Description

Title of Invention: RECOMBINANT YEAST TRANSFORMANT AND PROCESS FOR PREPARING IMMUNOGLOBULIN FC FRAGMENT EMPLOYING THE SAME

Technical Field

[1] The present invention relates to a recombinant yeast transformant, and a process for preparing an immunoglobulin Fc fragment using the same. More particularly, the present invention relates to a transformant prepared by introducing an expression vector comprising a polynucleotide coding for a human immunoglobulin Fc fragment into *Pichia sp.* yeast, a method for producing an immunoglobulin Fc fragment, comprising culturing the transformant and recovering the immunoglobulin Fc fragment from the culture, and an immunoglobulin Fc fragment, prepared by the above method for use as a drug carrier.

Background Art

[2] With the advance of bioengineering and biotechnology, many bioactive polypeptides (proteins) and peptide medicines have been developed as therapeutic options for various diseases. Due to their low stability, however, such polypeptides or peptide medicines readily denature and thus are highly prone to renal or hepatic clearance. Accordingly, protein medicines comprising polypeptides as medicinally active ingredients suffer from the disadvantage of frequent necessary administration to patients to maintain appropriate serum levels and titers thereof. It is thus essential for the development of protein medicines that allow for them to be maintained at a proper level in the body without frequent administration.

[3] To solve these problems, a lot of effort has been devoted to improving the serum stability of protein drugs and maintaining high drug concentration level in blood for a prolonged period of time for the maximization of the pharmaceutical efficacy of the drugs, thus improving change of protein formulations, fusion with other proteins or binding polymer have been attempted. One of the most favored methods has been focused on the fusion of immunoglobulins to proteins in recent years.

[4] There have been many attempts made to increase the stability of protein medicines by use of immunoglobulins and their fragments, as described in U.S. Patent No. 5,045,312 wherein human growth hormone is conjugated to bovine serum albumin or mouse immunoglobulin via a cross-linking agent. The conjugates have enhanced activity compared with unmodified growth hormone. Other various fusion proteins are also prepared as expressed in mammals after the Fc fragment of immunoglobulin is linked to interferon (Korean Patent Publication No. 10-2003-0009464), interleukin-4

receptor, interleukin-7 receptor or erythropoietin (Korean Patent No. 10-249572). PCT Patent Publication No. WO 01/03737 discloses a fusion protein in which a cytokine or a growth factor is linked through an oligopeptide linker to an Fc fragment of immunoglobulin. Also, U. S. Patent No. 5,116,964 describes a protein which is fused to the amino or carboxy end of an immunoglobulin Fc fragment using a genetic recombination technique. U. S. Patent No. 5,349,053 discloses a fusion protein in which IL-2 is linked to an immunoglobulin Fc fragment via a peptide linkage.

[5] Many other Fc fusion proteins constructed using genetic recombination techniques have been disclosed, examples of which include a fusion protein of an immunoglobulin Fc fragment with interferon-beta or a derivative thereof (PCT Patent Publication No. WO 00/23472), and an immunoglobulin Fc fragment with an IL-5 receptor (U. S. Patent No. 5,712,121). Further, an immunoglobulin Fc fragment has been used as a carrier rather than a fusion partner, as disclosed in U. S. Patent No. 7,736,653.

[6] Production of immunoglobulins or immunoglobulin Fc fragments has been carried out predominantly in *E. coli*. The American company Amgen Inc. described, in U.S. Pat. No. 6,660,843 and U.S. Pat. Publication Nos. 2004-0044188 and 2004-0053845, 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*, and 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 unnaturally-occurring abnormal amino acid sequence at the connection region between two proteins, the fusion protein could potentially be recognized as a foreign matter by the immune system, and thus induce immune responses.

[7] As described above, the use of *E. coli* is advantageous in that therapeutically effective proteins can be expressed as aglycosylated forms at high yield thanks to the

rapid growth rate of *E. coli* and the accumulated technology of fermentation and bio-engineering, but disadvantageous in that the recombinant proteins have methionine as the first amino terminal residue, as opposed to native proteins, and require a complex purification process in consideration of the removal of *E. coli*-derived pyrogens (endotoxins) and protein refolding.

[8] On the other hand, the use of animal cells advantageously allows for the production of fusion proteins as glycosylated proteins akin to native immunoglobulin forms, but suffers from the disadvantage of having high production cost, and being high prone to contamination with animal-derived viruses or proteins. There is therefore an increasing demand for solutions to the above-mentioned problems. Recommended is a strategy of utilizing yeasts having the advantages of both *E. coli* and animal cells as host cells.

[9]

[10] Representative among the yeast used for protein production is Saccharomyces cerevisiae. In addition to being safe for the human body, the eukaryote Saccharomyces cerevisiae is easy to genetically manipulate and to culture on a large scale. Also, various expression systems for the eukaryote have been developed. When producing higher cell-derived proteins, such as human proteins, using a recombinant method, this microorganism moreover provides the proteins with the ability to be secreted outside the cells and be post-translationally modified, such as via glycosylation. In addition, the recombinant protein of the yeast undergoes folding, and disulfide bond formation, glycosylation during secretion signal-driven extracellular secretion, thus evolving to a fully bioactive form. The yeast is also economically beneficial because it does not require cell lysis and protein refolding, which are of low efficiency. What has been shown as a problem with the protein secretion system of Saccharomyces cerevisiae is, however, the great variance in secretion rate depending on the kind of human protein. Often, proteins for use as human medicines of high value are difficult to express and secrete in Saccharomyces cerevisiae (Korea Patent No. 10-0798894).

[11]

Disclosure of Invention

Technical Problem

[12] Leading to the present invention, intensive and thorough research into the use of yeast in producing proteins for human medicines, conducted by the present inventors, resulted in the finding that

[13] *Pichia sp.* yeast, a species of methylotrophic yeast, can be used to produce a secretory immunoglobulin Fc fragment, useful as a drug carrier, at a high expression level with neither an additional refolding process, nor N-terminal modification with an additional amino acid, and that the secretary immunoglobulin Fc fragment can be

purified using a simple process, with the minimum load of endotoxin or animal-derived foreign pathogens thereto.

[14]

Solution to Problem

[15] It is an object of the present invention to provide a transformant prepared by introducing an expression vector comprising a polynucleotide encoding for a human immunoglobulin Fc fragment into a *Pichia sp.* yeast.

[16] It is another object of the present invention to provide a method for producing an immunoglobulin Fc fragment, comprising culturing the transformant, and recovering the immunoglobulin Fc fragment from the culture.

[17] It is a further object of the present invention to provide an immunoglobulin Fc fragment, produced by the method, for use as a drug carrier.

[18]

Advantageous Effects of Invention

[19] The recombinant transformant of the present invention can overcome the problems associated with the use of *E. coli* or animal cells as host cells in producing an immunoglobulin Fc fragment, is useful as a drug carrier, and can find applications in the effective and economical production of drugs.

[20]

Brief Description of Drawings

[21] FIG. 1 is a schematic view illustrating processes of constructing the recombinant expression vector pPIC9K-IgG4Fc.

[22] FIG. 2 is a schematic view illustrating processes of constructing the recombinant expression vector pPIC9K-mPSCFc.

[23] FIG. 3 is a PCR image showing the incorporation of genes of interest to the genomic DNA of multi-copy clones selected with 3mg/ml and 4 mg/ml Geneticin.

Best Mode for Carrying out the Invention

[24] In one embodiment of this aspect , the present invention addresses a transformant, modified by introducing an expression vector comprising a polynucleotide encoding for a human immunoglobulin Fc fragment into *Pichia* sp. yeast,

[25] As used herein, the term “immunoglobulin,” also known as “antibody,” refers to a protein that is produced by the immune system in response to an antigen stimulus and specifically binds to a specific antigen during vigilance through blood and lymph to exert an antigen-antibody reaction. Immunoglobulins are fundamentally composed of two identical full-length light chains and two identical full-length heavy chains, with connections by disulfide bonds between the heavy chains and between the heavy chains and the light chains. There are five distinct types of heavy chains based on dif-

ferences in the amino acid sequences of their constant regions: gamma (γ), mu (μ), alpha (α), delta (δ) and epsilon (ε), and the heavy chains include the following subclasses: gamma 1 (γ1), gamma 2 (γ2), gamma 3 (γ3), gamma 4 (γ4), alpha 1 (α1) and alpha 2 (α2). According to the features of the constant regions of the heavy chains, immunoglobulins are classified into five isotypes: IgG, IgA, IgD, IgE and IgM. The representative isotype IgG is further divided into IgG1, IgG2, IgG3 and IgG4 subclasses. For the purpose of the present invention, the term "immunoglobulin" includes a functional fragment of an immunoglobulin molecule as well as a whole immunoglobulin molecule. This functional fragment means a fragment retaining an antigen binding function, and includes Fab, F(ab'), F(ab')2, Fv, scFv, Fd, and Fc.

[26] Among the immunoglobulin fragments, Fab contains the variable regions of the light chain and the heavy chain, the constant region of the light chain, and the first constant region (CH1) of the heavy chain, and has a single antigen-binding site. The Fab' fragments differ from the Fab fragments in terms of having the hinge region containing one or more cysteine residues at the C-terminus of the heavy chain C_H1 domain. The F(ab')2 fragments are produced as a pair of Fab fragments by disulfide bonding formed between cysteine residues of the hinge regions of the Fab fragments. Fv is the minimum antibody fragment that contains only the heavy-chain variable region and the light-chain variable region. The scFv (single-chain Fv) fragments comprise the heavy-chain variable region and the light-chain variable region that are linked to each other by a peptide linker and thus are present in a single polypeptide chain. Also, the Fd fragments comprise only the variable region and C_H1 domain of the heavy chain. These functional fragments of immunoglobulin molecules can be obtained using proteolytic enzymes (for example, a whole antibody is digested into Fab by digestion with papain and into F(ab')2 by digestion with pepsin) or through genetic recombination technology.

[27]

[28] As used herein, the term “immunoglobulin Fc fragment” refers to an immunoglobulin fragment that is composed of two heavy chains that contribute two constant domains (CH2 and CH3), devoid of the variable domains of light and heavy chains, the constant domain 1 of the heavy chain (CH1) and the constant domain of the light chain (CL1). Optionally, the immunoglobulin Fc fragment may further comprise a hinge region attached to the constant domain of the heavy chain. Since it is a biodegradable polypeptide that can be metabolized in vivo, an immunoglobulin Fc fragment is safe for use as a drug carrier. In addition, an immunoglobulin Fc fragment is advantageous over a whole immunoglobulin molecule in terms of the preparation, purification and yield of a conjugate because of its lower molecular weight. Free of the Fab fragment, which is heterogeneous because it differs in amino acid sequence from

one antibody to another, the immunoglobulin Fc fragment is expected to greatly increase the homogeneity of the conjugate while decreasing the likelihood of provoking the blood antigenicity of the conjugate.

[29] The immunoglobulin Fc fragment of the present invention may be an extended Fc fragment which comprises a part of or the entirety of the constant domain 1 of the heavy chain (CH1) and/or the constant domain 1 of the light chain (CL1) devoid of the variable regions of the heavy and the light chains, so long as it shows effects substantially identical or superior to those of the classical Fc fragment. Further, the immunoglobulin Fc fragment of the present invention may be comprised of CH2 and/or CH3 that lacks a significant part of the amino acid sequence. Consequently, the immunoglobulin Fc fragment of the present invention may be composed of 1) CH1 domain, CH2 domain, CH3 domain and CH4 domain, 2) CH1 domain and CH2 domain, 3) CH1 domain and CH3 domain, 4) CH2 domain and CH3 domain, 5) a combination of one or more constant domains and an immunoglobulin hinge region (or a partial hinge region), or 6) a dimer of each constant domain of the heavy chain and the constant domain of the light chain.

[30] In addition, the immunoglobulin Fc fragment of the present invention is intended to cover not only native amino acid sequences but also mutants thereof. The amino acid sequence mutant means an amino acid sequence different from the native sequence by deletion, insertion, non-conservative or conservative substitution of one or more amino acid residues or combinations thereof. For example, the amino acid residues at positions 214 to 238, 297 to 299, 318 to 322, or 327 to 331 of IgG Fc, which are known to play an important role in antibody binding, may be modified so as to be used as suitable binding sites. In addition, various mutants which, for example, lack a residue forming a disulfide bond or several N-terminal amino acids of the native Fc, or have an additional methionine residue at the N terminus of the native Fc are possible. Further, effector functions may be eliminated by removing a complement binding motif, e.g., C1q binding motif, or an ADCC (antibody-dependent cell mediated cytotoxicity) motif. Reference may be made to PCT Publication Nos. 97/34631 and 96/32478 concerning the preparation of amino acid sequence mutants of immunoglobulin Fc fragments. The Fc fragment may be a native form isolated from humans and other animals including cows, goats, pigs, mice, rabbits, hamsters, rats and guinea pigs, or may be a recombinant or a derivative thereof, obtained from transformed animal cells or microorganisms. In the former case, whole immunoglobulin is isolated from humans or animals, followed by enzymatic treatment. When treated with papain, the whole immunoglobulin is divided into Fab and Fc. Pepsin cleaves whole immunoglobulin into pF'c and F(ab). From these fragments, Fc or pF'c can be separated using size-exclusion chromatography. Preferred is a re-

combinant immunoglobulin Fc domain derived from the human Fc domain in microorganisms.

[31]

[32] For the purposes of the present invention, the immunoglobulin Fc fragment may be one derived from IgG, such as an IgG1 Fc fragment, an IgG2 Fc fragment, an IgG3 Fc fragment, an IgG4 Fc fragment and the like, preferably an IgG2 Fc fragment or an IgG4 Fc fragment, more preferably an IgG4 Fc fragment, and most preferably an IgG4 Fc fragment encoded by the nucleotide sequence represented by SEQ ID NO: 1 or SEQ ID NO: 2, or an IgG4 Fc fragment comprising the amino acid sequence represented by SEQ ID NO: 10 or SEQ ID NO: 11.

[33] The immunoglobulin Fc fragment may undergo an amino acid substitution which does not alter the activity of native proteins or peptides as a whole. Most typical substitutions occur between 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. As needed, the amino acids may be modified by, for example, phosphorylation, sulfation, acrylation, glycosylation, methylation, farnesylation, acetylation, amidation, etc. These modification methods are known in the art (H. Neurath, R. L. Hill, The Proteins, Academic Press, New York, 1979).

[34]

[35] As used herein, the term "transformation" or "modification" refers to the genetic alteration of host cells (inclusive of prokaryotes, eukaryotes, animal cells and plant cells) resulting from the introduction of exogenous genetic material, whether carried by a plasmid or not, using a genetic manipulation technique. The term "transformant" means a cell which stably retains and expresses an exogenous genetic material introduced from the outside, whether carried by a plasmid or not, even though the cell divides many times. Any process by which a nucleic acid material can be introduced into an organism, a cell, a tissue, an organ, or a nucleic acid material may be employed to carry out transformation in the present invention. Preferably, standard methods known in the art may be selected according to host cells. Transformation into yeast hosts may be typically performed using a method described by Van Solingen (J. Bact., 1977, 130:946) and Hsiao et al. (Proc. Natl. Acad. Sci.(USA), 1979, 76:3829). Methods of transforming yeasts include, but are not limited to, electroporation using an electric device, and spheroplasting using a spheroplast devoid of cell walls. For the purposes of the present invention, the transformant may be in the form of a multi-copy clone resulting from the incorporation of an immunoglobulin G Fc gene to the genome of the yeast host. For the purposes of the present invention, no particular limitations are imparted to the transformant so long as it harbors an expression vector which comprises a polynucleotide encoding for a human immunoglobulin Fc fragment. By

way of example, *Pichia* (Komagataella) pastoris HMC041 (Accession No. KCCM11348P) which has the expression vector pPIC9K-IgG4Fc comprising an immunoglobulin Fc fragment-encoding polynucleotide, or *Pichia* (Komagataella) pastoris HMC042 (Accession No. KCCM11350P) which has the expression vector pPIC9K-mPSCFc comprising an immunoglobulin Fc fragment-encoding polynucleotide may be used.

[36]

[37] As used herein, the term "host cell" means a target cell to which an exogenous genetic material is introduced. Examples of the host cells useful in the present invention include, but are not limited to, yeasts (e.g., Pichia pastoris, *Saccharomyces* cerevisiae, *Schizosaccharomyces sp.*, *Neurospora* crassa)). Preferably, yeasts are employed as host cells because they can express the recombinant immunoglobulin structurally similar to a native form, with neither N-terminal modification with an additional amino acid, nor the necessity of a refolding process, and can be safe against contamination with a matter of animal origin. More preferable are *Pichia* sp. yeasts, with most preference for *Pichiapastoris*.

[38]

[39] The term "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. No particular limitations are imparted to the expression vector if it carries a polynucleotide encoding for the immunoglobulin Fc fragment of the present invention. For the purposes of the present invention, the expression vector is configured to express an immunoglobulin Fc fragment after transformation into a *Pichia* sp. yeast. For example, it may be pPIC9K-IgG4Fc, represented by the cleavage map of FIG. 1, or pPIC9K-mPSCFc, represented by the cleavage map of FIG. 2.

[40]

[41] The term "operably linked," as used herein, refers to a functional linkage between a nucleic acid expression regulatory sequence and a nucleic acid sequence encoding 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.

[42]

[43] According to one embodiment of the present invention, the expression vectors pPIC9K-IgG4Fc and pPIC9K-mPSCFc, each comprising an immunoglobulin Fc fragment-encoding polynucleotide, are constructed. The expression vectors

pPIC9K-IgG4Fc and pPIC9K-mPSCFc were transformed into *Pichia* pastoris to prepare transformants which were designated "*Pichia* (Komagataella) pastoris HMC041" and "*Pichia* (Komagataella) pastoris HMC042", and deposited at the Korean Culture Center of Microorganisms (located at 361-221, Hongje-1 dong, Seodaemungu, Seoul) on January 7, 2013, with Accession Nos. KCCM11348P and KCCM11350P, respectively.

[44]

[45] The term "multi-copy clone," as used herein, refers to a transformant in which multiple copies of an exogenous gene are randomly incorporated to the genome of the host cell by recombination or rearrangement upon the crossing of the exogenous gene with a certain genomic gene or site of the host cell after the introduction of the exogenous gene to the host cell by genetic manipulation. On the whole, the multi-copy clone may be a transformant in which one or more copies of a gene selectable by a Geneticin marker are inserted to the genome of the host cells, and preferably in which five or more copies of a gene selectable by a Geneticin marker of 3 mg/ml or higher are inserted to the genome.

[46]

[47] In accordance with another aspect thereof, the present invention addresses a method for preparing an immunoglobulin Fc fragment, comprising culturing the transformant; and recovering the immunoglobulin Fc fragment from the culture. And the method might be characterized by no requiding an additional protein refolding process.

[48]

[49] The recombinant *Pichia* pastoris yeast may be cultured in a suitable medium under a proper condition known in the art. The culturing process may be readily adjusted by those skilled in the art depending on the strain used.

[50]

[51] The immunoglobulin Fc fragment recovered from the culture may be used without further purification, or purified using dialysis, salting-out, and chromatography. Of them, chromatography is the most widely used. There are no rules that can be universally applied irrespective of the kind and order of the columns employed. According to the features of the target proteins of the antibody, and the culturing process, selection may be made from among ion exchange chromatography, size exclusion chromatography, affinity chromatography, hydrophobic chromatography, and protein-A affinity column.

[52]

[53] In accordance with a further aspect thereof, the present invention addresses an immunoglobulin Fc fragment, prepared by the method, for use as a drug carrier. Serving as a carrier, the immunoglobulin Fc fragment forms a conjugate with a drug and can

increase the bioavailability of the drug.

[54]

[55] As used herein, the term "drug" indicates a substance that exerts a therapeutic effect on humans or animals when administered thereto. As non-limitative examples, peptides, polypeptides, compounds, extracts, and nucleic acids fall within the scope of the drug, with preference for peptides or polypeptides.

[56]

[57] As used herein, the term "carrier" refers to a substance that is linked to a drug, with the aim of minimizing a decrease in the physiological activity of the drug and increasing the in vivo stability of the drug. That is, the present invention provides many possible IgG1, IgG2, IgG3 and IgG4 Fc fragments for increasing in vivo sustainability of the drug and minimizing a decrease in the in vivo activity of the drug. Preferably, IgG2 Fc and IgG4 Fc fragments are provided. More preferably, the present invention provides an IgG4 Fc fragment. However, so long as it has an FcRn receptor-binding site necessary for in vivo sustainability, any Fc fragment may fall within the scope of the present invention.

[58]

[59] The immunoglobulin Fc fragment prepared using the method of the present invention enjoys the advantage of requiring no additional refolding processes and being free of additional amino acid restudies at the N-terminus. Also, it can be expressed at an increased level, with a minimal load of endotoxin or pathogens of animal origin, and can be purified using a simple process. Since it is a biodegradable polypeptide that can be metabolized in vivo, the immunoglobulin Fc fragment with such advantages can be used as a drug carrier. In addition, the immunoglobulin Fc fragment is advantageous over a whole immunoglobulin molecule in terms of the preparation, purification and yield of a conjugate because of its lower molecular weight. Free of the Fab fragment, which is of heterogeneity because it differs in amino acid sequence from one antibody to another, the immunoglobulin Fc fragment is expected to greatly increase the homogeneity of the conjugate while decreasing the likelihood of provoking the blood antigenicity of the conjugate.

[60] Among the subclasses of IgG, IgG4 is the least prone to binding to a complement (C1q). Given lower affinity for the complement, the Fc fragment is less apt to mediate effector functions such as ADCC (antibody-dependent cell cytotoxicity) and CDC (complement-dependent cytotoxicity) and thus to provoke unnecessary immune responses in vivo. Affinity for C1q is lower in IgG2 and IgG4 Fc fragments than IgG1 Fc fragments, and the lowest in IgG4 Fc fragments. For use as a drug carrier, the Fc fragment conjugated to a drug preferably has to exhibit low effector functions such as ADCC and CDC. For the purposes of the present invention, hence, IgG2 Fc and IgG4

Fc fragments are useful, with higher preference for IgG4 Fc fragments. That is, the immunoglobulin Fc fragment useful as a drug carrier in accordance with the present invention may be an Fc fragment derived from human IgG4 Fc fragment or a derivative thereof lacking a part of the hinge, as represented by SEQ ID NO: 10 or SEQ ID NO: 11, which may have the same amino acid sequence as the protein produced by the *E.* coli transformant HM11201 (KCCM-10660P) disclosed in Korean Patent No. 10-824505.

[61]

Mode for the Invention

[62] 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 limiting the present invention.

[63]

[64] **EXAMPLE** 1: Construction of Expression Vector for Producing Immunoglobulin Fc Fragment in *Pichia pastoris*

[65]

[66] A recombinant expression vector for expressing a human immunoglobulin Fc fragment in *Pichiapastoris* yeast was constructed to anchor a nucleotide sequence (SEQ ID NO: 1) encoding a human immunoglobulin Fc fragment composed of an IgG4 hinge region and IgG4 constant domains CH2 and CH3, as follows.

[67] DNA of the human immunoglobulin Fc fragment was obtained by PCR, with the plasmid pBG4CH1-3 disclosed in Korean Patent No. 10-0725314 serving as a template. First, a forward primer (SEQ ID NO: 3) was designed to contain an SnaB I restriction site for fusion to an alpha factor secretion signal sequence while a reverse primer (SEQ ID NO: 4) was configured to have an EcoR I restriction site. A DNA encoding the immunoglobulin G4 Fc fragment was amplified by PCR using these primers. PCR was performed with 30 cycles of denaturing at 95℃ for 40 sec, annealing at 60℃ for 30 sec, and extending at 68℃ for 50 sec.

[68]

[69] 5'-GCTTACGTAGAGTCCAAATATGGTCCCCCATGCC-3'(SEQ ID NO: 3)

[70] 5'-CCGGAATTCTCATTTACCCAGAGACAGGGAGAGG-3'(SEQ ID NO: 4)

[71]

[72] The immunoglobulin G4 Fc fragment DNA (ca. 700 bp) thus obtained was cloned into a pPIC9K vector (Invitrogen). To be in frame with an alpha factor secretion signal sequence, the PCR product of the immunoglobulin G4 Fc fragment was digested with the restriction enzymes SnalB I and EcoR I, and then ligated in the presence of T4 ligase to a pPIC9K vector which was previously treated with the same restriction

enzymes. The resulting recombinant expression vector contained the immunoglobulin G4 Fc gene immediately downstream of the alpha factor secretion signal sequence, but the SnB I recognition site represented by the forward primer was also left on the vector, so that the immunoglobulin G4 Fc fragment, if expressed from the vector, would have two undesired amino acid residues at the N-terminus. To delete the SnaB I recognition site inserted into the junction between the alpha factor secretion signal sequence and the immunoglobulin G4 Fc gene, site-directed mutagenesis was conducted using a pair of primers represented by SEQ ID NOS: 6 and 7, and the resulting expression vector was designated pPIC9K-IgG4Fc (FIG. 1).

[73]

[74] 5'-GAGAAAAGAGAGGCTGAAGCTGAGTCCAAATATGGTCCCCCA-3' (SEQ ID NO: 6)

[75] 5'-TGGGGGACCATATTTGGACTCAGCTTCAGCCTCTCTTTTCTC-3' (SEQ ID NO: 7)

[76]

[77] FIG. 1 is a schematic view illustrating processes of constructing the recombinant expression vector pPIC9K-IgG4Fc. As can be seen in FIG. 1, the pPIC9K-IgG4Fc expression vector contains a DNA of SEQ ID NO: 1 under the control of 5' alcohol oxidase 1 (AOX1) gene promoter, and can be integrated to the genomic DNA of a host cell through the 3' alcohol oxidase 1 (AOX1) gene located downstream of the immunoglobulin G4 Fc gene.

[78]

[79] **EXAMPLE** 2: Construction of Expression Vector for Producing Immunoglobulin Fc Fragment Partially Devoid of Hinge in *Pichia pastoris*

[80]

[81] An expression vector for expressing an immunoglobulin Fc fragment partially devoid of the hinge region in *Pichiapastoris* was constructed to anchor a nucleotide sequence (SEQ ID NO: 2) human immunoglobulin Fc fragment composed of a part of a hinge of IgG4, and constant domains CH2 and CH3 of IgG4, according to the same strategy as described in Example 1.

[82] A DNA encoding the human immunoglobulin Fc fragment partially devoid of a hinge region was obtained by PCR, with the plasmid pBG4CH1-3 described in Korean Patent No. 10-0725314 serving as a template.

[83] First, a forward primer (SEQ ID NO: 5) was designed to contain an SnaB I restriction site for fusion to an alpha factor secretion signal sequence while a reverse primer (SEQ ID NO: 4) was configured to have an EcoR I restriction site. A DNA encoding the immunoglobulin G4 Fc fragment was amplified by PCR using these primers. PCR was performed with 30 cycles of denaturing at 95°C for 40 sec, annealing at 60°C for 30

sec, and extending at 68℃ for 50 sec.

[84]

[85] 5'-GCTTACGTACCATCATGCCCAGCACCTGAGTTCC-3' (SEQ ID NO: 5)

[86]

[87] The immunoglobulin G4 Fc fragment DNA (ca. 680 bp) thus obtained was cloned into a pPIC9K vector (Invitrogen). To be in frame with an alpha factor secretion signal sequence, the PCR product of the immunoglobulin G4 Fc fragment was digested with the restriction enzymes SnalB I and EcoR I, followed by ligation in the presence of T4 ligase to a pPIC9K vector which was previously treated with the same restriction enzymes. The resulting recombinant expression vector contained the immunoglobulin G4 Fc gene immediately downstream of the alpha factor secretion signal sequence, but the SnB I recognition site represented by the forward primer was also left on the vector, so that the immunoglobulin G4 Fc fragment, if expressed from the vector, would have undesired two amino acid residues at the N-terminus. To delete the SnaB I recognition site inserted into the junction between the alpha factor secretion signal sequence and the immunoglobulin G4 Fc gene, site-directed mutagenesis was conducted using a pair of primers represented by SEQ ID NOS: 8 and 9, and the resulting expression vector was designated pPIC9K-mPSCFc (FIG. 2).

[88]

[89] 5'-TCTCTCGAGAAAAGAGAGGCTGAAGCTCCATCATGCCCAGCACCTGAG TTCCTG-3' (SEQ ID NO: 8)

[90] 5'-CAGGAACTCAGGTGCTGGGCATGATGGAGCTTCAGCCTCTCTTTTCTCG AGAGA-3' (SEQ ID NO: 9)

[91]

[92] FIG. 2 is a schematic view illustrating processes of constructing the recombinant expression vector pPIC9K-mPSCFc. As can be seen in FIG. 2, the pPIC9K-mPSCFc expression vector contains a DNA of SEQ ID NO: 2 under the control of 5 alcohol oxidase 1 (AOX1) gene promoter, and can be integrated to the genomic DNA of a host cell through the 3 alcohol oxidase 1 (AOX1) gene located downstream of the immunoglobulin G4 Fc gene. The host cell transformed with the expression vector can secrete the immunoglobulin Fc fragment partially devoid of a hinge region to a culture medium, as driven by the alpha factor secretion signal sequence.

[93]

[94] **EXAMPLE** 3: Transformation to *Pichia pastoris* Yeast and Selection of Multi-Copy Clone

[95]

[96] For use in stable transformation and integration to genomic DNA, the recombinant vectors pPIC9K-IgG4Fc and pPIC9K-mPSCFc, obtained respectively in Examples 1

and 2, were linearized by digestion with the restriction enzyme SalI. The linearized recombinant IgG Fc fragment expression vector was designed to be integrated into the genomic DNA of a host by recombination with the alcohol oxidase gene site of the host. Yeast *Pichiapastoris* KM71 and GS115 were used as hosts to be transformed with the recombinant expression vectors obtained in Examples 1 and 2. Transformation was carried out by electroporation, which is known to be the most popular and efficient for yeast. For use in transformation, spheroplasts of *Pichiapastoris* KM71 and GS115 were prepared according to the protocol provided by Invitrogen USA.

[97] After 10 ug of each of the linearized recombinant vectors was well mixed with 80 ul of the spheroplast of KM 71 or GS115, the mixture was incubated for 5 min in a 0.2 cm-gap-cuvette on ice. Then, an electric shock was applied to the cuvette at 2000V, 200Ω 25uF in a Gene Pulser electroporation device (BIO-Rad) to induce transformation. The reaction mixture was added with 1 ml of chilled 1 M sorbitol, and spread over an MD (minimal dextrose) agar plate which was then incubated at 28℃ for 3 days to select His+ positive transformants. To discriminate multi-copy clones, each of the His+ positive transformants formed on the MD agar plate was homogeneously suspended in 1 ml of sterile distilled water and the suspensions were spread in an amount corresponding to 10^5 cells over YPD agar plates containing various concentrations of Geneticin (0.5, 1.0, 2.0, 3.0, 4.0 mg/ml), followed by incubation at 28℃ for 5 days. Of the Geneticin-resistant, multi-copy clones thus selected, the colonies formed on the YPD agar plates containing 3.0 mg/ml and 4.0 mg/ml were subjected to colony PCR to examine whether the immunoglobulin fragment genes were incorporated to the genomic DNA. Briefly, PCR employed a pair of primers of SEQ ID NOS: 3 and 4 to examine the incorporation of the immunoglobulin Fc fragment gene, and a pair of primers of SEQ ID NOS: 5 and 4 to examine the incorporation of the gene encoding the immunoglobulin Fc fragment partially devoid of the hinge region. PCR was performed with 30 cycles of denaturing at 95℃ for 40 sec, annealing at 60℃ for 30 sec, and extending at 68℃ for 50 sec (FIG. 3). FIG. 3 is a PCR image showing the incorporation of the genes of interest to the genomic DNA of multi-copy clones selected with 3mg/ml and 4 mg/ml Geneticin. As can be seen in FIG. 3, the PCR demonstrated the incorporation of the immunoglobulin Fc fragment genes to the genomes of host cells.

[98]

[99] The transformants prepared by transforming *Pichiapastoris* strains with the expression vectors pPIC9K-IgG4Fc and pPIC9K-mPSCFc, which carried respective immunoglobulin Fc fragment genes, were designated "*Pichia* (*Komagataella*) pastoris HMC041" and "*Pichia* (*Komagataella*) pastoris HMC042", and deposited at the Korean Culture Center of Microorganisms (located at 361-221, Hongje-1 dong, Seo-

daemungu, Seoul) on January 7, 2013, with Accession Nos. KCCM11348P and KCCM11350P, respectively.

[100]

[101] Although the preferred embodiments of the present invention have been disclosed for illustrative purposes, those skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.

[102]

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

INTERNATIONAL FORM

To Hanmi Pharm. Co., Ltd.
893-5 Hajeo-ri, Paltan-myeon,
Hwaseong-si, Gyeonggi-do, 445-913,
KOREA

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : *Pichia (Komagataella) pastoris* HMC041	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM11348P
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☐ a scientific description ☐ a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on January. 07. 2013. (date of the original deposit)[1]	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : 361-221, Yurim B/D Hongje-1-dong Seodaemun-gu SEOUL 120-091 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: January. 07. 2013.

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

Form BP/4 Sole page

한국미생물보존센터
KOREAN CULTURE CENTER OF MICROORGANISMS

[103]

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

INTERNATIONAL FORM

To. Hanmi Pharm. Co., Ltd.
893-5 Hajeo-ri, Paltan-myeon,
Hwaseong-si, Gyeonggi-do, 445-913,
KOREA

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : *Pichia (Komagataella) pastoris* HMC042	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM11350P
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☐ a scientific description ☐ a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on January. 10. 2013. (date of the original deposit)[1]	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : 361-221, Yurim B/D Hongje-1-dong Seodaemun-gu SEOUL 120-091 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: January. 10. 2013.

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

Form BP/4 Sole page

한국미생물보존센터
KOREAN CULTURE CENTER OF MICROORGANISMS

Claims

1. A *Pichia* sp. yeast comprising an expression vector comprising a polynucleotide encoding a human immunoglobulin Fc fragment into *Pichia* sp. Yeast, wherein the expression vector is pPIC9K-IgG4Fc, as represented by the cleavage map of FIG. 1, or pPIC9K-mPSCFc, as represented by the cleavage map of FIG. 2, and the immunoglobulin Fc fragment comprises an amino acid sequence represented by SEQ ID NO: 10 or SEQ ID NO: 11.

2. The *Pichia* sp. yeast of claim 1, wherein the *Pichia* sp. yeast is *Pichia pastoris*.

3. The *Pichia* sp. yeast of claim 1, wherein the expression vector is pPIC9K-mPSCFc, as represented by the cleavage map of FIG. 2, and the immunoglobulin Fc fragment comprises an amino acid sequence represented by SEQ ID NO: 11.

4. The *Pichia* sp. yeast of claim 1, wherein the polynucleotide comprises a nucleotide sequence represented by SEQ ID NO: 1 or SEQ ID NO: 2.

5. The *Pichia* sp. yeast of claim 1, being *Pichia (Komagataella) pastoris* HMC041 (Accession No. KCCM11348P) or *Pichia (Komagataella) pastoris* HMC042 (Accession No. KCCM11350P).

6. A method for producing an immunoglobulin Fc fragment, comprising:
   (a) culturing the *Pichia* sp. yeast of any one of claims 1 to 5; and
   (b) recovering the immunoglobulin Fc fragment from the culture.

7. An immunoglobulin Fc fragment, prepared by the method of claim 6, for use as a drug carrier.