchored to the bottom of the well plate, were bound to FcγRI, a 1:100000 dilution of an HRP-conjugated goat anti-human heavy chain antibody (Chemicon, AP309P) in an assay buffer was placed at a volume of 100 μl in each well and allowed to react at 25°C for 2 hours with shaking at a

constant rate. After washing six times with a washing buffer, 100 μl of a substrate (BD bioscience, Cat. no. 555214), which was able to react with the HRP conjugated with the antibody, was placed in each well and reacted at 25°C for 20 min. The reaction was terminated with 2N sulfuric acid and color intensity was measured with an ELISA reader (Molecular Devices, microplate reader) at 450nm. As seen in FIG. 3, almost none of the Fc proteins produced in E. coli bound to FcγRI while human IgG and Fc, both glycosylated, were strongly associated with FcγRI.

<5-4> Assay for binding to FcγRIII

With human serum IgG and the Fc separated by the treatment of human serum IgG with papain serving as controls, HM11200 and the HM11201 product purified in Example <1-2> were diluted to a concentration of 9 μg/ml in respective carbonate buffer (pH 9.0), followed by repeating a 1:3 serial dilution with the carbonate buffer seven times. 100 μl of the dilution was placed on each well of a 96-well plate and incubated at 4°C for 18 hours so that they were attached to the bottom of the 96-well plate. Each well of the 96-well plate was washed three times with 300 μl of a washing buffer consisting of PBS (pH 7.4) containing 0.05% Tween-20 (Amresco, Cat. no. 0777). Then, 300 μl of an assay buffer consisting of PBS (pH 7.4) containing 0.1% Tween-20 and 5% non-fat dry milk (Difco,

Cat. No. 232100) was added to each well to prevent the undesirable attachment of other substances onto the bottom of the well, and was incubated at 37°C for 1 hour, followed by complete removal of the reaction solution. The FcγRIII purified in Example <4-2> was diluted to a concentration of 1 μg/ml in the assay solution. 100 μl of the dilution was placed in each well of a 96-well plate and allowed to react at 25°C for 2 hours with shaking at a constant rate. The wells were washed six times with a washing buffer. A rabbit anti-GST antibody (Chemicon, AB3282), which was able to bind to the GST (glutathione S-transferase) of FcγRIII associated with the HM11200, the HM11201 product and the controls, was diluted 1:10000 in the assay buffer, and 100 μl of the dilution was placed in each well and allowed to react at 25°C for 2 hours with shaking at a constant rate. Subsequently, after washing the wells six times with a washing buffer, 100 μl of a 1:7500 dilution of the antibody against the rabbit antibody in the assay buffer was placed on each well.

Following reaction at 25°C for 2 hours with shaking at constant rate, the 96-well plate was washed six times with a washing buffer. A substrate was added in the same manner as in Example <5-3> and color intensity was measured with an ELISA reader. As seen in FIG. 4, almost none of the FcγRIII proteins produced in E. coli bound to FcγRI while human IgG and Fc, both glycosylated, were strongly

associated with FcγRIII.

<5-5> Assay for binding to FcRnαβ_2

With human serum IgG, and the Fc separated by the treatment of human serum IgG with papain serving as controls, HM11200 and the HM11201 product purified in Example <1-2> were diluted to a concentration of 20 μg/ml in respective carbonate buffer (pH 9.0), followed by repeating a 1:3 serial dilution with the carbonate buffer seven times. 100 μl of the dilution was placed on each well of a 96-well plate and incubated at 4°C for 18 hours so that they were attached onto the bottom of the 96-well plate. Each well of the 96-well plate was washed three times with 300 μl of a washing buffer consisting of PBS (pH 7.4) containing 0.05% Tween-20 (Amresco, Cat. no. 0777). Then, 300 μl of an assay buffer consisting of PBS (pH 7.4) containing 0.1% Tween-20 and 0.5% BSA (Amresco, Cat. No. 0332) was added to each well to prevent the undesirable attachment of other substances to the bottom of the well, and was incubated at 37°C for 1 hour, followed by complete removal of the reaction solution. The FcRnαβ_2 purified in Example <5-2> was diluted to a concentration of 3 μg/ml in the assay solution. 100 μl of the dilution was placed in each well of a 96-well plate and allowed to react at 25°C for 2 hours with shaking at a constant rate. The wells were washed six times with the washing buffer. A rabbit anti-GST

antibody (Chemicon, AB3282), which was able to bind to the GST (glutathione S-transferase) of FcRn$\alpha\beta_2$ associated with the HM11200, the HM11201 product and the controls, was diluted 1:10000 in the assay buffer, and 100 μl of the dilution was placed in each well and allowed to react at 25°C for 2 hours with shaking at a constant rate. Subsequently, after washing the wells six times with a washing buffer, 100 μl of a 1:7500 dilution of an antibody against the rabbit antibody in the assay buffer was placed in each well.

Following reaction at 25°C for 2 hours with shaking at a constant rate, the 96-well plate was washed six times with a washing buffer. A substrate was added in the same manner as in Example 5-2, and color intensity was measured with an ELISA reader. Like human IgG and glycosylated Fc, as seen in FIG. 5, the Fc proteins produced in E. coli strongly bind to FcRn$\alpha\beta_2$.

EXAMPLE 6: Preparation and Pharmacokinetic Analysis of Human EPO Conjugate

<6-1> Preparation of human EPO

To prepare a human EPO (erythropoietin) conjugate, first, an EPO gene was amplified through RT-PCR using total RNA isolated from blood cells and cloned into a pBluscript II (Stratagen) vector, thus generating a pBlueEP vector. To

transfer the cloned EPO gene into an animal cell expression vector pCMV/dhfr-(pCDNA3.1 (Invitrogen Company) containing a dhfr gene), the pBlueEP was digested with HindIII and BamHI, and the EPO gene-containing fragment thus obtained was inserted into the animal cell expression vector treated with the same restriction enzymes, thus providing pcmvEP. This expression vector carrying an EPO gene was transfected into CHO cells, a protein expression strain, using a Lipofectamine reagent (Gibco). The cells were treated with gradually increasing concentrations of MTX to 120 nM to elevate expression levels thereof. EPO was expressed at high levels, higher than 100 mg per liter.

<6-2> Preparation of human EPO-PEG complex

ALD-PEG-ALD (Shearwater), a 3.4-kDa polyethylene glycol having an aldehyde reactive group at both ends, was mixed with amounts of a 100 mM phosphate buffer containing the EPO prepared in <6-1> at a concentration of 5 mg/ml appropriate to form an EPO: PEG molar ratio of 1:1, 1:2.5, 1:5, 1:10 and 1:20. To this mixture, a reducing agent, sodium cyanoborohydride ($NaCNBH_3$, Sigma), was added at a final concentration of 20 mM and was allowed to react at 4°C for 2 hrs with gentle agitation to allow PEG to selectively link to the amino terminal end of EPO. To obtain a 1:1 complex of PEG and EPO, the reaction mixture was subjected to size exclusion chromatography using a Superdex^R column

(Pharmacia). The EPO-PEG complex was eluted from the column using 10 mM potassium phosphate buffer (pH 6.0) as an elution buffer, and EPO not linked to PEG, unreacted PEG and dimer byproducts, where PEG was linked to two EPO molecules were removed. The purified EPO-PEG complex was concentrated to 5 mg/ml. Through this experiment, the optimal reaction molar ratio for EPO to PEG, providing the highest reactivity and generating the smallest amount of byproducts such as dimers, was found to be 1:2.5 to 1:5.

<6-3> Preparation of conjugate of human EPO-PEG complex and recombinant immunoglobulin Fc region

The EPO-PEG complex prepared in Example <6-2> was linked to an immunoglobulin Fc region produced using the HM11201 in Example <1-3>. In detail, the immunoglobulin Fc region fragment (about 53 kDa) prepared in Example <1-3> was dissolved in 10 mM phosphate buffer and mixed with the EPO-PEG complex at an EPO-PEG complex: Fc region molar ratio of 1:1, 1:2, 1:4 and 1:8. After the phosphate buffer concentration of the reaction solution was adjusted to 100 mM, a reducing agent, $NaCNBH_3$, was added to the reaction solution at a final concentration of 20 mM and was allowed to react at 4°C for 20 hrs with gentle agitation. Through this experiment, the optimal reaction molar ratio for EPO-PEG complex to Fc region fragment, providing the highest reactivity and generating the fewest byproducts such as

dimers, was found to be 1:2.

After the coupling reaction, the reaction mixture was subjected to high-pressure liquid chromatography so as to eliminate unreacted substances and byproducts. The coupling reaction solution was desalted using a HiPrep 26/10 desalting column (Pharmacia) with 10 mM Tris buffer (pH 8.0). Then, the reaction solution was loaded onto 50 ml of a Q HP 26/10 column (Pharmacia) at a flow rate of 8 ml/min, and this column was eluted with a linear NaCl gradient of 0 M-0.2 M to obtain desired fractions. The collected fractions were again loaded onto a polyCAT 21.5x250 column equilibrated with 10 mM acetate buffer (pH 5.2) at a flow rate of 15 ml/min, and this column was eluted with a linear NaCl gradient of 0.1-0.3 M, thus providing highly pure fractions.

<6-4> Pharmacokinetic analysis

The native EPO prepared in Example <5-1>, Aranesp (Amgen) having a greater sialic acid content so as to increase the half-life thereof, and the EPO-PEG-Fc conjugate (test group) prepared in Example <5-3> were subcutaneously injected at a dose of 100 μg/kg into five SD rats per group. After the subcutaneous injection, blood samples were collected at 0.5, 1, 2, 4, 6, 12, 24 and 48 hrs in the control groups and at 1, 12, 24, 30, 48, 72, 96, 120, 144, 168, 192, 240, 288, 336 and 384 hrs in the test

groups. The blood samples were collected in 1.5 ml tubes, coagulated, and centrifuged for 10 min using an Eppendorf high-speed micro centrifugator to remove blood cells. Serum protein levels were measured by ELISA using an antibody specific to EPO.

Table 6, below, and FIG. 6 show serum half-lives of the native protein and the protein conjugate. The EPO-PEG-Fc (E. coli) protein conjugate, prepared using the immunoglobulin Fc region produced according to the present invention as a carrier, exhibited a much longer serum half-life than that of the native EPO. This extended half-life was found to be higher than that of Aranesp, known to be a second generation EPO having a long serum half-life.

TABLE 6

	EPO	EPO-PEG-Fc conjugate	Aranesp
C_{max}[1] (ng/ml)	30.4	192.8	96.8
T_{max}[2] (hr)	12.0	48.0	12.0
$T_{1/2}$[3] (hr)	6.1	47.0	16.4
AUC[4] (ng.hr/ml)	713	20436	4064
MRT[5] (hr)	15.1	88	32

[1]Maximal serum concentration
[2]Time taken to reach the maximal drug concentration
[3]Serum half-life of a drug
[4]Area under the serum concentration versus time curve
[5]Mean time that a drug molecule resides in the body

Industrial Applicability

As described hitherto, the method according to the

present invention allows the mass production of an immunoglobulin Fc region in an inclusion body form in E. coli using a recombinant immunoglobulin Fc region comprising a hinge region. When linked to a physiologically active protein, the produced immunoglobulin Fc region can be effectively used to enhance the serum half-life and physiological activity of the physiologically active protein with no risk of inducing immune responses.

Claims

1. A method of producing an immunoglobulin Fc region free of an initial methionine residue on a mass scale, comprising:

preparing a recombinant expression vector including a nucleotide sequence coding for a recombinant immunoglobulin Fc region composed of an immunoglobulin Fc region linked at an N-terminus thereof to an immunoglobulin hinge region via a peptide bond;

transforming a prokaryotic cell with the recombinant expression vector to create a transformant;

culturing the transformant to express the immunoglobulin Fc region as an inclusion body; and

isolating and purifying the immunoglobulin Fc region.

2. The method according to claim 1, wherein the immunoglobulin Fc region is isolated in a monomeric or dimeric form.

3. The method according to claim 1, wherein the hinge region has two or more consecutive amino acid sequences derived from the hinge region of IgG, IgA, IgM, IgE, or IgD.

4. The method according to claim 3, wherein the hinge

region has two or more consecutive amino acid sequences, each including at least one cystein residue.

5. The method according to claim 3, wherein the IgG is selected from the group consisting of IgG1, IgG2, IgG3 and IgG4.

6. The method according to claim 5, wherein the hinge region has an amino acid sequence represented by SEQ ID NO. 18, 19, 20, 21, 48, 49, 50, 51, 53, 54, 55, 56, 57, 58, 59 or 60.

7. The method according to claim 1, wherein the immunoglobulin Fc region is selected from the group consisting of Fc regions from IgG, IgA, IgM, IgE, IgD, and combinations and hybrids thereof.

8. The method according to claim 7, wherein the immunoglobulin Fc region is the Fc region of the IgG selected from the group consisting IgG1, IgG2, IgG3, IgG4, and combinations and hybrids thereof.

9. The method according to claim 1, wherein the immunoglobulin Fc region is composed of one to four domains selected from the group consisting of C_H1, C_H2, C_H3, C_H4 and C_L domains.

10. The method according to claim 1, wherein the immunoglobulin Fc region has an amino acid sequence represented by SEQ ID NO. 7, 9, 11, 13, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, or 47.

11. The method according to claim 1, wherein the recombinant expression vector is pmSCPFc, pmPSCFc, pmCPSFc, pmCPFc, pMEPKFC1, pMSCKFc1, pMDKTFc1, pMCPAFc1, pMPKSFc1, pMCPPFc1, pMPPCFc, pMPCPFc, pmPPCG2Fc, pmPCPG2Fc, pmCPG2Fc, pmCCVG2Fc, or pmCVE2Fc.

12. The method according to claim 1, wherein the prokaryotic cell is E. coli.

13. The method according to claim 1, wherein the transformant is *E. coli* BL21/pSTIIG1CH1_3(HM10935), BL21/pSTIIdCG1Fc (HM10927), BL21/pSTIIdCG1SFc (HM10928), BL21/pSTIIdCG1SFFc (HM10929), BL21/pSTIIG1Mo (HM10930), BL21/pSTIIdCG2Fc (HM10936), BL21/pSTIIdCG4Fc (HM10932), BL21/pSTIIG4CH1_3(HM10931), BL21/pSTIIG4Mo (HM10933), or BL21/pSTIIG4H_K (HM10934).

14. A monomeric or dimeric immunoglobulin Fc region free of initial methionine residues, prepared by the method of claim 1.