DESCRIPTION

Title of the Invention: CHROMATOGRAPHIC SUPPORT FOR PURIFYING FC FUSION PROTEIN AND FC FUSION PROTEIN PURIFICATION METHOD USING SAME

Technical Field

[0001]

The present invention relates to a chromatographic support for purifying an Fc fusion protein, and a method of purifying an Fc fusion protein using the same.

Background Art

[0002]

In the production process of biopharmaceuticals including antibody drugs and the like, it is necessary to separate a target pharmaceutical protein such as an antibody from an expression medium and purify the protein to a purity acceptable as a therapeutic agent or a diagnostic agent. Pharmaceutical proteins are generally produced via purification by affinity chromatography. The affinity support used for such chromatographic purification is required to improve the binding capacity to the target protein, and reduce non-specific adsorption of impurities, for example, components in a medium.

[0003]

Patent Literature 1 discloses that porous crosslinked particles obtained by polymerizing divinylbenzene, ethyldivinylbenzene, and glycidyl methacrylate and then crosslinking them are sieved to have a predetermined particle size distribution, and then protein ligands are bound thereto to produce a support for affinity chromatography, and that the dynamic binding capacity of the

support to an antibody is improved.

Citation List

Patent Literature

[0004]

Patent Literature 1: WO 2022/202466 A

Summary of Invention

Technical Problem

[0005]

An Fc fusion protein is a protein obtained by fusing a protein having a specific binding ability to a target molecule (for example, an extracellular domain of a receptor) to an Fc fragment of an immunoglobulin, and is clinically used as a molecularly targeted therapeutic agent. An Fc fusion protein is purified by affinity chromatography, similarly to an antibody drug. However, since a conventional affinity support is produced primarily for the purpose of purifying antibodies, when such a support is used for purification of an Fc fusion protein, performance equivalent to that obtained in antibody purification cannot be achieved in terms of dynamic binding capacity (DBC), contamination with impurities, or other factors. It is desired to separate an Fc fusion protein having higher purity with higher efficiency. The present invention provides a chromatographic support that makes it possible to separate an Fc fusion protein having a high DBC for the Fc fusion protein and higher purity with fewer contaminants, and a method of purifying an Fc fusion protein using the same.

Solution to Problem

[0006]

The present invention provides the following as representative embodiments.

[1] A chromatographic support for purifying an Fc fusion protein, including:

porous particles having a ligand immobilized thereon,

in which the porous particles are synthetic polymer-based or natural polymer-based porous particles,

the ligand is at least one selected from the group consisting of Protein A, Protein G, Protein L, and analogs thereof, and

an average pore diameter of the porous particles having the ligand immobilized thereon is 75 to 120 nm.

[2] The chromatographic support according to [1], in which an average pore diameter of the porous particles having the ligand immobilized thereon is 101 to 110 nm.

[3] The chromatographic support according to [1] or [2], in which the Fc fusion protein has a molecular weight of 160 to 300 kDa.

[4] The chromatographic support according to any one of [1] to [3], in which the porous particles having the ligand immobilized thereon have a volume average particle diameter of 45 to 100 μm and a porosity of 70 to 95% (v/v).

[5] The chromatographic support according to any one of [1] to [4], in which the Fc fusion protein is at least one selected from the group consisting of etanercept, rilonacept, and efmoroctocog alfa.

[6] The chromatographic support according to any one of [1] to [5], in which the Fc fusion protein is an Fc fusion protein consisting of the amino acid sequence of SEQ ID NO:4 or SEQ ID NO:5, or an amino acid sequence having 85% or more identity to any of these sequences.

[7] The chromatographic support according to any one of [1] to [6], in which the porous particles are a polymer of

- a monomer having a functional group capable of immobilizing a ligand and a polymerizable unsaturated group, and
- a monomer having a polymerizable unsaturated group and not having the functional group.

[8] The chromatographic support according to [7], in which the monomer having a polymerizable unsaturated group and not having the functional group includes a non-crosslinking monomer and a crosslinking monomer.

[9] A method of purifying an Fc fusion protein, including:

(Step 1) a step of passing a solution containing an Fc fusion protein through a column containing the chromatographic support according to any one of [1] to [8], to bind the Fc fusion protein to the support;

(Step 2) a step of washing the support; and

(Step 3) a step of recovering the Fc fusion protein from the support.

[10] The method according to [9], further including:

(Step 4) a step of passing an alkaline liquid through the column after the Step 3.

[11] The method according to [10], including repeating the Steps 1 to 4 a plurality of times.

Advantageous Effects of Invention

[0007]

The chromatographic support of the present invention has a large DBC for an Fc fusion protein, and is excellent in performance of separating a target Fc fusion protein from contaminants. Therefore, according to the method of purifying an Fc fusion protein using the chromatographic support of the present invention, an Fc fusion protein having higher purity can be separated with

higher efficiency.

Description of Embodiments

[0008]

All patent documents, non-patent documents, and other publications cited in the present specification are incorporated herein by reference in their entirety.

[0009]

In the present specification, a notation representing a numerical range such as “A to B” is synonymous with “A or more and B or less”, and includes A and B in the numerical range.

[0010]

As used herein, the term “ligand” used in relation to affinity chromatography refers to a molecule that binds to a target substance of affinity chromatography. “Protein ligand” represents a ligand in which a portion that binds to the target substance is composed of a protein.

[0011]

In the present specification, “Fc” and “Fc region” refer to an Fc region or an Fc fragment of an immunoglobulin. In the present specification, “Fc-binding domain” represents a functional unit of a polypeptide having a binding activity to an Fc by itself. In the present specification, “Fc-binding protein” refers to a protein that contains the “Fc-binding domain” and has an affinity specific to the Fc of an immunoglobulin.

[0012]

In the present specification, “Fc fusion protein” is a protein in which a heterologous protein is fused to an Fc fragment of an immunoglobulin. The heterologous protein may be of human or non-human origin, but is a protein other than an immunoglobulin Fab,

and in this respect, the Fc fusion protein is distinguished from antibodies and fragments thereof, including immunoglobulins such as IgG, IgA, IgD, IgE, and IgM, chimeric antibodies, and the like. Typically, the heterologous protein is a protein having a specific binding ability to a target molecule, such as an extracellular domain of a receptor, a receptor antagonist, or an enzyme. In clinical practice, Fc fusion proteins are used as molecularly targeted therapeutic agents for the treatment of inflammatory diseases such as rheumatoid arthritis and hemorrhagic diseases such as hemophilia. Examples of molecularly targeted therapeutic agents using an Fc fusion protein include etanercept, rilonacept, and efmoroctocog alfa.

[0013]

In the present specification, "85% or more identity" with respect to an amino acid sequence refers to preferably 90% or more, more preferably 94% or more, still more preferably 96% or more, further preferably 98% or more, and even more preferably 99% or more identity. Amino acid sequence identity can be determined using the BLAST algorithm (Pro.Natl.Acad.Sci.USA.,1993,90:5873-5877). Based on this BLAST algorithm, programs called BLASTN, BLASTX, BLASTP, TBLASTN, and TBLASTX have been developed (J.Mol.Biol.,1990,215:403-410). When these programs are used, default parameters of each program can be used. Specific methods of these analysis methods are known (refer to [www.ncbi.nlm.nih.gov]).

[0014]

In the present specification, the "corresponding position" on the amino acid sequence can be determined by aligning the target sequence and the reference sequence (for example, the amino acid sequence of SEQ ID NO: 1) to give the maximum homology to the

conserved amino acid residues present in each amino acid sequence. Alignment can be performed using known algorithms, the procedures of which are known to those skilled in the art. For example, the alignment can be performed by using Clustal W multiple alignment program (Thompson J.D et al., Nucleic AcidsRes.,1994,22:4673-4680) with default settings. Clustal W can be utilized, for example, on the website of the DNA Data Bank of Japan (DDBJ [www.ddbj.nig.ac.jp/index.html]), which is operated by the National Institute of Genetics. The position of the target sequence aligned to any position of the reference sequence by the above-described alignment is regarded as a "corresponding position" to that position.

[0015]

In the present specification, amino acid residues are also described by the following abbreviations: alanine (Ala or A), arginine (Arg or R), asparagine (Asn or N), aspartic acid (Asp or D), cysteine (Cys or C), glutamine (Gln or Q), glutamic acid (Glu or E), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), leucine (Leu or L), lysine (Lys or K), methionine (Met or M), phenylalanine (Phe or F), proline (Pro or P), serine (Ser or S), threonine (Thr or T), tryptophan (Trp or W), tyrosine (Tyr or Y), valine (Val or V), and any amino acid residue (Xaa or X). In addition, in the present specification, the amino acid sequence of the peptide is described such that the amino terminus (hereinafter referred to as N-terminus) is positioned on the left side and the carboxyl terminus (hereinafter referred to as C-terminus) is positioned on the right side in accordance with a conventional method.

[0016]

[Chromatographic Support for Purifying Fc Fusion Protein]

In one embodiment, the present invention provides a chromatographic support for purifying an Fc fusion protein. The chromatographic support of the present invention includes porous particles having a ligand having a binding ability to an Fc fusion protein immobilized thereon, and the support has an adjusted pore diameter.

[0017]

(Porous Particles)

The porous particles used for the chromatographic support of the present invention may be natural polymer-based porous particles or synthetic polymer-based porous particles, but are preferably synthetic polymer-based porous particles in order to adjust the pore diameter of the support. Further, the porous particles are preferably water-insoluble. The porous particle can be produced by suspending a monomer composition together with a porogen in an aqueous medium and polymerizing the monomer. Hereinafter, a method for producing porous particles used for the chromatographic support of the present invention will be described.

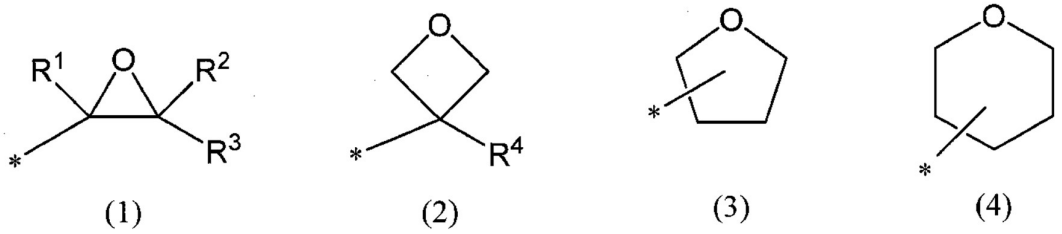
[0018]

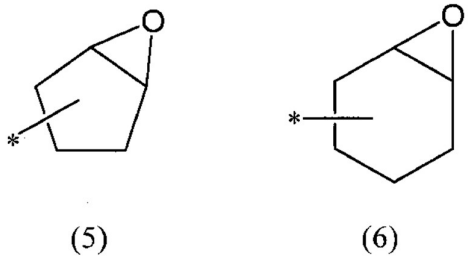
The monomer composition preferably contains a functional group-containing monomer. The functional group contained in the monomer is preferably one that can be used for an additional chemical reaction (for example, reaction with a crosslinking agent), and may be one capable of immobilizing a ligand. Examples of the functional group include a functional group selected from the group consisting of a cyclic ether group, a carboxy group, -C(=O)-O-C(=O)-, a succinimidyloxycarbonyl group, a formyl group, a hydroxyl group, and an isocyanate group. Among them, a cyclic ether group is preferable. Here, “cyclic ether group” is preferably a cyclic ether group having 3 to 7 atoms constituting a ring. The cyclic

ether group may have an alkyl group as a substituent. Examples of the cyclic ether group include cyclic ether groups represented by the following Formulas (1) to (6), and among them, the cyclic ether group represented by the Formula (1), (3), or (6) is preferable, and the cyclic ether group represented by the Formula (1) is more preferable.

[0019]

[Chemical Formula 1]





[0020]

[In the formula, R^1 to R^4 each independently represent a hydrogen atom or an alkyl group, and * represents a linking group.]

[0021]

The number of carbon atoms of the alkyl group represented by R^1 to R^4 is preferably 1 to 4, and more preferably 1 or 2. The alkyl group may be linear or branched, and examples thereof include a methyl group, an ethyl group, a n-propyl group, an isopropyl group, a n-butyl group, a sec-butyl group, and a tert-butyl group. Further, R^1 to R^4 are preferably hydrogen atoms.

[0022]

The functional group-containing monomer is preferably a monomer having a functional group capable of immobilizing a ligand and a polymerizable unsaturated group. Examples of such a monomer include (meth)acrylate-based monomers having a cyclic ether group, such as glycidyl (meth)acrylate, 3-oxiranylpropyl (meth)acrylate, 4-oxiranylbutyl (meth)acrylate, 5-oxiranylpentyl (meth)acrylate, 6-oxiranylhexyl (meth)acrylate, 7-oxiranylheptyl (meth)acrylate, 8-oxiranyloctyl (meth)acrylate, (3-methyloxiranyl)methyl (meth)acrylate, 4-hydroxybutyl (meth)acrylate glycidyl ether, glycerin mono(meth)acrylate glycidyl ether, 3,4-epoxycyclohexylmethyl (meth)acrylate, 3,4-epoxycyclohexylethyl (meth)acrylate, 3,4-epoxycyclohexylpropyl (meth)acrylate, α-(meth)acryl-ω-glycidyl polyethylene glycol, and tetrahydrofurfuryl (meth)acrylate; aromatic vinyl-based monomers having a cyclic ether group, such as (vinylbenzyl) glycidyl ether, (isopropenylbenzyl) glycidyl ether, (vinylphenethyl) glycidyl ether, (vinylphenylbutyl) glycidyl ether, (vinylbenzyloxyethyl) glycidyl ether, (vinylphenyl) glycidyl ether, (isopropenylphenyl) glycidyl ether, and 1,2-epoxy-3-(4-vinylbenzyl)propane; allyl ether-based monomers having a cyclic ether group, such as allyl glycidyl ether; (meth)acrylate-based monomers having an isocyanate group, such as isocyanatoethyl (meth)acrylate; unsaturated dicarboxylic acid anhydride-based monomers, such as maleic anhydride, methylmaleic anhydride, and glutaconic anhydride; and (meth)acrylic acid, 3,4-epoxy-1-butene, and 3,4-epoxy-3-methyl-1-butene. These monomers can be used singly or in combination of two or more thereof. Among these monomers, a (meth)acrylate-based monomer having a cyclic ether group is preferable, and glycidyl (meth)acrylate is more preferable.

[0023]

The total content of the functional group-containing monomer in

the monomer composition is preferably 35 parts by mass or more, more preferably 45 parts by mass or more, and still more preferably 55 parts by mass or more with respect to 100 parts by mass of the total amount of monomers in the monomer composition, and on the other hand, is preferably 99 parts by mass or less, more preferably 90 parts by mass or less, and still more preferably 85 parts by mass or less with respect to 100 parts by mass of the total amount of monomers in the monomer composition.

[0024]

The monomer composition may further contain a monomer (hereinafter also referred to as another monomer) other than the functional group-containing monomer in addition to the functional group-containing monomer. Examples of the other monomer include a polymerizable unsaturated group-containing monomer having no functional group capable of immobilizing a ligand. The other monomers are roughly classified into a non-crosslinking monomer and a crosslinking monomer, and one of these monomers may be used or used in combination.

[0025]

Examples of the non-crosslinking monomer usable as the other monomer include a (meth)acrylate-based non-crosslinking monomer, a (meth)acrylamide-based non-crosslinking monomer, an aromatic vinyl-based non-crosslinking monomer, a vinyl ketone-based non-crosslinking monomer, a (meth)acrylonitrile-based non-crosslinking monomer, and an N-vinylamide-based non-crosslinking monomer. These can be used singly or in combination of two or more thereof. Among the non-crosslinking monomers, a (meth)acrylate-based non-crosslinking monomer and an aromatic vinyl-based non-crosslinking monomer are preferable.

[0026]

Examples of the (meth)acrylate-based non-crosslinking monomer include methyl (meth)acrylate, ethyl (meth)acrylate, n-butyl (meth)acrylate, 4-tert-butyl (meth)acrylate, isobutyl (meth)acrylate, n-octyl (meth)acrylate, 2-ethylhexyl (meth)acrylate, cyclohexyl (meth)acrylate, methoxyethyl (meth)acrylate, hydroxyethyl (meth)acrylate, hydroxypropyl (meth)acrylate, glycerol mono (meth)acrylate, trimethylolethane mono (meth)acrylate, trimethylolpropane mono (meth)acrylate, butane triol mono (meth)acrylate, polyethylene glycol mono (meth)acrylate, methoxy polyethylene glycol (meth)acrylate, pentaerythritol mono (meth)acrylate, dipentaerythritol mono (meth)acrylate, and inositol mono (meth)acrylate. These can be used singly or in combination of two or more thereof.

[0027]

Examples of the (meth)acrylamide-based non-crosslinking monomer include (meth)acrylamide, dimethyl (meth)acrylamide, hydroxyethyl (meth)acrylamide, (meth)acryloylmorpholine, and diacetone (meth)acrylamide. These can be used singly or in combination of two or more thereof.

[0028]

Examples of the aromatic vinyl-based non-crosslinking monomer include styrenes such as styrene, α-methylstyrene, halogenated styrene, 4-methylstyrene, 2,4-dimethylstyrene, 2,4,6-trimethylstyrene, ethylvinylbenzene, 4-isopropylstyrene, 4-n-butylstyrene, 4-isobutylstyrene, and 4-tert-butylstyrene; and vinylnaphthalenes, such as 1-vinylnaphthalene and 2-vinylnaphthalene. These can be used singly or in combination of two or more thereof.

[0029]

Examples of the vinyl ketone-based non-crosslinking monomer

include ethyl vinyl ketone, propyl vinyl ketone, and isopropyl vinyl ketone. These can be used singly or in combination of two or more thereof.

[0030]

Examples of the (meth)acrylonitrile-based non-crosslinking monomer include acrylonitrile and methacrylonitrile. These can be used singly or in combination of two or more thereof.

[0031]

Examples of the N-vinylamide-based non-crosslinking monomer include N-vinylacetamide and N-vinylpropionamide. These can be used singly or in combination of two or more thereof.

[0032]

The total content of the non-crosslinking monomer in the monomer composition is preferably 0.01 parts by mass or more, more preferably 0.05 parts by mass or more, and still more preferably 0.1 parts by mass or more with respect to 100 parts by mass of the total amount of monomers in the monomer composition, and on the other hand, is preferably 30 parts by mass or less, more preferably 15 parts by mass or less, and still more preferably 5 parts by mass or less with respect to 100 parts by mass of the total amount of monomers in the monomer composition.

[0033]

Examples of the crosslinking monomer that can be used as the other monomer include (meth)acrylate-based crosslinking monomers, aromatic vinyl-based crosslinking monomers, and allyl-based crosslinking monomers. These can be used singly or in combination of two or more thereof. As the crosslinking monomer, a bifunctional to pentafunctional crosslinking monomer is preferable, and a bifunctional or trifunctional crosslinking monomer is more preferable. Further, among the crosslinking monomers, a

(meth)acrylate-based crosslinking monomer and an aromatic vinyl-based crosslinking monomer are preferable.

[0034]

Examples of the (meth)acrylate-based crosslinking monomer include ethylene glycol di(meth)acrylate, diethylene glycol di(meth)acrylate, triethylene glycol di(meth)acrylate, tetraethylene glycol di(meth)acrylate, polyethylene glycol di(meth)acrylate, propylene glycol di(meth)acrylate, dipropylene glycol di(meth)acrylate, tripropylene glycol di(meth)acrylate, tetrapropylene glycol di(meth)acrylate, polypropylene glycol di(meth)acrylate, 1,4-butanediol di(meth)acrylate, 1,6-hexanediol di(meth)acrylate, glycerin di(meth)acrylate, trimethylolethane di(meth)acrylate, trimethylolpropane di(meth)acrylate, trimethylolpropane tri(meth)acrylate, butanetriol di(meth)acrylate, pentaerythritol di(meth)acrylate, pentaerythritol tri(meth)acrylate, pentaerythritol tetra(meth)acrylate, glucose di(meth)acrylate, glucose tri(meth)acrylate, glucose tetra(meth)acrylate, dipentaerythritol di(meth)acrylate, dipentaerythritol tri(meth)acrylate, dipentaerythritol tetra(meth)acrylate, dipentaerythritol penta(meth)acrylate, inositol di(meth)acrylate, inositol tri(meth)acrylate, inositol tetra(meth)acrylate, mannitol di(meth)acrylate, mannitol tri(meth)acrylate, mannitol tetra(meth)acrylate, and mannitol penta(meth)acrylate. These can be used singly or in combination of two or more thereof.

[0035]

Examples of the aromatic vinyl-based crosslinking monomer include divinylbenzene, trivinylbenzene, divinyltoluene, divinylxylene, divinylethylbenzene, and divinylnaphthalene. These can be used singly or in combination of two or more thereof.

[0036]

Examples of the allyl-based crosslinking monomer include diallyl phthalate, diallyl isophthalate, diallyl terephthalate, diallyl maleate, diallyl fumarate, diallyl itaconate, diallyl trimellitate, triallyl trimellitate, triallyl cyanurate, diallyl isocyanurate, and triallyl isocyanurate. These can be used singly or in combination of two or more thereof.

[0037]

Further, examples of the crosslinking monomer include, in addition to those exemplified above, dehydration-condensation products of an amino alcohol, such as diaminopropanol, tris(hydroxymethyl)aminomethane, or glucosamine, with (meth)acrylic acid, and conjugated diolefins, such as butadiene and isoprene.

[0038]

The total content of the crosslinking monomer in the monomer composition is preferably 1 part by mass or more, more preferably 5 parts by mass or more, and still more preferably 10 parts by mass or more with respect to 100 parts by mass of the total amount of monomers in the monomer composition, and on the other hand, is preferably 50 parts by mass or less, more preferably 40 parts by mass or less, and still more preferably 30 parts by mass or less with respect to 100 parts by mass of the total amount of monomers in the monomer composition.

[0039]

Examples of the aqueous medium used for producing the porous particle include a water-soluble polymer aqueous solution. Examples of the water-soluble polymer include hydroxyethyl cellulose, polyvinyl alcohol, carboxymethyl cellulose, polyvinyl pyrrolidone, starch, and gelatin. The total amount of the aqueous medium used in the production of the porous particles is usually

about 200 parts by mass or more and 7000 parts by mass or less with respect to 100 parts by mass of the total amount of monomers. When water is used as the dispersion medium of the aqueous medium, for example, a dispersion stabilizer such as sodium carbonate, calcium carbonate, sodium sulfate, calcium phosphate, or sodium chloride may be used.

[0040]

In the production of the porous particles, the porogen is present as a non-polymerized component during the polymerization of the monomer, and has a role of forming pores in the particles obtained by the polymerization. The porogen is not particularly limited as long as it can be easily removed from the surface of the produced porous particles. Examples of the porogen include various organic solvents and linear polymers soluble in a mixed monomer, and these may be used in combination.

[0041]

Examples of the porogen include aliphatic hydrocarbons, such as hexane, heptane, octane, nonane, decane, and undecane; alicyclic hydrocarbons, such as cyclopentane and cyclohexane; aromatic hydrocarbons, such as benzene, toluene, xylene, naphthalene, ethylbenzene, and polystyrene; halogenated hydrocarbons, such as carbon tetrachloride, 1,2-dichloroethane, tetrachloroethane, and chlorobenzene; aliphatic alcohols, such as butanol, pentanol, hexanol, heptanol, 4-methyl-2-pentanol, and 2-ethyl-1-hexanol; alicyclic alcohols, such as cyclohexanol; aromatic alcohols, such as 2-phenylethyl alcohol and benzyl alcohol; ketones, such as diethyl ketone, methyl isobutyl ketone, diisobutyl ketone, acetophenone, 2-octanone, and cyclohexanone; ethers, such as dibutyl ether, diisobutyl ether, anisole, ethoxybenzene, polyethylene glycol, and polypropylene glycol; esters, such as

isopentyl acetate, butyl acetate, 3-methoxybutyl acetate, and diethyl malonate; and linear polymers, such as polysaccharides including methyl cellulose, ethyl cellulose, acetyl cellulose, cellulose acetate, cellulose triacetate, and alkyl cellulose, and homopolymers of non-crosslinking vinyl monomers. These porogens can be used singly or in combination of two or more thereof.

[0042]

The porogen preferably has a weight average molecular weight of 40000 or more, and more preferably 50000 or more, and on the other hand, preferably 180000 or less, and more preferably 100000 or less. Further, the total amount of the porogen used in the production of the porous particles is preferably 40 parts by mass or more, and more preferably 70 parts by mass or more, and on the other hand, preferably 600 parts by mass or less, and more preferably 400 parts by mass or less, with respect to 100 parts by mass of the total amount of monomers. The weight average molecular weight of the porogen and the use amount thereof can affect the pore diameter of the ligand-immobilized porous particles described later. For example, when the weight average molecular weight of the porogen decreases or the amount thereof used decreases, the pore diameter of the ligand-immobilized porous particles tends to decrease. The pore diameter of the porous particles can be adjusted by adjusting the molecular weight and the use amount of the porogen to be used.

[0043]

Specific methods for producing the porous particles used in the present invention include, for example, a method in which a polymerization initiator is dissolved in a mixed solution (monomer solution) containing the monomer composition and the porogen, the mixed solution is suspended in the aqueous medium, and the

suspension is heated to a predetermined temperature to carry out polymerization; a method in which a polymerization initiator is dissolved in a mixed solution (monomer solution) containing the monomer composition and the porogen, and the mixed solution is added to the aqueous medium heated to a predetermined temperature to carry out polymerization; and a method in which a mixed solution (monomer solution) containing the monomer composition and the porogen is suspended in the aqueous medium and heated to a predetermined temperature, and then a polymerization initiator is added thereto to carry out polymerization. When a polysaccharide such as ethyl cellulose is used as the porogen, it is preferable that the porogen be heated and stirred in advance, then mixed with the monomer composition to prepare a monomer solution, and subsequently a polymerization initiator be added thereto.

[0044]

As the polymerization initiator, a radical polymerization initiator is preferable. Examples of the radical polymerization initiator include azo-based initiators, peroxide-based initiators, and redox-based initiators. Specific examples include azobisisobutyronitrile, dimethyl azobisisobutyrate, azobis-2,4-dimethylvaleronitrile, benzoyl peroxide, di-tert-butyl peroxide, and benzoyl peroxide-dimethylaniline. The total amount of the polymerization initiator used in the production of the porous particles is usually about 0.01 to 10 parts by mass with respect to 100 parts by mass of the total amount of monomers.

[0045]

In the production of the porous particles, various surfactants may be used, including anionic surfactants such as alkyl sulfate salts, alkylaryl sulfate salts, alkyl phosphate salts, and fatty acid salts. In addition, polymerization inhibitors such as nitrite

salts (for example, sodium nitrite), iodide salts (for example, potassium iodide), tert-butylpyrocatechol, benzoquinone, picric acid, hydroquinone, copper chloride, and ferric chloride may also be used. Further, a polymerization regulator such as dodecyl mercaptan may be used.

[0046]

In the production of the porous particles, the polymerization temperature of the monomer may be determined according to the polymerization initiator used, but is usually about 2 to 100°C, and preferably 50 to 100°C. Further, the polymerization time of the monomer is usually 5 minutes to 48 hours, and preferably 10 minutes to 24 hours.

[0047]

The obtained porous particles may be reacted with at least one selected from the group consisting of a crosslinking agent and a hydrophilizing agent. When both a crosslinking agent and a hydrophilizing agent are used, the hydrophilization reaction may be performed after performing the crosslinking reaction, or the crosslinking reaction may be performed after performing the hydrophilization reaction. In addition, the crosslinking reaction and the hydrophilization reaction may be performed simultaneously. Alternatively, each of the crosslinking reaction and the hydrophilization reaction of the porous particles may be performed in parallel with the polymerization reaction of the monomer by the polymerization initiator. When a monomer composition containing a functional group-containing monomer is used as the monomer composition, the crosslinking reaction causes the crosslinking agent to undergo an addition reaction with a portion of the functional groups present in polymer molecules of the porous particles, thereby introducing a partial structure derived from the

crosslinking agent. As a result, residues of the functional groups are crosslinked to one another via the partial structure derived from the crosslinking agent. Further, when a monomer composition containing a functional group-containing monomer is used as the monomer composition, the hydrophilization reaction causes the hydrophilizing agent to undergo an addition reaction with a portion of the functional groups present in polymer molecules of the porous particles, thereby introducing a partial structure derived from the hydrophilizing agent.

[0048]

The crosslinking agent used in the crosslinking reaction may be any crosslinking agent that can react with a functional group capable of immobilizing a ligand to introduce a crosslinked structure. A crosslinking agent that can react with a functional group capable of immobilizing a ligand to introduce a crosslinked structure, and contains at least two groups represented by -C(=O)-NH- in the molecule, is preferable.

[0049]

When the porous particles have a cyclic ether group, preferably, a crosslinking agent containing at least two groups represented by $-C(=O)-NH-NH_2$ in the molecule as crosslinkable groups, a crosslinking agent containing at least two groups represented by -C(=O)-NH- in the molecule and at least two carboxy groups in the molecule as crosslinkable groups, or the like can be used. Further, when the porous particles have a carboxy group, -C(=O)-O-C(=O)-, a succinimidyloxycarbonyl group, a formyl group, or an isocyanate group, preferably, a crosslinking agent containing at least two groups represented by $-C(=O)-NH-NH_2$ in the molecule as crosslinkable groups, or the like can be used.

[0050]

Examples of the crosslinking agent containing at least two groups represented by -C(=O)-NH-NH_2 in a molecule include dicarboxylic acid dihydrazides, such as oxalyldihydrazide, malonic acid dihydrazide, succinic acid dihydrazide, 2,3-dihydroxysuccinic acid dihydrazide, glutaric acid dihydrazide, adipic acid dihydrazide, pimelic acid dihydrazide, octanedioic acid dihydrazide, nonanedioic acid dihydrazide, sebacic acid dihydrazide, dodecanedioic acid dihydrazide, phthalic acid dihydrazide, isophthalic acid dihydrazide, terephthalic acid dihydrazide, and quinolinic acid dihydrazide; and tricarboxylic acid trihydrazides, such as cyclohexanetricarboxylic acid trihydrazide. Examples of the crosslinking agent containing at least two groups represented by -C(=O)-NH- in the molecule and at least two carboxy groups in the molecule as crosslinkable groups include (alkylenebisimino)bis(oxoalkanoic acids), such as N1,N1-(ethane-1,2-diyl)bis(succinic monoamide). These crosslinking agents can be used singly or in combination of two or more thereof. Among these crosslinking agents, dicarboxylic acid dihydrazides and (alkylenebisimino)bis(oxoalkanoic acids) are preferable, and dicarboxylic acid dihydrazides are more preferable, in order to improve the flow properties of the chromatographic support, or the pressure resistance characteristics and fouling resistance during liquid passage.

[0051]

In the crosslinking reaction, a crosslinking agent other than the above-described crosslinking agent can also be used. Examples of such a crosslinking agent include polyfunctional isocyanate-based crosslinking agents, polyfunctional epoxy-based crosslinking agents, polyfunctional aldehyde-based crosslinking agents, polyfunctional thiol-based crosslinking agents, polyfunctional

oxazoline-based crosslinking agents, polyfunctional aziridine-based crosslinking agents, and metal chelate-based crosslinking agents.

[0052]

In the crosslinking reaction, the total amount of the crosslinking agent used is preferably 0.01 molar equivalent or more, more preferably 0.05 molar equivalent or more, and still more preferably 0.1 molar equivalent or more, and on the other hand, preferably ~0.8 molar equivalent or less, more preferably 0.7 molar equivalent or less, and still more preferably 0.6 molar equivalent or less, with respect to 1 mole of the functional groups derived from the functional group-containing monomer used in the production of the porous particles.

[0053]

As the hydrophilizing agent used in the hydrophilization reaction, in order to improve the fouling resistance property or ligand low-leakage property of the chromatographic support, a compound having a total of 2 or more of at least one hydrophilic group selected from a hydroxy group and a mercapto group in the molecule is preferable, and a compound having a total of 2 to 4 of at least one hydrophilic group selected from a hydroxy group and a mercapto group in the molecule is more preferable. Examples of the hydrophilizing agent include alcohols having a mercapto group in a molecule, such as mercaptoethanol and thioglycerol; and polyhydric alcohols, such as glycerol and diglycerol. These hydrophilizing agents can be used singly or in combination of two or more thereof. Among these, in order to improve the fouling resistance property or ligand low-leakage property of the support, alcohols having a mercapto group in a molecule are preferable, and thioglycerol is more preferable.

[0054]

The total amount of the hydrophilizing agent used in the hydrophilization reaction is preferably 0.5 mol equivalent or more, more preferably 1 mol equivalent or more, still more preferably 2 mol equivalent or more, and on the other hand, preferably 10 mol equivalent or less, more preferably 8 mol equivalent or less, still more preferably 6 mol equivalent or less, with respect to 1 mol of the functional group derived from the functional group-containing monomer used for producing the porous particles.

[0055]

The crosslinking reaction or the hydrophilization reaction may be performed in the presence of a basic catalyst. Examples of the basic catalyst include triethylamine, N,N-dimethyl-4-aminopyridine, sodium hydroxide, and diisopropylethylamine, and one thereof can be used singly or in combination of two or more thereof.

[0056]

The reaction time of the crosslinking reaction or the hydrophilization reaction is not particularly limited, but is usually about 0.5 to 72 hours, and preferably 0.5 to 48 hours. The reaction temperature may be appropriately selected at a temperature equal to or lower than the boiling point of the solvent, and is usually about 2 to 100°C.

[0057]

(Ligand)

The ligand immobilized on the porous particles is a protein ligand having a binding ability to an Fc fusion protein, and is preferably one or more protein ligands selected from the group consisting of Protein A, Protein G, Protein L, and analogs thereof. The ligand is preferably Protein A or an analog of Protein A, and more preferably an analog of Protein A.

[0058]

Protein A includes five domains of E, D, A, B, and C, which are capable of binding to the Fc of an immunoglobulin and are thus Fc-binding domains. Preferably, the analog of Protein A used as a ligand in the present invention is an Fc-binding protein containing an Fc-binding domain of Protein A or a modified Fc-binding domain thereof. Preferably, the ligand of the present invention includes one or more Fc-binding domains selected from the group consisting of the B domain, the C domain or modified domains thereof of Protein A. More preferably, the ligand includes one or more Fc-binding domains selected from the group consisting of the C domain of Protein A consisting of the amino acid sequence of SEQ ID NO: 1 and modified domains thereof. More preferably, the Fc-binding domain contained in the ligand is a modified domain of the C domain of Protein A consisting of the amino acid sequence of SEQ ID NO: 1.

[0059]

In order to improve the DBC of a chromatographic support for an Fc fusion protein or to suppress leakage of the ligand, the ligand preferably includes an Fc-binding domain consisting of an amino acid sequence having one or more substitutions selected from the group consisting of the following (a) to (i), with respect to the amino acid sequence of SEQ ID NO: 1 or an amino acid sequence having 85% or greater identity thereto (the amino acid sequence of a parent Fc-binding domain):

(a) substitution of the amino acid residue at a position corresponding to position 1 of the amino acid sequence of SEQ ID NO: 1 with a valine residue;

(b) substitution of the amino acid residue at a position corresponding to position 3 of the amino acid sequence of SEQ ID NO: 1 with an alanine residue;

(c) substitution of the amino acid residue at a position

corresponding to position 6 of the amino acid sequence of SEQ ID NO: 1 with an alanine residue or an aspartic acid residue;

(d) substitution of the amino acid residue at a position corresponding to position 9 of the amino acid sequence of SEQ ID NO: 1 with an alanine residue;

(e) substitution of the amino acid residue at a position corresponding to position 11 of the amino acid sequence of SEQ ID NO: 1 with an alanine residue, a glutamine residue, or a glutamic acid residue;

(f) substitution of the amino acid residue at a position corresponding to position 23 of the amino acid sequence of SEQ ID NO: 1 with a leucine residue;

(g) substitution of the amino acid residue at a position corresponding to position 29 of the amino acid sequence of SEQ ID NO: 1 with an alanine residue;

(h) substitution of the amino acid residue at a position corresponding to position 43 of the amino acid sequence of SEQ ID NO: 1 with an alanine residue; and

(i) substitution of the amino acid residue at a position corresponding to position 49 of the amino acid sequence of SEQ ID NO: 1 with an arginine residue.

[0060]

Preferably, the Fc-binding domain contained in the ligand consists of an amino acid sequence in which 3 or more substitutions, more preferably 3 to 9 substitutions, further preferably 3 to 6 substitutions, and further preferably 3 to 5 substitutions selected from the group consisting of (a) to (i) above are made with respect to the amino acid sequence of the parent Fc-binding domain.

[0061]

Preferable examples of the substitution of the Fc-binding domain contained in the ligand include the following:

three or more substitutions selected from the group consisting of (a), (b), (c), (d), (g), (h) and (i);

three or more substitutions including a combination of two or more substitutions selected from the group consisting of (a), (b), (c), (d), (g), (h), and (i) and one or more substitutions selected from the group consisting of (e) and (f);

four or more substitutions including a combination of three or more substitutions selected from the group consisting of (a), (b), (c), (d), (g), (h), and (i) and one or more substitutions selected from the group consisting of (e) and (f); and

three or more substitutions including a combination of one or more substitutions selected from the group consisting of (a) and (d), one or more substitutions selected from the group consisting of (e) and (f), and one or more substitutions selected from the group consisting of (b), (c), (g), (h), and (i).

Specific examples of such substitutions include the following:

a combination of (a), (b), and (g);

a combination of (a), (c), and (g);

a combination of (a), (b), (c), and (g);

a combination of (a), (d), and (g);

a combination of (a), (e), and (g);

a combination of (d), (e), and (h);

a combination of (a), (e), (f), and (g);

a combination of (a), (e), (g), and (i);

a combination of (a), (b), (c), (e), and (g); and

a combination of (a), (b), (c), (e), (g), and (i).

[0062]

The Fc-binding domain contained in the ligand used in the

present invention can be prepared in accordance with a known technique. For example, the Fc-binding domain can be prepared by expressing a polynucleotide encoding the same. The polynucleotide encoding the Fc-binding domain may be isolated from a cell or chemically synthesized. For example, a polynucleotide encoding an Fc-binding domain consisting of the amino acid sequence of SEQ ID NO: 1 can be isolated from a microorganism expressing Protein A (for example, Staphylococcus aureus). A desired mutation can also be added to the isolated polynucleotide by known means. For example, an Fc-binding domain including the above-described amino acid residue substitution can be prepared by introducing a mutation into a polynucleotide encoding a parent domain (for example, an Fc-binding domain consisting of the amino acid sequence of SEQ ID NO: 1 or an amino acid sequence having 85% or more identity thereto) such that a desired amino acid residue substitution is generated. Specific methods for introducing a mutation into a polynucleotide include site-directed mutagenesis, homologous recombination, and SOE (splicing by overlap extension)-PCR (Gene,1989,77:61-68), and detailed procedures therefor are well known to those skilled in the art.

[0063]

The ligand used in the present invention may contain one or more of the Fc-binding domains, but preferably contains 2 or more, more preferably 2 to 12, and still more preferably 4 to 7 Fc-binding domains. When the ligand includes two or more of the Fc-binding domains, each of the domains may be the same or different. Preferably, the ligand is an Fc-binding protein in which the amino acid sequences of two or more of the Fc-binding domains are linearly linked. Here, the amino acid sequence being “linearly linked” means a structure in which two or more amino acid sequences

are linked in series via a linker or without a linker. For example, in the case of using a linker, "linearly linked" means a structure in which the C-terminus of one amino acid sequence and the N-terminus of another amino acid sequence are linked in series via a linker, and on the other hand, in the case of not using a linker, "linearly linked" means a structure in which the C-terminus of one amino acid sequence and the N-terminus of another amino acid sequence are linked in series by a peptide bond.

[0064]

The ligand can be produced using a known genetic recombination technique described in Current Protocols In Molecular Biology by Frederick M. Ausbel et al. or Molecular Cloning (Cold Spring Harbor Laboratory Press, 3rd edition, 2001) edited by Sambrook et al. For example, an expression vector containing a polynucleotide encoding the Fc-binding protein is transformed into a host cell, and the obtained recombinant is cultured in an appropriate liquid medium, whereby a target protein ligand can be obtained from the cultured cell. As a preferable expression vector, any known vector capable of replicating in a host cell can be used, and examples thereof include plasmids described in US 5,151,350, and plasmids described in Molecular Cloning (Cold Spring Harbor Laboratory Press, 3rd edition, 2001) edited by Sambrook et al. In addition, the host for transformation is not particularly limited, and known hosts used for expression of recombinant proteins, such as bacteria including *Escherichia coli*, fungal cells, insect cells, and mammalian cells, can be used. For the introduction of a polynucleotide for transforming a host, any method known in the art may be used depending on each host, and for example, a known method described in Molecular Cloning (Cold Spring Harbor Laboratory Press, 3rd edition, 2001) edited by Sambrook et al. or the like can be used.

A method for culturing the obtained transformant (preferably a cell such as a bacterium) and recovering the expressed protein is well known to those skilled in the art. Alternatively, the Fc-binding protein of the present invention may be expressed using a cell-free protein synthesis system.

[0065]

(Immobilization of Ligand on Porous Particles)

Immobilization of the ligand on the porous particles may be performed in accordance with a conventional method. As a method of immobilizing a ligand on the particles, a chemical binding method is preferable, and examples thereof include a method of binding a ligand to a functional group capable of immobilizing the ligand contained in the porous particles. Specific examples include a method of binding a cyclic ether group, a carboxy group, a -C(=O)-O-C(=O)- group, or a formyl group contained in the porous particles to an amino group or the like of the ligand, for example. This method may be performed with reference to the description in, for example, WO 2015/119255 A and WO 2015/041218 A.

[0066]

The ligand can be immobilized on the porous particles via a linker (spacer). The introduction of the linker into the porous particles and the immobilization of the ligand using the linker may be performed with reference to US 5,260,373, JP 2010-133733 A, and JP 2010-133734 A. Examples of compounds providing the linker include diglycidyl ethers of aliphatic polyhydroxy compounds, such as ethylene glycol diglycidyl ether, diethylene glycol diglycidyl ether, polyethylene glycol diglycidyl ether, 1,2-propylene glycol diglycidyl ether, dipropylene glycol diglycidyl ether, polypropylene glycol diglycidyl ether, 1,4-butanediol diglycidyl ether, neopentyl glycol diglycidyl ether, 1,6-hexanediol diglycidyl

ether, and glycerol diglycidyl ether; and polyglycidyl ethers of aliphatic polyhydroxy compounds, such as sorbitol polyglycidyl ether, glycerol polyglycidyl ether, trimethylolpropane polyglycidyl ether, diglycerol polyglycidyl ether, and polyglycerol polyglycidyl ether. Among these, when the above-described hydrophilization reaction of the porous particles is performed, diglycidyl ethers of aliphatic polyhydroxy compounds are preferable.

[0067]

The linker introduction reaction and the ligand immobilization reaction to the porous particles are preferably performed in a buffer at pH 7 to 14 in order to increase the reaction efficiency. In addition, in the linker introduction reaction, the reaction time is not particularly limited, but is usually about 0.5 to 72 hours, and the reaction temperature may be appropriately selected at a temperature equal to or lower than the boiling point of the solvent, but is usually about 2 to 100°C. In addition, in the ligand immobilization reaction, the reaction time is not particularly limited, but is usually about 0.1 to 72 hours, and the reaction temperature may be appropriately selected at a temperature equal to or lower than the boiling point of the solvent, but is usually about 2 to 100°C.

[0068]

Alternatively, the ligand may be immobilized on the porous particle using, for example, a method of controlling the orientation of the ligand (U.S. Patent No. 6,399,750, Ljungquist C et al., Eur.J.Biochem.,1989,186:557-561), or a method of accumulating the ligand on the porous particles by an associative group (JP 2011-256176 A).

[0069]

The amount of the ligand immobilized on the porous particles is

preferably 10 mg or more, and more preferably 25 mg or more, and on the other hand, preferably 300 mg or less, and more preferably 150 mg or less per 1 g of dry weight of the porous particles in order to increase the DBC.

[0070]

The obtained ligand-immobilized porous particles are preferably hydrophilized in order to improve the fouling resistance property or ligand low-leakage property of the chromatographic support. In the hydrophilization reaction, the ligand-immobilized porous particles are reacted with a compound having a hydrophilic group. As the compound having the hydrophilic group, a compound having, in total, 2 to 4 hydrophilic groups in a molecule, the hydrophilic groups being at least one selected from the group consisting of a hydroxy group and a mercapto group, is preferable. Examples of such compounds include alcohols having a mercapto group in a molecule, such as mercaptoethanol and thioglycerol; and polyhydric alcohols, such as glycerol and diglycerol. These compounds can be used singly or in combination of two or more thereof. Among these compounds, in order to improve the fouling resistance property or ligand low-leakage property of the support, an alcohol having a mercapto group in the molecule is preferable, and thioglycerol is more preferable.

[0071]

In the hydrophilization reaction, the total amount of the compound having a hydrophilic group used is preferably 1 part by mass or more, more preferably 10 parts by mass or more, and still more preferably 100 parts by mass or more, and on the other hand, preferably 1000 parts by mass or less, more preferably 800 parts by mass or less, and still more preferably 600 parts by mass or less, with respect to 100 parts by mass of the ligand-immobilized porous

particles (dry weight).

[0072]

The hydrophilization reaction may be performed in the presence of a basic catalyst. Examples of the basic catalyst include triethylamine, N,N-dimethyl-4-aminopyridine, sodium hydroxide, and diisopropylethylamine, and these can be used singly or in combination of two or more thereof.

[0073]

In the hydrophilization reaction, the reaction time is not particularly limited, but is usually about 0.5 to 72 hours, preferably 0.5 to 48 hours, and the reaction temperature may be appropriately selected at a temperature equal to or lower than the boiling point of the solvent, but is usually about 2 to 100°C.

[0074]

The obtained ligand-immobilized porous particles can be purified by separation means such as filtration or washing. In addition, the ligand-immobilized porous particles may be classified.

[0075]

The volume average particle diameter of the ligand-immobilized porous particles is preferably 40 to 150 μm, more preferably 45 to 100 μm, still more preferably 50 to 100 μm, and still more preferably 50 to 80 μm. When the volume average particle diameter of the ligand-immobilized porous particle is in the above range, the DBC for an Fc fusion protein and the pressure resistance characteristics during liquid passage are improved. The coefficient of variation of the volume average particle diameter is preferably 40% or less, and more preferably 30% or less. In addition, the chromatographic support of the present invention preferably has a specific surface area of 1 to 500 m^2/g, and more

preferably 10 to 300 m^2/g. In the present specification, the volume average particle diameter of the ligand-immobilized porous particles refers to a volume average particle diameter measured by a laser diffraction scattering method in accordance with JIS Z 8825(2013). Specifically, the volume average particle diameter of the particles can be determined by measuring the volume-based particle diameter distribution of the particles with a laser diffraction scattering particle size distribution analyzer (for example, LS13320 manufactured by Beckman Coulter, Inc.) in accordance with JIS Z 8825(2013) as described in Examples described later. In addition, in the present specification, the specific surface area of the chromatographic support refers to a value measured by, for example, laser diffraction/scattering particle diameter distribution measurement.

[0076]

(Application to Fc Fusion Protein Purification)

The ligand-immobilized porous particles for use in the present invention are tuned to have a specific range of average pore diameter, thereby improving the ability to purify an Fc fusion protein. The average pore diameter of the ligand-immobilized porous particles is preferably 75 nm or more, and more preferably 101 nm or more, and on the other hand, preferably 120 nm or less, and more preferably 110 nm or less. When the average pore diameter of the ligand-immobilized porous particle is in the above range, the DBC for an Fc fusion protein and the pressure resistance characteristics during liquid passage are improved.

[0077]

Herein, the average pore diameter is calculated in accordance with the method described in Hagel et al. (Journal of Chromatography A,1996,Vol.743:32-42). Specifically, the average

pore diameter may be calculated in accordance with a method described in Examples described later. That is, a plot of the molecular size and the distribution coefficient Kd of pullulan is created, an approximate straight line is drawn, and Kd = ε (1 - molecular size/average pore size)^(1/2) is established, such that the average pore diameter is calculated from the intercept and the slope. The distribution coefficient Kd of pullulan is expressed by Kd = (elution volume of pullulan - elution volume of dextran)/(elution volume of sodium chloride - elution volume of dextran). The elution volumes of pullulan, dextran, and sodium chloride can be measured by passing a 20 mM sodium phosphate/150 mM sodium chloride aqueous solution containing pullulan, a 20 mM sodium phosphate/150 mM sodium chloride aqueous solution containing dextran, and a 500 mM sodium chloride aqueous solution, respectively, through a column packed with the ligand-immobilized porous particles.

[0078]

In addition, the ligand-immobilized porous particles preferably have a porosity of 70 to 95% (v/v), and more preferably 75 to 85% (v/v) in a wet state. When the porosity of the ligand-immobilized porous particle is in the above range, the DBC for an Fc fusion protein and the pressure resistance characteristics during liquid passage are improved. In the present specification, the porosity of the ligand-immobilized porous particles in a wet state refers to the ratio of the pore volume (the volume of the pore portions) to the particle volume (the total volume of the non-pore portions of the porous particles, the pore portions of the porous particles, and the ligand portions) in the ligand-immobilized porous particles when measured in a wet state. For example, the porosity can be determined based on the elution volume of a 500 mM sodium chloride

aqueous solution from a column packed with ligand-immobilized porous particles and the elution volume of a 20 mM sodium phosphate/150 mM sodium chloride aqueous solution containing dextran from the column.

[0079]

Specifically, the average pore diameter and the porosity in a wet state of the ligand-immobilized porous particles may be measured according to the method described in Examples described later.

[0080]

When the ligand-immobilized porous particle is used for purification of an Fc fusion protein, the ligand-immobilized porous particle can exhibit high performance in terms of the height of the DBC and the small amount of non-specific binding (impurity contamination). This indicates that the chromatographic support of the present invention has a high ability to purify an Fc fusion protein considering that high performance was not obtained when the conventional support was used for Fc fusion protein purification. Therefore, the ligand-immobilized porous particles can be used as a chromatographic support for purifying an Fc fusion protein.

[0081]

[Chromatography Column]

A chromatography column including the chromatographic support of the present invention is provided. The chromatography column of the present invention is the same as an ordinary chromatography column except that it contains the chromatographic support of the present invention. Specific examples thereof include a chromatography column including a column container and the chromatographic support of the present invention packed in the column container. The chromatography column of the present

invention is suitable for use in affinity chromatography.

[0082]

[Method of purifying an Fc fusion protein]

In one embodiment, the present invention provides a method of purifying an Fc fusion protein using the chromatographic support of the present invention described above. The method of purifying an Fc fusion protein according to the present invention can be carried out in accordance with general procedures for antibody purification by affinity chromatography, except that the chromatographic support of the present invention is used.

[0083]

In one preferable embodiment, the method of purifying an Fc fusion protein according to the present invention includes: a step of passing a solution containing an Fc fusion protein through a column containing the chromatographic support of the present invention, to bind the Fc fusion protein to the support (Step 1); a step of washing the support (Step 2); and a step of recovering the Fc fusion protein from the support (Step 3).

[0084]

In the Step 1, a solution (sample solution) containing an Fc fusion protein is caused to flow through a column containing the chromatographic support under the condition that the Fc fusion protein binds to a protein ligand of the support. Here, examples of the sample solution include an extract of a culture of Fc-fusion protein-expressing cells or a buffer containing the extract. Conditions for binding the Fc fusion protein include, for example, a protein concentration of 0.1 to 10 g/L, a pH of the solution of 5 to 9, a residence time in the column of 0.5 to 50 minutes, and a temperature of 0 to 40°C.

[0085]

In the Step 1, most of the substances other than the Fc fusion protein in the sample solution pass through the column. In the Step 2, contaminants (for example, proteins other than the Fc fusion protein in the sample solution) remaining in the column are removed by washing. For the washing, a neutral buffer containing a salt such as NaCl may be used for the packing material, for example, a sodium phosphate/sodium chloride aqueous solution, a sodium dihydrogen phosphate/disodium hydrogen phosphate solution, a citric acid/disodium hydrogen phosphate solution, a hydrochloric acid/tris(hydroxymethyl)aminomethane solution, or a HEPES/sodium hydroxide solution.

[0086]

In the Step 3, the purified Fc fusion protein is recovered by allowing an eluent to flow through the column and eluting the Fc fusion protein from the column. As the eluent, a buffer having a pH of 2 to 5 can be used, for example, a citric acid/sodium citrate solution, an acetic acid/sodium acetate solution, or a hydrochloric acid/glycine solution.

[0087]

The Fc fusion protein contained in the eluate eluted from the column in the Step 3 may be further purified. Purification of an Fc fusion protein can be performed using, for example, cation-exchange chromatography, anion-exchange chromatography, mixed-mode chromatography, hydrophilic interaction chromatography, hydrophobic interaction chromatography, and size-exclusion chromatography, singly or in an appropriate combination.

[0088]

The type of Fc fusion protein to be targeted in the method of purifying an Fc fusion protein according to the present invention is not particularly limited, but Fc fusion proteins having a

molecular weight of 160 to 300 kDa are preferable. Preferable examples of target Fc fusion proteins include etanercept, rilonacept, and efmoroctocog alfa, and among them, etanercept is more preferable. Other preferable examples of the target Fc fusion protein include a biosimilar of etanercept, rilonacept, or efmoroctocog alfa. Examples thereof include an Fc fusion protein having 85% or more amino acid sequence identity to etanercept, rilonacept, or efmoroctocog alfa, and having a binding property to the ligand contained in the chromatographic support of the present invention in a region corresponding to the Fc fragment thereof. The amino acid sequence of etanercept can be represented by SEQ ID NO: 4. The amino acid sequence of rilonacept can be represented by SEQ ID NO: 5. Therefore, an Fc fusion protein consisting of the amino acid sequence of SEQ ID NO: 4 or SEQ ID NO: 5, or an amino acid sequence having 85% or more identity to any one of these sequences, may be mentioned as a preferable example of the target Fc fusion protein.

[0089]

The method of purifying an Fc fusion protein according to the present invention may further include a step of passing an alkaline liquid through the chromatographic support (Step 4) after the Step 3. Examples of the alkaline liquid used in the Step 4 include an aqueous sodium hydroxide solution, an aqueous potassium hydroxide solution, triethylamine, and tetrabutylammonium hydroxide. The molar concentration of the alkali salt in the alkaline solution used in the Step 4 is preferably 0.01 to 4.0 M, and more preferably 0.1 to 2.0 M or more. The pH of the alkaline solution used is preferably pH 11.0 to 15.0, and more preferably pH 12.0 to 14.0.

[0090]

After the Step 4, the chromatographic support can be used again

for purification of an Fc fusion protein. Thus, in one embodiment, the method of purifying an Fc fusion protein according to the present invention includes repeating the Step 1 to Step 4 a plurality of times, preferably 50 times or more, and more preferably 100 times or more. The chromatographic support of the present invention can maintain a high DBC for an Fc fusion protein and a state with less non-specific binding (impurity contamination) even when repeatedly used. In addition, the chromatographic support of the present invention is excellent in pressure resistance characteristics during liquid passage after being packed into a column, which is also an advantage for repeated use.

Examples

[0091]

Hereinafter, the present invention will be described in detail with reference to Examples. However, the present invention is not limited to these Examples.

[0092]

(Preparation Example 1) Preparation of Ligand

Fc-binding proteins PrA-1 and PrA-2 were prepared. PrA-1 and PrA-2 are Fc-binding proteins containing a homopentamer in which Fc-binding domains are linked in series. The Fc-binding domains contained in PrA-1 and PrA-2 are modified domains of the C domain of Protein A (SEQ ID NO: 1), and each of the Fc-binding domains is composed of an amino acid sequence in which a mutation described in Table 1 is introduced into the parent domain of SEQ ID NO: 1.

[0093]

[Table 1]

Fc-binding protein	Fc-binding domain			Number of domains
	Parent domain	Introduced mutation	SEQ ID NO:	

PrA-1	ADNKFNKEQQNAFYEILHLPNLTEEQR NGFIQSLKDDPSVSKEILAEAKKLNDA QAPK (SEQ ID NO: 1)	A1V, N3A, N6D, N11Q, G29A, K49R	2	5
PrA-2		A1V, N11E, G29A,	3	5

[0094]

Expression and purification of PrA-1 and PrA-2 were performed as follows. Escherichia coli BL21(DE3) was transformed using plasmids encoding PrA-1 and PrA-2, respectively, and the obtained transformants were cultured in a rich medium at 37°C until the logarithmic growth phase was reached. Thereafter, isopropyl-β-thiogalactopyranoside (manufactured by Wako Pure Chemical Industries, Ltd.) was added to the medium to a final concentration of 1 mM, and the culture was further cultured at 37°C for 4 hours, thereby expressing a target protein. Subsequently, the culture solution was centrifuged to remove a supernatant, and a 30 mM Tris buffer (pH 9.5) containing lysozyme derived from egg white (manufactured by Wako Pure Chemical Industries, Ltd.) and polyoxyethylene (10) octylphenyl ether (manufactured by Wako Pure Chemical Industries, Ltd.) was added to the obtained bacterial cells to disrupt the bacterial cells. The recombinant Fc-binding protein was purified from the obtained cell lysate by cation-exchange chromatography (SP-Sepharose FF, manufactured by GE Healthcare Biosciences) and anion-exchange chromatography (Q-Sepharose FF, manufactured by GE Healthcare Biosciences). Purified Fc-binding proteins were dialyzed against 10 mM citrate buffer at pH 6.0. The purity of the recombinant Fc-binding protein confirmed by SDS-PAGE was 95% or more.

[0095]

(Example 1) Production of Chromatographic Support and Purification of Fc Fusion Protein

(1) Preparation of Porous Particles

Step (a): To 448 g of pure water, 2.69 g of polyvinyl alcohol (PVA-217 manufactured by Kuraray Co., Ltd.) was added, and heated and stirred to dissolve polyvinyl alcohol, thereby preparing an aqueous solution S. Ethyl cellulose (ETHOCEL™ 14, manufactured by Dow Chemical Company) (2.94 g) was added to 26.44 g of 2-octanone (manufactured by Toyo Gosei Co., Ltd.), and the mixture was heated and stirred to dissolve the ethyl cellulose, thereby preparing an organic solution P. Subsequently, a monomer composition consisting of 3.63 g of divinylbenzene (manufactured by Wako Pure Chemical Industries, Ltd.), 0.36 g of 1-ethyl-4-vinylbenzene (manufactured by ChemSampCo), and 14.15 g of glycidyl methacrylate (manufactured by Mitsubishi Gas Chemical Company, Inc.) was dissolved in the organic solution P, thereby preparing a monomer solution.

[0096]

Step (b): The entire amount of the aqueous solution S was charged into a separable flask, a thermometer, a stirring blade, and a cooling tube were attached, the separable flask was set in a hot water bath, and stirring was started at a stirring speed of 470 rpm under a nitrogen atmosphere. The entire amount of the monomer solution was charged into the separable flask, and heated with a warm water bath. When the internal temperature reached 85°C, 1.34 g of 2,2′-azobis(methyl isobutyrate) (manufactured by Wako Pure Chemical Industries, Ltd.) was added thereto, and the internal temperature was adjusted to 86°C. Thereafter, while maintaining the solution at 86°C, 86.38 g of thioglycerol (manufactured by Tokyo Chemical Industry Co., Ltd.) and 10.15 g of diisopropylethylamine (manufactured by Tokyo Chemical Industry Co., Ltd.) were added, and the mixture was stirred for a total of 3 hours. The obtained reaction solution was cooled and then filtered

to recover particles, and the particles were washed with pure water and ethanol. The washed particles were dispersed in pure water, and decantation was performed three times to remove small particles. Next, the particles were dispersed in pure water such that the concentration of the particles was 10% by mass to obtain a dispersion of porous particles.

[0097]

Step (c): Ethylene glycol diglycidyl ether was reacted with thioglycerol-derived hydroxy groups contained in the porous particles in the obtained dispersion. That is, 8.8 g of pure water, 0.91 g of sodium sulfate (manufactured by Wako Pure Chemical Industries, Ltd.), and 0.08 g of sodium carbonate (manufactured by Wako Pure Chemical Industries, Ltd.) were mixed to obtain a carbonate buffer (pH 9.5). To the carbonate buffer were added 0.27 g of ethylene glycol diglycidyl ether (DENACOL EX810, manufactured by Nagase ChemteX Corporation) and 8 mL of the porous particles, followed by shaking and stirring at 23°C for 16 hours. Next, the particles were dispersed in pure water such that the concentration of the particles was 50% by volume to obtain a porous particle dispersion. The porous particles contained in this dispersion are referred to as “porous particles 1”.

[0098]

(2) Preparation of Ligand-Immobilized Porous Particles

A ligand was immobilized on the porous particle 1. That is, 28.8 g of pure water, 5.4 g of sodium sulfate (manufactured by Wako Pure Chemical Industries, Ltd.), 0.2 g of sodium hydrogen carbonate (manufactured by Wako Pure Chemical Industries, Ltd.), and 0.16 g of sodium carbonate (manufactured by Wako Pure Chemical Industries, Ltd.) were mixed to obtain a carbonate buffer (pH 9.3). To 25 mL of the carbonate buffer were added 0.17 g of the Fc fusion protein

PrA-1 prepared in Preparation Example 1 and 8 mL of the porous particles 1, followed by shaking and stirring at 23°C for 1.5 hours. The obtained reaction solution was then filtered to recover the particles. A buffer was obtained by mixing 8.8 g of pure water, 0.1 g of sodium sulfate (manufactured by Wako Pure Chemical Industries, Ltd.), and 0.03 g of sodium hydroxide (manufactured by Wako Pure Chemical Industries, Ltd.), and then 4.5 g of thioglycerol (manufactured by Tokyo Chemical Industry Co., Ltd.) was added to prepare a hydrophilization reaction solution. The hydrophilization reaction solution was added to the particles, and the mixture was shaken and stirred at 23°C for 16 hours to perform a hydrophilization reaction. Next, the particles were dispersed in pure water such that the concentration of the particles was 50% by volume to obtain a ligand-immobilized porous particle dispersion. The average pore diameter of the ligand-immobilized porous particles contained in this dispersion was calculated by the following procedure. This ligand-immobilized porous particle is referred to as a “support 1”.

[0099]

<Calculation of Volume Average Particle Diameter>

The volume average particle diameter of the support 1 was measured by a laser diffraction scattering type particle size distribution analyzer (LS13320 manufactured by Beckman Coulter, Inc.) in accordance with JIS Z 8825(2013). Here, the refractive index of the solvent (water) was set to 1.333, and the refractive index of the ligand-immobilized porous particles was set to 1.50.

[0100]

<Calculation of Porosity>

The porosity of the support 1 in a wet state was measured by the following method. The support 1 was packed into a 4 mL volume

(1) column (5 mmφ × 200 mm in length) using a 20 mM sodium phosphate/150 mM sodium chloride aqueous solution. 50 μL of a 500 mM sodium chloride aqueous solution was injected into the column, and the elution volume (2) was measured. In addition, 50 μL of a 20 mM sodium phosphate/150 mM sodium chloride aqueous solution containing dextran (molecular weight: 5000000 to 40000000, manufactured by Wako Pure Chemical Industries, Ltd.) was injected into the column, and the elution volume (3) was measured. The value obtained by subtracting the elution volume (3) (particle void volume) of the dextran-containing aqueous solution from the column volume was defined as "particle volume", and the value obtained by subtracting the elution volume (3) of dextran from the elution volume of the sodium chloride aqueous solution (2) was defined as "pore volume". According to the following formula, the ratio (%) of the pore volume to the particle volume was calculated as "porosity in a wet state".

Porosity (%) = pore volume/particle volume × 100 = {(2) - (3)}/{(1) - (3)} × 100

(1): Column volume

(2): Elution volume of 500 mM sodium chloride aqueous solution

(3): Elution volume of dextran-containing aqueous solution

[0101]

<Calculation of Average Pore Diameter>

The average pore diameter in the wet state of the support 1 was calculated in accordance with the method described in Hagel et al. (Journal of Chromatography A,1996,Vol.743:32-42). The support 1 was packed into a column having a volume of 4 mL (5 mmφ × 200 mm in length) using a 20 mM sodium phosphate/150 mM sodium chloride aqueous solution. 50 μL of a 500 mM sodium chloride aqueous solution was injected into the column, and the elution volume (1)

of the sodium chloride aqueous solution was measured. In addition, 50 μL of a 20 mM sodium phosphate/150 mM sodium chloride aqueous solution containing dextran (molecular weight: 5000000 to 40000000, manufactured by Wako Pure Chemical Industries, Ltd.) was injected into the column, and the elution volume (2) of the dextran-containing aqueous solution was measured. In addition, 50 μL of a 20 mM sodium phosphate/150 mM sodium chloride aqueous solution containing a standard sample of pullulan (P-82 manufactured by Showa Denko K.K.) was injected into the column, and the elution volume (3) of the pullulan-containing aqueous solution was measured.

The distribution coefficient Kd of pullulan was determined according to the following formula.

Kd of pullulan = {(3) - (2)}/{(1) - (2)}

(1): Elution volume of 500 mM sodium chloride aqueous solution

(2): Elution volume of dextran-containing aqueous solution

(3): Elution volume of pullulan-containing aqueous solution

A plot of the molecular size and the distribution coefficient Kd of pullulan is created, an approximate straight line is drawn, and Kd = ε (1 - molecular size/average pore size)^(1/2) is established, such that the average pore diameter of the support 1 is calculated from the intercept and the slope.

[0102]

The volume average particle diameter, porosity, and average pore diameter of the supports 2 to 8 and the support 01 described later were also calculated in accordance with the same procedure as described above.

[0103]

(3) Purification of Fc Fusion Protein by Chromatography

The Fc fusion protein was purified by chromatography using the

support 1. 4 mL of the support 1 was packed in a Tricorn 50/200 column manufactured by Cytiva, and this column was connected to AKTA avant25 manufactured by Cytiva. As a sample solution, a culture solution (etanercept concentration: 1.1 mg/mL) of Fc fusion protein (etanercept)-expressing cells was prepared.

One cycle consisted of the following steps (i) to (iv), and this cycle was repeated 100 times.

(i) The column was equilibrated by passing 3 CV (Column Volume: packed column volume) of a 20 mM sodium phosphate/150 mM sodium chloride aqueous solution (pH 7.5) through the column at a flow rate of 1 mL/min under an environment of 23°C.

(ii) 36.36 mL of the sample solution was loaded onto the column at a residence time of 4 minutes, and then the column was washed with a washing solution (20 mM sodium phosphate/500 mM sodium chloride aqueous solution: pH 7.5).

(iii) The eluent (100 mM aqueous sodium acetate solution, pH 3.3) was passed through the column, and after the eluate was recovered, the column was equilibrated with a 20 mM sodium phosphate/150 mM sodium chloride aqueous solution (pH 7.5).

(iv) After 3 CV of an alkaline liquid (0.1 M aqueous sodium hydroxide solution) was passed through the column at a flow rate of 1 mL/min, the column was equilibrated with a 20 mM sodium phosphate/150 mM sodium chloride aqueous solution (pH 7.5).

[0104]

(Example 2)

Porous particles (referred to as “porous particles 2”) were prepared in accordance with the same procedure as in Example 1(1), except that ethyl cellulose (ETHOCEL™ 14, manufactured by Dow Chemical Company) contained in the organic solution P was changed to ethyl cellulose (ETHOCEL™ 10, manufactured by Dow Chemical

Company). Using the porous particles 2, ligand-immobilized porous particles (referred to as "support 2") were prepared in accordance with the same procedure as in Example 1 (2). Purification of the Fc fusion protein was performed using the support 2 in accordance with the same procedure as in Example 1(3).

[0105]

(Example 3)

Porous particles (referred to as "porous particles 3") were prepared in accordance with the same procedure as in Example 1(1), except that ethyl cellulose (ETHOCEL™ 14, manufactured by Dow Chemical Company) contained in the organic solution P was changed to ethyl cellulose (ETHOCEL™ 20, manufactured by Dow Chemical Company). Using the porous particles 3, ligand-immobilized porous particles (referred to as "support 3") were prepared in accordance with the same procedure as in Example 1(2). Purification of the Fc fusion protein was performed using the support 3 in accordance with the same procedure as in Example 1(3).

[0106]

(Example 4)

Porous particles (referred to as "porous particles 4") were prepared in accordance with the same procedure as in Example 1(1), except that the stirring speed in the Step (b) was changed to 490 rpm. Using the porous particles 4, ligand-immobilized porous particles (referred to as "support 4") were prepared in accordance with the same procedure as in Example 1(2). Purification of the Fc fusion protein was performed using the support 4 in accordance with the same procedure as in Example 1(3).

[0107]

(Example 5)

Porous particles (referred to as "porous particles 5") were

prepared in accordance with the same procedure as in Example 1(1), except that the stirring speed in the Step (b) was changed to 230 rpm. Using the porous particles 5, ligand-immobilized porous particles (referred to as "support 5") were prepared in accordance with the same procedure as in Example 1(2). Purification of the Fc fusion protein was performed using the support 5 in accordance with the same procedure as in Example 1(3).

[0108]

(Example 6)

Porous particles (referred to as "porous particles 6") were prepared in accordance with the same procedure as in Example 1(1), except that the amounts of the reagents were changed such that, in Step (a), 2-octanone (manufactured by Toyo Gosei Co., Ltd.) was changed to 23.70 g, ethyl cellulose (ETHOCEL™ 14, manufactured by Dow Chemical Company) was changed to 2.64 g, divinylbenzene (manufactured by Wako Pure Chemical Industries, Ltd.) was changed to 4.35 g, 1-ethyl-4-vinylbenzene (manufactured by ChemSampCo, Inc) was changed to 0.43 g, and glycidyl methacrylate (manufactured by Mitsubishi Gas Chemical Company, Inc.) was changed to 16.94 g; and, in Step (b), 2,2'-azobis(methyl isobutyrate) (manufactured by Wako Pure Chemical Industries, Ltd.) was changed to 1.60 g, thioglycerol (manufactured by Tokyo Chemical Industry Co., Ltd.) was changed to 103.39 g, and diisopropylethylamine (manufactured by Tokyo Chemical Industry Co., Ltd.) was changed to 12.15 g. Using the porous particles 6, ligand-immobilized porous particles (referred to as "support 6") were prepared in accordance with the same procedure as in Example 1(2). Purification of the Fc fusion protein was performed using the support 6 in accordance with the same procedure as in Example 1(3).

[0109]

(Example 7)

Porous particles (referred to as "porous particles 7") were prepared in accordance with the same procedure as in Example 1(1), except that the amounts of the reagents were changed such that, in Step (a), 2-octanone (manufactured by Toyo Gosei Co., Ltd.) was changed to 28.37 g, ethyl cellulose (ETHOCEL™ 14, manufactured by Dow Chemical Company) was changed to 3.15 g, divinylbenzene (manufactured by Wako Pure Chemical Industries, Ltd.) was changed to 3.12 g, 1-ethyl-4-vinylbenzene (manufactured by ChemSampCo, Inc) was changed to 0.31 g, and glycidyl methacrylate (manufactured by Mitsubishi Gas Chemical Company, Inc.) was changed to 12.17 g; and, in Step (b), 2,2'-azobis(methyl isobutyrate) (manufactured by Wako Pure Chemical Industries, Ltd.) was changed to 1.15 g, thioglycerol (manufactured by Tokyo Chemical Industry Co., Ltd.) was changed to 74.29 g, and diisopropylethylamine (manufactured by Tokyo Chemical Industry Co., Ltd.) was changed to 8.73 g. Using the porous particles 7, ligand-immobilized porous particles (referred to as "support 7") were prepared in accordance with the same procedure as in Example 1(2). Purification of the Fc fusion protein was performed using the support 7 in accordance with the same procedure as in Example 1(3).

[0110]

(Example 8)

Ligand-immobilized porous particles (referred to as "support 8") were prepared from the porous particles 1 in accordance with the same procedure as in Examples 1(1) and 1(2), except that the ligand was changed to PrA-2. Purification of the Fc fusion protein was performed using the support 8 in accordance with the same procedure as in Example 1(3).

[0111]

(Example 9)

Purification of an Fc fusion protein (rilonacept) was performed using the support 1 in accordance with the same procedure as in Example 1(3), except that the sample solution was changed to a culture solution of rilonacept expressing cells (rilonacept concentration: 1.1 mg/mL), and that, in Step (ii), 29.09 mL of the sample solution was loaded onto a column for a residence time of 4 minutes.

[0112]

(Example 10)

Purification of an Fc fusion protein (efmoroctocog alfa) was performed using the support 1 in accordance with the same procedure as in Example 1(3), except that the sample solution was changed to a culture solution of efmoroctocog alfa expressing cells (efmoroctocog alfa concentration: 1.1 mg/mL), and that, in Step (ii), 22.59 mL of the sample solution was loaded onto the column for a residence time of 4 minutes.

[0113]

(Example 11)

Purification of an Fc fusion protein was performed using the support 1 in accordance with the same procedure as in Example 1(3), except that the washing solution used in the Step (ii) was changed to a 20 mM sodium phosphate/150 mM sodium chloride aqueous solution (pH 7.5).

[0114]

(Example 12)

Purification of an Fc fusion protein was performed using the support 1 in accordance with the same procedure as in Example 1(3), except that the washing solution used in the Step (ii) was changed to a 20 mM sodium phosphate/500 mM sodium chloride aqueous solution

(pH 6.5).

[0115]

(Example 13)

Purification of an Fc fusion protein was performed using the support 1 in accordance with the same procedure as in Example 1(3), except that the alkaline liquid used in the Step (iv) was changed to a 0.5 M aqueous NaOH solution.

[0116]

(Comparative Example 1)

Porous particles (referred to as "porous particles 01") were prepared in accordance with the same procedure as in Example 1(1), except that 29.44 g of 2-octanone (manufactured by Toyo Gosei Co., Ltd.) was used as the organic solution P. Using the porous particles 01, ligand-immobilized porous particles (referred to as "support 01") were prepared in accordance with the same procedure as in Example 1(2). Purification of the Fc fusion protein was performed using the support 01 in accordance with the same procedure as in Example 1(3).

[0117]

(Test Example 1) Measurement of Dynamic Binding Capacity (DBC)

The DBC for the target protein at the first cycle and the 100th cycle of chromatography in Examples 1 to 13 and Comparative Example 1 was measured. That is, the DBC was determined from the amount of the target protein captured at 10% breakthrough at the leading edge of elution and the packed column volume. The DBC (mg/mL) of the first cycle and the percentage (%DBC) of the DBC of the 100th cycle with respect to the first cycle were calculated. The results are shown in Tables 2 and 3.

[0118]

(Test Example 2) Measurement of Host Cell Protein (HCP) Amount

The residual HCP content (HCP residual ratio: HCP/target) per target protein content in the eluates obtained at the first cycle and the 100th cycle of chromatography in Examples 1 to 13 and Comparative Example 1 was calculated. The residual HCP content in the eluate was measured by an HCP ELISA kit (F550-1). The content of the target protein in the eluate was measured based on the absorbance. The residual HCP content (ppm) at the first cycle and the percentage (%[HCP/target]) of the HCP residual ratio at the 100th cycle with respect to the first cycle were calculated. The results are shown in Tables 2 and 3.

[0119]

(Test Example 3) Evaluation of Pressure Resistance Characteristics During Liquid Passage

Two Hiscale 26/20 columns (manufactured by Cytiva) were connected to prepare a column having an inner diameter of 26 mm and a packing height of 200 mm. The column was packed with any one of supports 1, 4, and 7. Each column was connected to an AKTA AVANT 150 (manufactured by Cytiva), and then pure water was passed through the column at a linear flow rate of 100 cm/hr for 30 minutes. Thereafter, the upper column was removed, and the adapter was brought into contact with the support layer, thereby packing the support into the column. Thereafter, pure water was passed through the column at a linear flow rate of 800 cm/hr, and the back pressure at that time was measured. The results are shown in Table 2. It can be said that the smaller the value of the back pressure, the better the pressure resistance characteristics.

[0120]

[Table 2]

	Example 1	Example 2	Example 3	Example 4	Example 5	Example 6	Example 7	Comparative Example 1
Support	Support 1	Support 2	Support 3	Support 4	Support 5	Support 6	Support 7	Support 01
Porous particles	Porous particles 1	Porous particles 2	Porous particles 3	Porous particles 4	Porous particles 5	Porous particles 6	Porous particles 7	Porous particles 01
Ligand	PrA-1	PrA-1	PrA-1	PrA-1	PrA-1	PrA-1	PrA-1	PrA-1
Volume average particle diameter (μm)	50	50	50	40	85	50	50	50
Porosity (%)	78	78	78	78	78	70	87	75
Average pore diameter (nm)	105	80	120	105	105	90	112	50
Fc fusion protein	Etanercept	Etanercept	Etanercept	Etanercept	Etanercept	Etanercept	Etanercept	Etanercept
Molecular weight (kDa)	150	150	150	150	150	150	150	150
Intermediate washing	20 mM phosphoric acid/500 mM NaCl	20 mM phosphoric acid/500 mM NaCl	20 mM phosphoric acid/500 mM NaCl	20 mM phosphoric acid/500 mM NaCl	20 mM phosphoric acid/500 mM NaCl	20 mM phosphoric acid/500 mM NaCl	20 mM phosphoric acid/500 mM NaCl	20 mM phosphoric acid/500 mM NaCl
Alkaline washing	0.1 MNaOH	0.1 MNaOH	0.1 MNaOH	0.1 MNaOH	0.1 MNaOH	0.1 MNaOH	0.1 MNaOH	0.1 MNaOH
DBC (mg/mL) in first cycle	15.0	14.2	14.0	16.4	10.7	11.8	15.8	10.3
%DBC	96%	93%	93%	94%	94%	93%	95%	86%
Back pressure (MPa) (linear flow rate 800 cm/hr)	0.41			0.68			0.59	
First cycle [HCP/target] (ppm)	6800	7200	7100	6900	7000	7100	6900	6900
%[HCP/target]	94%	97%	99%	95%	97%	94%	95%	86%

[0121]

[Table 3]

	Example 8	Example 9	Example 10	Example 11	Example 12	Example 13
Support	Support 8	Support 1		Support 1		
Porous particles	Porous particles 1	Porous particles 1		Porous particles 1		
Ligand	PrA-2	PrA-1		PrA-1		
Volume average particle diameter (μm)	50	50		50		
Porosity (%)	78	78		78		
Average pore diameter (nm)	105	105		105		
Fc fusion protein	Etanercept	Rilonacept	Efmoroctocog alfa	Etanercept		
Molecular weight (kDa)	150	250	220	150		
Intermediate washing	20 mM phosphoric acid/500 mM NaCl			20 mM phosphoric acid/150 mM NaCl	20 mM phosphoric acid/500 mM NaCl	20 mM phosphoric acid/500 mM NaCl
Alkaline washing	0.1 MNaOH			0.1 MNaOH		0.5 MNaOH
DBC (mg/mL) in first cycle	14.0	12.4	12.7	14.9	15.1	14.8
%DBC	95%	92%	93%	91%	94%	90%
First cycle [HCP/target] (ppm)	7200	3800	5500	8200	7400	7000
%[HCP/target]	91%	97%	93%	90%	94%	89%

CLAIMS

[Claim 1]

A chromatographic support for purifying an Fc fusion protein, comprising:

porous particles having a ligand immobilized thereon,

wherein the porous particles are synthetic polymer-based or natural polymer-based porous particles,

the ligand is at least one selected from the group consisting of Protein A, Protein G, Protein L, and analogs thereof, and

an average pore diameter of the porous particles having the ligand immobilized thereon is 75 to 120 nm.

[Claim 2]

The chromatographic support according to claim 1, wherein an average pore diameter of the porous particles having the ligand immobilized thereon is 101 to 110 nm.

[Claim 3]

The chromatographic support according to claim 1, wherein the Fc fusion protein has a molecular weight of 160 to 300 kDa.

[Claim 4]

The chromatographic support according to claim 1, wherein the porous particles having the ligand immobilized thereon have a volume average particle diameter of 45 to 100 μm and a porosity of 70 to 95% (v/v).

[Claim 5]

The chromatographic support according to claim 1, wherein the Fc fusion protein is at least one selected from the group consisting of etanercept, rilonacept, and efmoroctocog alfa.

[Claim 6]

The chromatographic support according to claim 1, wherein the Fc fusion protein is an Fc fusion protein consisting of the amino

acid sequence of SEQ ID NO:4 or SEQ ID NO:5, or an amino acid sequence having 85% or more identity to any of these sequences.

[Claim 7]

The chromatographic support according to claim 1, wherein the porous particles are a polymer of

- a monomer having a functional group capable of immobilizing a ligand and a polymerizable unsaturated group, and
- a monomer having a polymerizable unsaturated group and not having the functional group.

[Claim 8]

The chromatographic support according to claim 7, wherein the monomer having a polymerizable unsaturated group and not having the functional group includes a non-crosslinking monomer and a crosslinking monomer.

[Claim 9]

A method of purifying an Fc fusion protein, the method comprising:

(Step 1) a step of passing a solution containing an Fc fusion protein through a column containing the chromatographic support according to any one of claims 1 to 8, to bind the Fc fusion protein to the support;

(Step 2) a step of washing the support; and

(Step 3) a step of recovering the Fc fusion protein from the support.

[Claim 10]

The method according to claim 9, further comprising:

(Step 4) a step of passing an alkaline liquid through the column after the Step 3.

[Claim 11]

The method according to claim 10, comprising repeating the

Steps 1 to 4 a plurality of times.