



(51) International Patent Classification:

C07K 14/31 (2006.01) *C07K 1/22* (2006.01)
B01D 15/38 (2006.01)

(21) International Application Number:

PCT/SE2013/050335

(22) International Filing Date:

26 March 2013 (26.03.2013)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

1250304-1 28 March 2012 (28.03.2012) SE
1250493-2 15 May 2012 (15.05.2012) SE

(71) Applicant: **GE HEALTHCARE BIO-SCIENCES AB**
[SE/SE]; Patent Department, Björkgatan 30, S-751 84
Uppsala (SE).

(72) Inventors: **ANDER, Mats**; GE Healthcare, Björkgatan 30,
S-751 84 Uppsala (SE). **BAURÉN, Göran**; GE Health-
care, Björkgatan 30, S-751 84 Uppsala (SE). **BJÖRK-
MAN, Tomas**; GE Healthcare, Björkgatan 30, S-751 84
Uppsala (SE). **ABERG, Per-Mikael**; GE Healthcare,
Björkgatan 30, S-751 84 Uppsala (SE). **RODRIGO, Gust-
av**; GE Healthcare, Björkgatan 30, S-751 84 Uppsala (SE).

(74) Agents: **GE HEALTHCARE BIO-SCIENCES AB** et al.;
Patent Department, Björkgatan 30, S-751 84 Uppsala (SE).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP,
KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD,
ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI,
NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU,
RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ,
TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA,
ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments (Rule 48.2(h))

[Continued on next page]

(54) Title: AFFINITY CHROMATOGRAPHY MATRIX

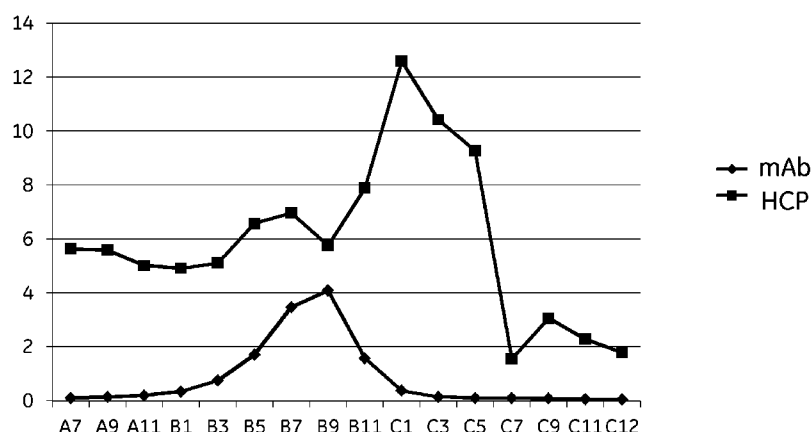
Z(H18S,P57I)₄

Figure 2

(57) Abstract: The invention discloses an immunoglobulin-binding protein comprising one or more mutated immunoglobulin-binding domains (monomers) of staphylococcal Protein A (E, D, A, B,C) or protein Z or a functional variant thereof, wherein in at least one of the one or more mutated monomers, the asparagine or histidine at the position corresponding to H18 of the B domain of Protein A or of Protein Z has been deleted or substituted with a first amino acid residue which is not proline or asparagine and wherein, if the amino acid residue at position 57 is proline and the amino acid residue at position 28 is asparagine, then the amino acid residue at the position corresponding to H18 of the B domain of protein A or of protein Z is not serine, threonine or lysine.



— with sequence listing part of description (Rule 5.2(a))

Affinity Chromatography Matrix

Field of the Invention

The present invention relates to the field of affinity chromatography, and more specifically to separation matrices containing ligands with one or more of a protein A domain (E, D, A, B, C), or protein Z, which have been mutated. The invention also relates to methods for the separation of proteins of interest on such separation matrices.

Background of the Invention

Immunoglobulins, in particular monoclonal antibodies (mAbs) represent the most prevalent biopharmaceutical products in either manufacture or development worldwide. The high commercial demand for and hence value of this particular therapeutic market has led to the intense efforts from pharmaceutical companies to maximise the productivity of their respective mAb manufacturing processes whilst controlling the associated costs.

Affinity chromatography is used in most cases, as one of the key steps in the purification of these immunoglobulin molecules, such as monoclonal or polyclonal antibodies. A particularly interesting class of affinity reagents is proteins capable of specific binding to invariable parts of an immunoglobulin molecule, such interaction being independent on the antigen-binding specificity of the antibody. Such reagents can be widely used for affinity chromatography recovery of immunoglobulins from different samples such as but not limited to serum or plasma preparations or cell culture derived feed stocks. An example of such a protein is staphylococcal protein A, containing domains capable of binding to the Fc and Fab portions of IgG immunoglobulins from different species.

Staphylococcal protein A (SpA) based reagents have due to their high affinity and selectivity found a widespread use in the field of biotechnology, e.g. in affinity chromatography

for capture and purification of antibodies as well as for detection. At present, SpA-based affinity medium probably is the most widely used affinity medium for isolation of monoclonal antibodies and their fragments from different samples including industrial feed stocks from cell cultures. Accordingly, various matrices comprising protein A ligands are commercially available, for example, in the form of native protein A (e.g. Protein A SEPHAROSE™, GE Healthcare, Uppsala, Sweden) and also comprised of recombinant protein A (e.g. rProtein A SEPHAROSE™, GE Healthcare). More specifically, the genetic manipulation performed in the commercial recombinant protein A product is aimed at facilitating the attachment thereof to a support.

These applications, like other affinity chromatography applications, require comprehensive attention to definite removal of contaminants. Such contaminants can for example be non-eluted molecules adsorbed to the stationary phase or matrix in a chromatographic procedure, such as non-desired biomolecules or microorganisms, including for example proteins, carbohydrates, lipids, bacteria and viruses. The removal of such contaminants from the matrix is usually performed after a first elution of the desired product in order to regenerate the matrix before subsequent use. Such removal usually involves a procedure known as cleaning-in-place (CIP), wherein agents capable of eluting contaminants from the stationary phase are used. One such class of agents often used is alkaline solutions that are passed over said stationary phase. At present the most extensively used cleaning and sanitising agent is NaOH, and the concentration thereof can range from 0.1 up to e.g. 1 M, depending on the degree and nature of contamination. This strategy is associated with exposing the matrix for pH-values above 13. For many affinity chromatography matrices containing proteinaceous affinity ligands such alkaline environment is a very harsh condition and consequently results in decreased capacities owing to instability of the ligand to the high pH involved.

An extensive research has therefore been focussed on the development of engineered

protein ligands that exhibit an improved capacity to withstand alkaline pH-values. For example, Gülich et al (Journal of Biotechnology 80 (2000), 169-178) suggested protein engineering to improve the stability properties of a Streptococcal albumin-binding domain (ABD) in alkaline environments. Gülich et al created a mutant of ABD, wherein all the four asparagine residues
5 have been replaced by leucine (one residue), aspartate (two residues) and lysine (one residue). Further, Gülich et al report that their mutant exhibits a target protein binding behaviour similar to that of the native protein, and that affinity columns containing the engineered ligand show higher binding capacities after repeated exposure to alkaline conditions than columns prepared using the parental non-engineered ligand. Thus, it is concluded therein that all four asparagine residues can
10 be replaced without any significant effect on structure and function.

Recent work show that changes can also be made to protein A (SpA) to effect similar properties. US patent application 2005/0143566 discloses that when at least one asparagine residue is mutated to an amino acid other than glutamine or aspartic acid, the mutation confers an increased chemical stability at pH-values of up to about 13-14 compared to the parental SpA,
15 such as the B-domain of SpA, or Protein Z, a synthetic construct derived from the B-domain of SpA (US 5,143,844). The authors show that when these mutated proteins are used as affinity ligands, the separation media as expected can better withstand cleaning procedures using alkaline agents. US patent application 2006/0194955 shows that the mutated ligands can better withstand proteases thus reducing ligand leakage in the separation process. Another US patent application
20 2006/0194950 shows that the alkali stable SpA domains can be further modified such that the ligands lacks affinity for Fab but retains Fc affinity, for example by a G29A mutation.

Historically the native protein A containing 5 IgG binding domains was used for production of all protein A affinity media. Using recombinant technology a number of protein A construct have been produced all containing 4 or 5 IgG binding domains. A recent study shows
25 that dimeric ligands have a similar, or increased binding capacity compared to tetrameric ligands

(WO 2010/080065).

It is well known that some antibodies are prone to aggregation or sensitive (e.g. they can lose activity) at low pH. There is still a need in this field to obtain a separation matrix containing protein ligands having an increased elution pH for antibody or related targets. Further there is a need for improved selectivity in the initial capture step of mAb processes, leading to improved clearance of host cell proteins and antibody aggregates.

Summary of the Invention

A first aspect of the invention is to provide a mutated immunoglobulin- or Fc-binding protein with improved dissociation properties, including release of bound immunoglobulins or Fc-containing proteins at an increased pH and selective release of monomeric immunoglobulins or Fc-containing proteins at pH values higher than for bound host cell proteins and/or aggregates of immunoglobulins or Fc-containing proteins. This is achieved with a mutated immunoglobulin- or Fc-binding protein as defined in Claim 1.

A second aspect of the present invention is to provide an affinity separation matrix with improved elution properties, including elution of bound immunoglobulins or Fc-containing proteins at an increased pH and selective elution of monomeric immunoglobulins or Fc-containing proteins at pH values higher than for bound host cell proteins and/or aggregates of immunoglobulins or Fc-containing proteins. This is achieved with an affinity separation matrix as defined in Claim 9.

A third aspect of the invention is to provide a method for separating one or more Fc-containing proteins, which method allows elution of bound Fc-containing proteins at an increased pH and selective elution of monomeric Fc-containing proteins at pH values higher than for bound host cell proteins and/or aggregates of Fc-containing proteins. This is achieved with a method

according to Claim 16.

A fourth aspect of the invention is to provide a method of selecting an elution buffer for matrices with adsorbed Fc-containing proteins, which method allows for selection of buffers allowing selective elution of monomeric Fc-containing proteins at pH values higher than for bound host cell proteins and/or aggregates of Fc-containing proteins. This is achieved with a method according to Claim 28.

Further suitable embodiments of the invention are described in the depending claims.

Brief Description of the Drawings

Figure 1 shows an alignment of the amino acid sequences from each of the five domains of protein A, along with three varieties of protein Z. The Asparagine or Histidine at the position corresponding to H18 of B domain of Protein A is denoted in bold. Also shown are the locations of the three alpha-helices (Graille et al, PNAS 2000, 97 (10): 5399-5404; Deisenhofer, Biochemistry 1981, 20 (9): 2361-2370).

Figure 2 shows an overlay of the pH-gradient elution chromatograms of monoclonal antibody and host cell proteins for prototype Z(H18S,P57I)₄.

Figure 3 shows an overlay of the pH-gradient elution chromatograms of monoclonal antibody and host cell proteins for comparative prototype Z₄.

Figure 4 is an overlay of representative chromatogram showing results for size exclusion chromatography on the mAb fractions B7, B12 and C2 from the experiment with Z(H18S,P57I)₄ described in Figure 2.

Detailed Description of the Invention

Definitions

The term “protein” is used herein to describe proteins as well as fragments thereof. Thus,

any chain of amino acids that exhibits a three dimensional structure is included in the term “protein”, and protein fragments are accordingly embraced.

The term “functional variant” of a protein means herein a variant protein, wherein the function, in relation to the invention defined as affinity and stability, are essentially retained.

5 Thus, one or more amino acids that are not relevant for said function may have been exchanged.

The term “parental molecule” is used herein for the corresponding protein in the form before a mutation according to the invention has been introduced.

The term “structural stability” refers to the integrity of three-dimensional form of a molecule, while “chemical stability” refers to the ability to withstand chemical degradation.

10 The term “Fc -binding” protein means that the protein is capable of binding to the Fc (also called “fragment, crystallisable”) region of an immunoglobulin. However, it is not excluded that an Fc -binding protein also can bind other regions, such as Fab regions of immunoglobulins.

The term “Fc-containing protein” means a protein comprising an Fc region or fragment of an immunoglobulin. The Fc-containing protein can be an immunoglobulin or a fusion protein or
15 conjugate comprising an Fc region/fragment or comprising an entire immunoglobulin.

In the present specification, if not referred to by their full names, amino acids are denoted with the conventional one-letter or three letter symbols.

Mutations are defined herein by the number of the position exchanged, preceded by the wild type or non-mutated amino acid and followed by the mutated amino acid. Thus, for
20 example, the mutation of a histidine (H) in position 18 to a glutamic acid (E) is denoted H18E. Deletion mutations are defined by the number of the position deleted, preceded by the wild-type or non-mutated amino acid and followed by “del”. Thus, for example, the deletion of a histidine in position 18 is denoted H18del.

25 In a first aspect the present invention discloses an immunoglobulin- or Fc-binding protein

which comprises one or more mutated immunoglobulin- or Fc-binding domains (also called monomers) of staphylococcal Protein A (i.e. the E, D, A, B and/or C domains) or protein Z or a functional variant thereof, such as the variants defined by SEQ ID NO: 7 or SEQ ID NO: 8. In at least one of the one or more mutated monomers, the Asparagine or Histidine at the position corresponding to H18 of the B domain of Protein A (SEQ ID NO:4) or of Protein Z (SEQ ID NO:6) has been substituted with a first amino acid residue which is not Proline or Asparagine. Further, if the amino acid residue at the position corresponding to P57 of the B domain of Protein A or of Protein Z is Proline and the amino acid residue at the position corresponding to N28 of the B domain of Protein A or of Protein Z is Asparagine, then the amino acid residue at the position corresponding to H18 of the B domain of Protein A or of Protein Z is not Serine, Threonine or Lysine. An advantage of this mutated protein is that it binds immunoglobulins and Fc-containing proteins and releases them at pH levels higher than immunoglobulin- or Fc-binding proteins not having the H18 mutation. The mutation of the Asparagine or Histidine residue at the position corresponding to H18 of B domain of Protein A or Protein Z unexpectedly increases the elution pH of immunoglobulins and Fc-containing proteins, such as IgG, IgA and/or IgM, or fusion proteins containing an Fc-fragment. Preferably, the elution pH increases by between 0.2 to over 1.0 pH. More preferably, the elution pH increases at least 0.3 pH, most preferably, the elution pH increases at least 0.4 pH. Alternatively, the elution pH is increased to >4.0, preferably pH >4.2, while the yield of the target molecule is at least 80% or preferably >95%. As immunoglobulins and other Fc-containing proteins are prone to aggregation at low pH levels, this is important for the use of the proteins in bioprocessing. The aggregates are potentially immunogenic and need to be removed before pharmaceutical use of the immunoglobulins or Fc-containing proteins. This adds significant cost to the processing.

A further advantage of the mutation of the Asparagine or Histidine residue at the position

corresponding to H18 of B domain of Protein A or Protein Z is that any host cell proteins (HCP) or aggregates of antibodies or Fc-containing proteins that adsorb to the matrix will elute at a higher pH than the antibody/Fc-containing protein monomers. It is then possible to recover the monomeric antibody or FC-containing protein at a higher purity by eluting it at a pH where HCP and aggregates are still bound to the matrix. On comparative matrices with ligands not having the H18 mutations, bound HCP and aggregates coelute with the antibody monomers.

In certain embodiments, the ligands are also rendered alkali-stable, such as by mutating at least one asparagine residue of at least one of the monomeric domains of the SpA domain B or protein Z to an amino acid other than glutamine. As discussed earlier, US patent application 2005/0143566 discloses that when at least one asparagine residue is mutated to an amino acid other than glutamine or aspartic acid, the mutation confers the ligand an increased chemical stability at high pH (e.g., N23T). Further, affinity media including these ligands can better withstand cleaning procedures using alkaline agents. US patent application 2006/0194955 shows that the mutated ligands can also better withstand proteases thus reducing ligand leakage in the separation process. The disclosures of these applications are hereby incorporated by reference in their entirety.

In some embodiments, the ligand(s) so prepared lack any substantial affinity for the Fab part/region of an antibody, while having affinity for the Fc part/region. Thus, in certain embodiments, at least one glycine of the ligands has been replaced by an alanine. US patent application 2006/0194950 shows that the alkali stable domains can be further modified such that the ligands lacks affinity for Fab but retains Fc affinity, for example by a G29A mutation. The disclosure of the application is hereby incorporated by reference in its entirety. The numbering used herein of the amino acids is the conventionally used in this field, exemplified by the position

on domain B of protein A, and the skilled person in this field can easily recognize the position to be mutated for each domain of E, D, A, B, C.

In certain embodiments, an asparagine residue located between a leucine residue and a glutamine residue has also been mutated, for example to a threonine residue. Thus, in one embodiment, the asparagine residue in position 23 of the sequence defined in SEQ ID NO: 6 has been mutated, for example to a threonine residue. In a specific embodiment, the asparagine residue in position 43 of the sequence defined in SEQ ID NO: 6 has also been mutated, for example to a glutamic acid. In the embodiments where amino acid number 43 has been mutated, it appears to most advantageously be combined with at least one further mutation, such as N23T.

Accordingly, in some embodiments, a mutated protein according to the invention comprises at least about 75%, such as at least about 80% or preferably at least about 95%, of the sequence as defined in SEQ ID NO: 4 or 6, with the proviso that the asparagine mutation is not in position 21.

In the present specification, SEQ ID NO: 4 defines the amino acid sequence of the B-domain of SpA:

ADNKFNKEQQNAFYEILHLPNLNEEQRNGFIQSLKDDPSQSANLLAEAKKLNDAQAPK

SEQ ID NO: 6 defines a protein known as protein Z:

VDNKFNKEQQNAFYEILHLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDAAQAPK

Protein Z is a synthetic construct derived from the B-domain of SpA, wherein the glycine in position 29 has been exchanged for alanine, see e.g. Ståhl et al, 1999: Affinity fusions in biotechnology: focus on protein A and protein G, in The Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation. M.C. Fleckinger and S.W. Drew,

editors. John Wiley and Sons Inc., New York, 8-22.

In one embodiment, the above described mutant protein is comprised of the amino acid sequence defined in SEQ ID NO: 4, 5, 6, 7 or 8, or is a functional variant thereof, with a substitution at Histidine at the position corresponding to H18 of B domain of Protein A or Protein Z. In another embodiment, the above described mutant protein is comprised of the amino acid sequence defined in SEQ ID NO: 1, 2 or 3, or is a functional variant thereof, with a substitution at Asparagine at the position corresponding to H18 of B domain of Protein A. The term “functional variant” as used in this context includes any similar sequence, which comprises one or more further variations in amino acid positions that have no influence on the mutant protein’s affinity to immunoglobulins or Fc-containing proteins or its improved chemical stability in environments of increased pH-values.

As mentioned above, in order to achieve a mutant protein useful as a ligand with high binding capacity for a prolonged period of time in alkaline conditions, mutation of the asparagine residue in position 21 is avoided. In one embodiment, the asparagine residue in position 3 is not mutated.

As the skilled person in this field will easily understand, the mutation of Asparagine or Histidine at position 18, the mutations at positions 57 and 28, the mutations to provide alkaline-stability, and the G to A mutation may be carried out in any order of sequence using conventional molecular biology techniques. Further, the ligands can be expressed by a vector containing a nucleic acid sequence encoding the mutated protein ligands. Alternatively, they can also be made by protein synthesis techniques. Methods for synthesizing peptides and proteins of predetermined sequences are well known and commonly available in this field.

In certain embodiments, amino acids with bulky side chains, e.g. Tyrosine, Tryptophan or Methionine can be avoided as substituents in the H18 position. Bulky side chains may perturb the tertiary structure of the protein, which could affect the immunoglobulin- or Fc-binding properties.

5

In some embodiments additionally at least one of the Proline at position 57 and the Asparagine at position 28 has been substituted with a second amino acid residue. An advantage of this is that even higher elution pH values can be achieved, in combination with higher binding capacities. In certain embodiments neither of the amino acid residues at positions 57 and 28 is Proline or As-

10 paragine.

In some embodiments the amino acid residue at position 18 is selected from the group consisting of Serine, Glutamic acid, Aspartic acid, Threonine and Lysine. With these amino acids, particularly high elution pH levels can be achieved.

15

In certain embodiments at least one of the amino acid residues at position 57 and 28 is selected from the group consisting of Alanine and Isoleucine.

In some embodiments the protein comprises one or more mutated monomers derived from the parental molecules defined by SEQ ID NO 5, 6, 7 or 8, wherein the mutation is selected from the group consisting of H18E; H18S,N28A; H18S,P57I; N28A,P57I and H18S,N28A,P57I.

20

In certain embodiments the protein comprises one or more mutated monomers defined by SEQ ID NO: 14, 16, 17 or 18.

25

In some embodiments the protein comprises at least two mutated monomers, such as 3, 4, 5 or 6 monomers, forming a multimeric protein. The monomers may be linked covalently, such as by forming parts of a single polypeptide chain. The mutated monomers may be identical, but they can also be different mutated monomers according to the invention. In addition to the monomers of the invention, the multimeric protein may also comprise other mutated or non-mutated IgG-binding domains of Protein A. In some embodiments the Asparagine or Histidine at the position corresponding to H18 of B domain of Protein A or Protein Z in at least one of the monomeric domains in a multimeric ligand is substituted. In other embodiments, the Asparagine or Histidine residue in all the monomeric domains in a multimeric ligand is substituted.

In some embodiments, the multimeric protein according to the invention comprises monomer units linked to each other by a stretch of amino acids preferably ranging from 0 to 15 amino acids, such as 0-5, 0-10 or 5-10 amino acids. The nature of such a link should preferably not destabilize the spatial conformation of the protein units. This can e.g. be achieved by avoiding the presence of proline in the links. Furthermore, said link should preferably also be sufficiently stable in alkaline environments not to impair the properties of the mutated protein units. For this purpose, it is advantageous if the links do not contain asparagine. It can additionally be advantageous if the links do not contain glutamine.

In certain embodiments, the present dimeric ligands comprise the sequence of SEQ ID NO: 9:

VDAKFDKEQQNAFYEILELPNLTEEQRNAFIQSLKDDPSQSANLLAEAKKLNDAQAPKV
DAKFDKEQQNAFYEILELPNLTEEQRNAFIQSLKDDPSQSANLLAEAKKLNDAQAPKC

In certain embodiments, the present tetrameric ligands comprise the sequence of SEQ ID NO: 11:

VDAKFDKEQQNAFYEILSLPNLTEEQRNAFIQSLKDDPSQSANLLAEAKKLNDAQAIKV
DAKFDKEQQNAFYEILSLPNLTEEQRNAFIQSLKDDPSQSANLLAEAKKLNDAQAIKVD

AKFDKEQQNAFYEILSLPNLTEEQRNAFIQSLKDDPSQSANLLAEAKKLNDQAQIKVDAK
FDKEQQNAFYEILSLPNLTEEQRNAFIQSLKDDPSQSANLLAEAKKLNDQAQIKC

In some embodiments, the present tetrameric ligands comprise the sequence of SEQ ID NO: 12:

5 VDAKFDKEQQNAFYEILSLPNLTEEQRAAFIQSLKDDPSQSANLLAEAKKLNDQAQPKV
DAKFDKEQQNAFYEILSLPNLTEEQRAAFIQSLKDDPSQSANLLAEAKKLNDQAQPKVD
AKFDKEQQNAFYEILSLPNLTEEQRAAFIQSLKDDPSQSANLLAEAKKLNDQAQPKVDAK
FDKEQQNAFYEILSLPNLTEEQRAAFIQSLKDDPSQSANLLAEAKKLNDQAQPKC

10 In certain embodiments, the present tetrameric ligands comprise the sequence of SEQ ID NO:
13:

15 VDAKFDKEQQNAFYEILSLPNLTEEQRAAFIQSLKDDPSQSANLLAEAKKLNDQAQIKV
DAKFDKEQQNAFYEILSLPNLTEEQRAAFIQSLKDDPSQSANLLAEAKKLNDQAQIKVDA
KFDKEQQNAFYEILSLPNLTEEQRAAFIQSLKDDPSQSANLLAEAKKLNDQAQIKVDAKFD
KEQQNAFYEILSLPNLTEEQRAAFIQSLKDDPSQSANLLAEAKKLNDQAQIKC

20 In certain embodiments the protein further comprises a C-terminal cysteine. The cysteine
can be directly linked to the C-terminal monomer of a protein according to the invention or it can
also be linked via a stretch of amino acids preferably ranging from 0 to 15 amino acids, such as 0-
5, 0-10 or 5-10 amino acids. This stretch should preferably also be sufficiently stable in alkaline
environments not to impair the properties of the mutated protein. For this purpose, it is
25 advantageous if the stretch does not contain asparagine. It can additionally be advantageous if the
stretch does not contain glutamine. An advantage of having a C-terminal cysteine is that endpoint
coupling of the protein can be achieved through reaction of the cysteine thiol with an
electrophilic group on a support. This provides excellent mobility of the coupled protein which is
important for the binding capacity.

30 In a second aspect the invention discloses an affinity separation matrix, which comprises a protein
according to any of the embodiments described above coupled to a support. This matrix is useful
for chromatographic separation of immunoglobulins and other Fc-containing proteins, where the

higher elution pH and the improved elution selectivity allow for better recoveries and lower impurity levels after the first capture step.

As the skilled person will understand, the expressed protein should be purified to an appropriate extent before been immobilized to a support. Such purification methods are well known in the field, and the immobilization of protein-based ligands to supports is easily carried out using standard methods. Suitable methods and supports will be discussed below in more detail.

The solid support of the matrix according to the invention can be of any suitable well-known kind. A conventional affinity separation matrix is often of organic nature and based on polymers that expose a hydrophilic surface to the aqueous media used, i.e. expose hydroxy (-OH), carboxy (-COOH), carboxamido (-CONH₂, possibly in N- substituted forms), amino (-NH₂, possibly in substituted form), oligo- or polyethylenoxy groups on their external and, if present, also on internal surfaces.

The ligand may be attached to the support via conventional coupling techniques utilising, e.g. amino and/or carboxy groups present in the ligand. Bisepoxides, epichlorohydrin, CNBr, N-hydroxysuccinimide (NHS) etc are well-known coupling reagents. Between the support and the ligand, a molecule known as a spacer can be introduced, which improves the availability of the ligand and facilitates the chemical coupling of the ligand to the support. Alternatively, the ligand may be attached to the support by non-covalent bonding, such as physical adsorption or biospecific adsorption.

In some embodiments the matrix comprises 5 – 15 mg/ml, such as 6 – 11 mg/ml of said protein coupled to said support. The amount of coupled protein can be controlled by the concentration of protein used in the coupling process, by the coupling conditions used and by the pore structure of the support used. As a general rule the absolute binding capacity of the matrix increases with the amount of coupled protein, at least up to a point where the pores become significantly constricted by the coupled protein. The relative binding capacity per mg coupled protein will decrease at high coupling levels, resulting in a cost-benefit optimum within the ranges specified above.

In certain embodiments the protein is coupled to the support via a thioether bridge.

Methods for performing such coupling are well-known in this field and easily performed by the skilled person in this field using standard techniques and equipment.

In some embodiments the protein is coupled via a C-terminal cysteine provided on the protein as described above. This allows for efficient coupling of the cysteine thiol to electrophilic groups, e.g. epoxide groups, halohydrin groups etc. on a support, resulting in a thioether bridge coupling.

In certain embodiments the support comprises a polyhydroxy polymer, such as a polysaccharide. Examples of polysaccharides include e.g. dextran, starch, cellulose, pullulan, agar, agarose etc. Polysaccharides are inherently hydrophilic with low degrees of nonspecific interactions, they provide a high content of reactive (activatable) hydroxyl groups and they are generally stable towards alkaline cleaning solutions used in bioprocessing.

In some embodiments the support comprises agar or agarose. The supports used in the present invention can easily be prepared according to standard methods, such as inverse suspension gelation (S Hjertén: *Biochim Biophys Acta* 79(2), 393-398 (1964). Alternatively, the base

matrices are commercially available products, such as SEPHAROSE™ FF (GE Healthcare). In an embodiment, which is especially advantageous for large-scale separations, the support has been adapted to increase its rigidity using the methods described in US6602990 or US7396467, and hence renders the matrix more suitable for high flow rates.

5

In certain embodiments the support is crosslinked, such as with hydroxyalkyl ether crosslinks. Crosslinker reagents producing such crosslinks can be e.g. epihalohydrins like epichlorohydrin, diepoxides like butanediol diglycidyl ether, allylating reagents like allyl halides or allyl glycidyl ether. Crosslinking is beneficial for the rigidity of the support and improves the chemical stability.

10 Hydroxyalkyl ether crosslinks are alkali stable and do not cause significant nonspecific adsorption.

Alternatively, the solid support is based on synthetic polymers, such as polyvinyl alcohol, polyhydroxyalkyl acrylates, polyhydroxyalkyl methacrylates, polyacrylamides,

15 polymethacrylamides etc. In case of hydrophobic polymers, such as matrices based on divinyl and monovinyl-substituted benzenes, the surface of the matrix is often hydrophilised to expose hydrophilic groups as defined above to a surrounding aqueous liquid. Such polymers are easily produced according to standard methods, see e.g. "Styrene based polymer supports developed by suspension polymerization" (R Arshady: Chimica e L'Industria 70(9), 70-75 (1988)).

20 Alternatively, a commercially available product, such as SOURCE™ (GE Healthcare) is used. In another alternative, the solid support according to the invention comprises a support of inorganic nature, e.g. silica, zirconium oxide etc.

In yet another embodiment, the solid support is in another form such as a surface, a chip,
25 capillaries, or a filter.

As regards the shape of the matrix according to the invention, in one embodiment the matrix is in the form of a porous monolith. In an alternative embodiment, the matrix is in beaded or particle form that can be porous or non-porous. Matrices in beaded or particle form can be used as a
5 packed bed or in a suspended form. Suspended forms include those known as expanded beds and pure suspensions, in which the particles or beads are free to move. In case of monoliths, packed bed and expanded beds, the separation procedure commonly follows conventional chromatography with a concentration gradient. In case of pure suspension, batch-wise mode will be used.

10

In a third aspect the present invention relates to a method of separating Fc-containing proteins from other substances, comprising the steps of:

- a) providing an affinity separation matrix according to any of the embodiments described above;
- 15 b) providing a solution comprising an Fc-containing protein and at least one other substance;
- c) contacting said solution with said separation matrix;
- d) optionally washing said separation matrix with at least one wash buffer;
- e) eluting said separation matrix with an elution buffer, or an elution buffer gradient of decreasing pH, to obtain an eluate comprising said Fc-containing protein and;
- 20 f) recovering said eluate.

If an elution buffer with essentially constant pH is used (step elution), the elution buffer has a pH of 4.0 - 6.0 and if an elution buffer gradient is used (gradient elution) at least part of the elution buffer gradient is within the pH range of 6.0 - 4.0.

Regular protein A media based on native or recombinant protein A (e.g. MABSELECT™ and rProtein A SEPHAROSE™ 4 Fast Flow) normally elute at pH 3.1 – 4.0 (measured at peak apex) mainly depending on its VH3 binding (see e.g. Ghose, S. et al. Biotechnology and Bioengineering 92 665-673 [2005]). The alkaline stabilized product MABSELECT SURE™, derived from the B-domain of protein A, essentially lacks the VH3 binding giving a higher elution pH: 3.7-4.0. For a general review of the principles of affinity chromatography, see e.g. Wilchek, M., and Chaiken, I. 2000. An overview of affinity chromatography. Methods Mol. Biol. **147**: 1-6.

The substitution of the Asparagine or Histidine residue at the position corresponding to H18 of B domain of Protein A or Protein Z unexpectedly increases the elution pH of immunoglobulins, or fusion proteins containing an Fc-fragment. The substitution thus provides a ligand which allows for elution above pH 4.0, preferably above 4.2, while the yield of target molecule is at least 80% or preferably >95%. This results in gentler elution conditions which minimize the risk for aggregation or inactivation of the target molecule.

Aggregate separation from monomers has been a challenge in antibody purification, especially at higher resolution. The present ligands show an improved separation between monomers and aggregates, making this a viable approach for aggregate removal in the capture step, even at large scale (see Figures 5 and 6). It is also possible to remove other contaminants with the method of the invention, such as e.g. DNA, non-desirable antibody isoforms (including certain charge isoforms and glycoforms), endotoxins, viruses, fermentation additives (e.g. antibiotics) etc.

In certain embodiments of the invention, the conditions for the adsorption step may be

any conventionally used, appropriately adapted depending on the properties of the target antibody such as the pI thereof. The optional wash step can be performed using a buffer commonly used such as a PBS buffer. It may also be performed with more than one wash buffer, e.g. of different pH, ionic strength or additives. Additives like solvents, salts or detergents or mixtures thereof may be used in one or more wash buffers.

The present method is useful to capture target antibodies, such as a first step in a purification protocol of antibodies which are e.g. for therapeutic or diagnostic use. In one embodiment, at least 75% of the antibodies are recovered. In an advantageous embodiment, at least 80%, such as at least 90%, and preferably at least 95% of the antibodies are recovered using an eluent having a suitable pH for the particular ligand system. Thus, in a specific embodiment, more than about 98% of the antibodies are recovered. The present method may be followed by one or more additional steps, such as other chromatography steps. The additional step(s) may comprise e.g. cation exchange chromatography, anion exchange chromatography, multimodal chromatography (with either positively or negatively charged matrices), thiophilic chromatography, hydroxyapatite chromatography and/or gel filtration. Additional chromatography steps can be performed in either bind-elute or flow-through mode and they can be performed in packed beds or with membrane adsorbers or monolithic columns. Hold tanks may or may not be used between steps and it is possible to use e.g. in-line conditioning in order to avoid hold tanks between steps.

In some embodiments the method further comprises a step g) of stripping said separation matrix with a stripping buffer having a pH of at least 0.1 pH units lower than the pH of the elution buffer or the pH of the elution buffer gradient at the end of step e). When the bound host cell proteins, aggregates and other impurities elute at a lower pH, a low pH stripping will efficiently remove

these substances from the matrix. Even if an alkaline cleaning step is applied afterwards to remove further impurities, the low pH strip can increase the efficiency of the alkaline cleaning and may prevent any fouling during the alkaline step.

- 5 In certain embodiments the solution comprising an Fc-containing protein and at least one other substance comprises host cell proteins (HCP), such as CHO cell or E Coli proteins. Contents of CHO cell and E Coli proteins can conveniently be determined by immunoassays directed towards these proteins, e.g. the CHO HCP or E Coli HCP ELISA kits from Cygnus Technologies.
- 10 In some embodiments the host cell proteins or CHO cell/E Coli proteins are desorbed during step g).

- In certain embodiments the solution comprising an Fc-containing protein and at least one other substance comprises aggregates of said Fc-containing protein, such as at least 1%, at least 5% or
- 15 at least 10% aggregates calculated on the total amount of the Fc-containing protein in said solution. In many cases an antibody or other Fc-containing protein contains aggregates already in the fermentation broth. The presence of significant aggregate amounts creates major difficulties during the subsequent purification and it is an advantage of the invention that aggregates can be removed directly in the capture step. Aggregate levels can conveniently be determined e.g. by ana-
- 20 lytical gel filtration, where aggregates elute before the monomeric Fc-containing protein.

In some embodiments aggregates are desorbed during step g).

- In certain embodiments the aggregate content in the recovered eluate is less than 1%, such as less
- 25 than 0.5% or less than 0.2% aggregates calculated on the total amount of the Fc-containing pro-

tein in said recovered eluate. As described above, a significant clearance of aggregates in the capture step facilitates the subsequent purification steps in the process. It may even allow the use of feeds that would otherwise not be feasible for production due to too high aggregate contents.

5 The elution may be performed by using any suitable solution used for elution from Protein A media. In some embodiments the elution buffer or the elution buffer gradient comprises at least one anion species selected from the group consisting of acetate, citrate, glycine, succinate, phosphate, and formate.

10 In certain embodiments the elution buffer or the elution buffer gradient comprises at least one elution additive, such as arginine or urea. The use of such additives can further increase the pH levels for elution of the Fc-containing protein.

In some embodiments, the eluate of step f) is collected in a pool and the pH of said pool is at
15 least 0.5 pH units higher than the pH of said elution buffer or the pH of said elution buffer gradient at the end of step e). The pH level of the pool can be at least 4.5, such as at least 5.0 or at least 5.5 or between 4.5 and 6.5 or between 5.0 and 6.5. A high pH level in the pool is important in order to prevent aggregate formation from the eluted monomeric Fc-containing protein, as the protein is exposed to the pool conditions for significant periods of time (at least several hours).

20

In some embodiments the method further comprises a step h), optionally performed after step g), wherein the matrix is cleaned by application of an alkaline solution of pH 13 – 14, such as a solution comprising 0.1 M to 0.5 M sodium hydroxide. As discussed earlier, for either SpA (domain E, D, A, B, C) or protein Z ligand, when at least one asparagine residue is mutated to an
25 amino acid other than glutamine or aspartic acid, affinity media including these mutant ligands

can better withstand cleaning procedures using alkaline agents (US patent application 2005/0143566). The increased stability means that the mutated protein's initial affinity for immunoglobulin is essentially retained for a prolonged period of time. Thus its binding capacity will decrease more slowly than that of the parental molecule in an alkaline environment. The environment can be defined as alkaline, meaning of an increased pH-value, for example above about 10, such as up to about 13 or 14, i.e. from 10-13 or 10-14, in general denoted alkaline conditions. Alternatively, the conditions can be defined by the concentration of NaOH, which can be up to about 1.0 M, such as 0.7 M or specifically about 0.5 M, accordingly within a range of 0.7-1.0 M.

Thus, the affinity to immunoglobulin i.e. the binding properties of the ligand, in the presence of the asparagine mutation as discussed, and hence the capacity of the matrix, is not essentially changed in time by treatment with an alkaline agent. Conventionally, for a cleaning in place treatment of an affinity separation matrix, the alkaline agent used is NaOH and the concentration thereof is up to 0.75 M, such as 0.5 M. Thus, its binding capacity will decrease to less than about 70 %, preferably less than about 50% and more preferably less than about 30%, such as about 28%, after treatment with 0.5 M NaOH for 7.5 h.

In certain embodiments the method further comprises a step i) of applying said eluate or pool to a cation exchange resin without any adjustment of the pH of said eluate or pool. As the eluate (and thus the pool) produced by the method of invention is higher than for conventional protein A media, it is possible to apply the eluate/pool directly to a cation exchanger without any intermediate pH adjustment. This simplifies the process and allows for straight-through processing.

In some embodiments the method further comprises, before step i), a step of solvent-detergent virus inactivation. The two commonly used virus inactivation methods are low pH inactivation and solvent-detergent inactivation. As exposure to low pH causes aggregation, it is less desirable with the current method which enables separation without exposing the protein to low pH. However, solvent-detergent inactivation is amenable to application on the pool/eluate before application to e.g. a cation exchange step. In this method a detergent (e.g. 0.3-1.5 % of a nonionic surfactant like TritonTM X-100 from Dow Chemical or TweenTM 80 from Uniqema) and a solvent (e.g. 0.1-0.5 % tri n-butyl phosphate) are applied to the eluate/pool and allowed to act for 30-90 min, such as about 45 min.

The present invention relates to a method of isolating an immunoglobulin, such as IgG, IgA and/or IgM, wherein a ligand or a matrix according to the invention is used. Thus, the invention encompasses a process of chromatography, wherein at least one target compound is separated from a liquid by adsorption to a ligand or matrix described above. The desired product can be the separated compound or the liquid. Thus, this aspect of the invention relates to affinity chromatography, which is a widely used and well-known separation technique. In brief, in a first step, a solution comprising the target compounds, preferably antibodies as mentioned above, is passed over a separation matrix under conditions allowing adsorption of the target compound to ligands present on said matrix. Such conditions are controlled e.g. by pH and/or salt concentration i.e. ionic strength in the solution. Care should be taken not to exceed the capacity of the matrix, i.e. the flow should be sufficiently slow to allow a satisfactory adsorption. In this step, other components of the solution will pass through in principle unimpeded.

In a fourth aspect the invention discloses a method of selecting an elution buffer for a matrix according to any embodiment described above, with an adsorbed Fc-containing protein, comprising the steps of:

- a) providing a solution comprising an Fc-containing protein and at least one other substance;
- 5 b) contacting said solution with said separation matrix under conditions where the Fc-containing protein and at least part of the other substance adsorb to the separation matrix;
- c) optionally washing said separation matrix with at least one wash buffer;
- d) eluting said separation matrix with an elution buffer gradient of decreasing pH, wherein at least part of said elution buffer gradient is within the pH range of 6.0-4.0;
- 10 e) measuring the content of said Fc-containing protein and said other substance in the eluate obtained in step d) and;
- f) selecting an elution buffer having the pH of the buffer gradient where at least 90%, such as at least 95% of the Fc-containing protein is eluted and where less than 25%, such as less than 10% of the adsorbed other substance is eluted.

15

In certain embodiments the other substance comprises host cell proteins, such as CHO cell proteins, or aggregates of said Fc-containing protein.

Examples

- 20 Below, the present invention will be described by way of examples, which are provided for illustrative purposes only and accordingly are not to be construed as limiting the scope of the present invention as defined by the appended claims. All references given below and elsewhere in this application are hereby included herein by reference.

25 **Mutagenesis of protein**

Site-directed mutagenesis was performed by a two-step PCR using oligonucleotides coding for the defined amino acid replacement. As template a plasmid containing a single domain of either wild type Z (SEQ ID NO: 6) or mutated Z (to create two or more mutations) was used. The PCR fragments were ligated into an *E. coli* expression vector (pGO). DNA sequencing was used to verify the correct sequence of inserted fragments.

To form multimers of Z(H18E), Z(H18del), Z(H18S,P57I), Z(H18S,N28A) and Z(H18S,N28A,P57I) an *Acc* I site located in the starting codons (GTA GAC) of the C or Z domain was used, corresponding to amino acids VD. pGO Z(H18E)1, pGO Z(H18del)1, pGO Z(H18S,P57I)1, pGO Z(H18S,N28A)1 and pGO Z(H18S,N28A,P57I)1 were digested with *Acc* I and CIP treated. *Acc* I sticky-ends primers were designed, specific for each variant, and two overlapping PCR products were generated from each template. The PCR products were purified and the concentration was estimated by comparing the PCR products on a 2% agarose gel. Equal amounts of the pair wise PCR products were hybridized (90°C → 25°C in 45min) in ligation buffer. The resulting product consists approximately to ¼ of fragments likely to be ligated into an *Acc* I site (correct PCR fragments and/or the digested vector). After ligation and transformation colonies were PCR screened to identify constructs containing Z(H18E)2, Z(H18del)2, Z(H18S,P57I)4, Z(H18S,N28A)4 and Z(H18S,N28A,P57I)4. Positive clones were verified by DNA sequencing.

Construct expression and purification

The constructs were expressed in the bacterial periplasm by fermentation of *E. coli* K12 in standard media. After fermentation the cells were heat-treated to release the periplasm content into the media. The constructs released into the medium were recovered by microfiltration with a membrane having a 0.2 µm pore size.

Each construct, now in the permeate from the filtration step, was purified by affinity. The permeate was loaded onto a chromatography medium containing immobilized IgG. The loaded product was washed with phosphate buffered saline and eluted by lowering the pH.

- 5 The elution pool was adjusted to a neutral pH and reduced by addition of dithiothreitol. The sample was then loaded onto an anion exchanger. After a wash step the construct was eluted in a NaCl gradient to separate it from any contaminants. The elution pool was concentrated by ultra-filtration to 40-50 mg/ml.
- 10 The purified ligands were analyzed with LC-MS to determine the purity and to ascertain that the molecular weight corresponded to the expected (based on the amino acid sequence).

The mutant Z(H18del)2 (SEQ ID NO: 10) did not bind to the IgG medium in the affinity step. Hence it was concluded that this mutation caused a loss of the IgG-binding capability and no

- 15 further experiments were made with H18del mutants.

Activation

The base matrix used was rigid crosslinked agarose beads of 85 microns average diameter, prepared according to the methods of US6602990 and with a pore size corresponding to an inverse

20 gel filtration chromatography Kav value of 0.70 for dextran of Mw 110 kDa, according to the methods described in Gel Filtration Principles and Methods, Pharmacia LKB Biotechnology 1991, pp 6-13.

- 25 25 mL (g) of drained base matrix, 10.0 mL distilled water and 2.02 g NaOH (s) was mixed in a 100 mL flask with mechanical stirring for 10 min at 25°C. 4.0 mL of epichlorohydrin was added

and the reaction progressed for 2 hours. The activated gel was washed with 10 gel sediment volumes (GV) of water.

Coupling

- 5 To 20 mL of ligand solution (50 mg/mL) in a 50 ml Falcon tube, 169 mg NaHCO_3 , 21 mg Na_2CO_3 , 175 mg NaCl and 7 mg EDTA, was added. The Falcon tube was placed on a roller table for 5-10 min, and then 77 mg of DTE was added. Reduction proceeded for >45 min. The ligand solution was then desalted on a PD10 column packed with Sephadex G-25. The ligand content in the desalted solution was determined by measuring the 276 nm UV absorption.

10

The activated gel was washed with 3-5 GV {0.1 M phosphate/1 mM EDTA pH 8.6} and the ligand was then coupled according to the method described in US6399750. All buffers used in the experiments had been degassed by nitrogen gas for at least 5-10 min.

- 15 After immobilisation the gels were washed 3xGV with distilled water. The gels + 1 GV {0.1 M phosphate/1 mM EDTA/10% thioglycerol pH 8.6} was mixed and the tubes were left in a shaking table at room temperature over night. The gels were then washed alternately with 3xGV {0.1 M TRIS/0.15 M NaCl pH 8.6} and 0.5 M HAc and then 8-10xGV with distilled water. Gel samples were sent to an external laboratory for amino acid analysis and the ligand content (mg/ml
- 20 gel) was calculated from the total amino acid content.

Example 1

Prototypes

- Mutant Z(H18E)2 (SEQ ID NO: 9): ligand dimers containing two copies of protein Z, each containing the H18E substitution (Z(H18E)2), with ligand density of 5.2mg/ml.
- 25

- Comparative prototype Z2 (similar to SEQ ID NO: 9, except H18 are not substituted to E): ligand dimer containing two copies of protein Zvar2 (Z2), with ligand density of 5.9 mg/ml.
 - Mutant Z(H18S,P57I)4 (SEQ ID NO: 11):-ligand tetramer containing four copies of protein Z, each containing the H18S,P57I substitutions Z(H18S,P57I)4, with ligand density of 5.3 mg/ml.
 - Mutant Z(H18S,N28A)4 (SEQ ID NO: 12): ligand tetramer containing four copies of protein Z, each containing the H18S,N28A substitutions, with ligand density of 6.9 mg/ml.
 - Comparative prototype Z4 (similar to SEQ ID NO: 11, except H18 and P57 are not substituted to S and I respectively): ligand tetramer containing four copies of protein Zvar2 (Z2), with ligand density of 6.0 mg/ml.
 - Comparative prototype Z(H18S)4 (similar to SEQ ID NO:12, except N28 is not substituted to A): ligand tetramer containing four copies of protein Z, each containing the H18S substitution, with ligand density of 6.7 mg/ml.
 - Comparative prototype Z(N28A)4 (similar to SEQ ID NO:12, except H18 is not substituted to S): ligand tetramer containing four copies of protein Z, each containing the N28A substitution, with ligand density of 7.7 mg/ml.
- For each prototype, 2 ml of resin was packed in Tricorn 5 100 column.

Protein

Gammanorm 165 mg/ml (Octapharma), diluted to 1 mg/ml in Equilibration buffer.

Equilibration buffer

APB Phosphate buffer 20 mM +0.15 M NaCl , pH 7.4 (Elsichrom AB)

Elution buffers

Citrate buffer 0.1 M, pH 6.

5 Citrate buffer 0.1 M, pH 3.

CIP

0.1 M NaOH.

10 Experimental details and results:

The breakthrough capacity was determined with an ÄKTAExplorer 10 system at a residence time of 2.4 minutes. Equilibration buffer was run through the bypass column until a stable baseline was obtained. This was done prior to auto zeroing. Sample was applied through the bypass until the 100% UV signal was obtained. Then, equilibration buffer was applied to the column again until a stable baseline was obtained. Sample was loaded onto the column until a UV signal of 85% of maximum absorbance was reached. The column was then washed with equilibration buffer until a UV signal of 20% of maximum absorbance at flow rate 0.5 ml/min. The protein was eluted with a linear gradient over 10 column volumes starting at pH 6.0 and ending at pH 3.0 at a flow rate of 0.5 ml/min. Then the column was cleaned with 0.1 M NaOH at flow rate 0.5 ml/min and re-equilibrated with equilibration buffer prior to adding 20% ethanol. The last step was to check the sample concentration by loading sample through the bypass column until a 100% UV signal was obtained.

For calculation of breakthrough capacity at 10%, equation below was used. That is i.e. the amount of IgG that is loaded onto the column until the concentration of IgG in the column effluent is 10% of the IgG concentration in the feed.

$$q_{10\%} = \frac{C_0}{V_c} \left[V_{app} - V_{sys} - \int_{V_{sys}}^{V_{app}} \frac{A(V) - A_{sub}}{A_{100\%} - A_{sub}} * dv \right]$$

$A_{100\%}$ = 100% UV signal;

A_{sub} = absorbance contribution from non-binding IgG subclass;

$A(V)$ = absorbance at a given applied volume;

5 V_c = column volume;

V_{app} = volume applied until 10% breakthrough;

V_{sys} = system dead volume;

C_0 = feed concentration.

The dynamic binding capacity (DBC) at 10% breakthrough was calculated and the
 10 appearance of the curve was studied. The curve was also studied regarding binding, elution and CIP peak. The dynamic binding capacity (DBC) was calculated for 5, 10 and 80% breakthrough. Some results are shown in Table 1. Similar capacity was observed for ligands with H18S substitution as compared to the parental H18 ligand (Z2 or Z4).

IgG capacity and elution study for polyclonal human IgG and different mABs were done
 15 on Z(H18E)2, Z(H18S,P57I)4, Z(H18S,N28A)4, Z2 and Z4. Some examples are shown in Table 1.

Table 1. Capacity data for Z(H18E)2, Z(H18S,N28A)4, Z(H18S,P57I)4, Z2 and Z4, using 1 mg/ml IgG dissolved in 20 mM PBS+0.15 M NaCl buffer, pH 7.4.

prototype	Qb10% (mg/ml resin)	Qb80% (mg/ml resin)	Residence time (min)	Ligand density (mg/ml)
Z(H18S,N28A)4	35.2	56.0	2.4	6.9
Z(H18S,N28A)4	45.9	59.1	6	6.9
Z(H18S,P57I)4	35.6	56.0	2.4	5.3
Z(H18S,P57I)4	47.0	58.0	6	5.3
Z(H18S)4	37.0	62.2	2.4	6.7
Z(H18S)4	55.0	67.5	6	6.7
Z(N28A)4	37.4	62.5	2.4	7.7
Z(N28A)4	53.2	65.6	6	7.7
Z(H18E)2	26.6	41.3	2.4	5.2
Z(H18E)2	31.6	43.3	6	5.2

Z2	31.4	43.8	2.4	5.9
Z2	40.4	47.1	6	5.9
Z4	36.7	54.4	2.4	6.0
Z4	48.0	57.2	6	6.0

The elution studies were done for different cell culture supernatant (mAb C, mAb D) or polyclonal human IgG in phosphate buffer. The elution pH increases with 0.6 to 1.8 pH units for Z(H18E)2, Z(H18S,P57I)4 and Z(H18S,N28A)4 compared to Z2 and Z4.-The biggest difference is shown with polyclonal IgG (Gammanorm) while the mAb D shows somewhat smaller differences. In these elution studies approximately 10 mg cell culture supernatant (different cell culture supernatants were used, see Table 2) was loaded on the column. A gradient elution from pH 6 to pH 3 was done using 0.1 M citrate buffers. The pH at peak apex was noted (see Table 2.)

Table 2. Elution pH for polyclonal human IgG and two different mAbs.

prototype	pH hIgG (first peak)	pH hIgG (second peak)	pH mAb
Z(H18S,N28A)4	5.68	5.15	5.05
Z(H18S,P57I)4	5.44	4.91	4.71
Z(H18S)4	5.10	4.61	
Z(N28A)4	4.77	4.42	
Z(H18E)2	4.83	4.23	4.48
Z2	3.99	3.59	4.03
Z4	3.89	3.53	4.12

Note pH hIgG (first peak) and pH hIgG (second peak) are polyclonal IgG (Gammanorm).

Except for higher elution pH for Z(H18E)2, Z(H18S,P57I)4 and Z(H18S,N28A)4, they also have better separation properties than Z2 and Z4. Figures 2 and 3 show the elution of antibody E and adsorbed host cell proteins from Z4 (Fig. 3) and Z(H18S,P57I)4 (Fig. 2) in a decreasing pH gradient. On Z4 (as well as on other matrices not having the H18 mutations), the antibody and the HCP co-elute, meaning that HCP bound to the matrix will not be removed in

the chromatography step. On Z(H18S,P57I)4 the HCP elutes later in the gradient, which creates a possibility for selective elution to remove the bound HCP, either by gradient elution or by applying an elution buffer having a pH where the antibody elutes and then e.g. applying a stripping buffer at lower pH to remove the HCP.

5 Also antibody aggregates elute at a lower pH than the antibody monomer, as illustrated by Figure 4. This figure shows SEC chromatograms for the eluate fractions of a monoclonal antibody on Z(H18S,P57I)4 (the same fractions as shown in Fig. 2). It can be seen that the main antibody peak (fraction B7) contains no aggregates, while the aggregates start to appear in B12 and the later fraction C1 consists almost entirely of aggregates.

10 The above examples illustrate specific aspects of the present invention and are not intended to limit the scope thereof in any respect and should not be so construed. Those skilled in the art having the benefit of the teachings of the present invention as set forth above, can effect numerous modifications thereto. These modifications are to be construed as being encompassed within the scope of the present invention as set forth in the appended claims.

15

CLAIMS:

1. An immunoglobulin-binding protein comprising one or more mutated immunoglobulin-binding domains (monomers) of staphylococcal Protein A (E, D, A, B, C) or protein Z or a functional variant thereof, wherein in at least one of the one or more mutated monomers, the Asparagine or
5 Histidine at the position corresponding to H18 of the B domain of Protein A or of Protein Z has been substituted with a first amino acid residue which is not Proline or Asparagine and wherein, if the amino acid residue at position 57 is Proline and the amino acid residue at position 28 is Asparagine, then the amino acid residue at the position corresponding to H18 of the B domain of Protein A or of Protein Z is not Serine, Threonine or Lysine.

10

2. A protein according to claim 1, wherein additionally at least one of the Proline at position 57 and the Asparagine at position 28 has been substituted with a second amino acid residue.

15

3. A protein according to claim 1, wherein neither of the amino acid residues at positions 57 and 28 is Proline or Asparagine.

4. A protein according to any preceding claim, wherein the amino acid residue at position 18 is selected from the group consisting of Serine, Glutamic acid, Aspartic acid, Threonine and Lysine.

20

5. A protein according to any preceding claim, wherein at least one of the amino acid residues at position 57 and 28 is selected from the group consisting of Alanine and Isoleucine.

6. A protein according to any preceding claim, wherein the protein comprises one or more mutated monomers derived from the parental molecules defined by SEQ ID NO 5, 6, 7 or 8, wherein

the mutation is selected from the group consisting of H18E; H18S,N28A; H18S,P57I; N28A,P57I and H18S,N28A,P57I.

7. A protein according to any preceding claim, wherein the protein comprises one or more mutated monomers defined by SEQ ID NO: 14, 16, 17, or 18.

8. A protein according to any preceding claim, comprising at least two mutated monomers, such as 3, 4, 5 or 6 monomers.

9. A protein according to any preceding claim, further comprising a C-terminal cysteine.

10. An affinity separation matrix, comprising a protein according to any preceding claim coupled to a support.

11. A matrix according to claim 10, comprising 5 – 15 mg/ml, such as 6 – 11 mg/ml, of said protein coupled to said support.

12. A matrix according to claim 10 or 11, wherein said protein is coupled to the support via a thioether bridge.

13. A matrix according to claim 10 or 11, wherein said protein is coupled via a C-terminal cysteine.

14. A matrix according to any one of claims 10-13, wherein said support comprises a polyhydroxy polymer, such as a polysaccharide.

15. A matrix according to claim 14, wherein said support comprises agar or agarose.

16. A matrix according to any one of claims 10-15, wherein said support is crosslinked, such as
5 with hydroxyalkyl ether crosslinks.

17. A method of separating Fc-containing proteins from other substances, comprising the steps of:

g) providing an affinity separation matrix according to any one of claims 10-16;

10 h) providing a solution comprising an Fc-containing protein and at least one other substance;

i) contacting said solution with said separation matrix;

j) optionally washing said separation matrix with at least one wash buffer;

k) eluting said separation matrix with an elution buffer, or an elution buffer gradient of decreasing pH, to obtain an eluate comprising said Fc-containing protein and;

15 l) recovering said eluate.

wherein said elution buffer has a pH of 4.0 -6.0 or wherein at least part of said elution buffer gradient is within the pH range of 6.0-4.0.

18. The method of claim 17, further comprising a step g) of stripping said separation matrix with
20 a stripping buffer having a pH of at least 0.1 pH units lower than the pH of the elution buffer or the pH of the elution buffer gradient at the end of step e).

19. The method of claim 17 or 18, wherein said solution comprises host cell proteins, such as CHO cell or E Coli proteins.

20. The method of claim 19, wherein host cell proteins are desorbed during step g).

5

21. The method of any one of claims 17-20, wherein said solution comprises aggregates of said Fc-containing protein, such as at least 1%, at least 5% or at least 10% aggregates calculated on the total amount of the Fc-containing protein in said solution.

10 22. The method of claim 21, wherein aggregates are desorbed during step g).

23. The method of claim 21 or 22, wherein the aggregate content in the recovered eluate is less than 1%, such as less than 0.5% or less than 0.2% aggregates calculated on the total amount of the Fc-containing protein in said recovered eluate.

15

24. The method of any one of claims 17-23, wherein said elution buffer or said elution buffer gradient comprises at least one anion species selected from the group consisting of acetate, citrate, glycine, succinate, phosphate, and formate.

20 25. The method of any one of claims 17-24, wherein said elution buffer or said elution buffer gradient comprises at least one elution additive, such as arginine or urea.

26. The method of any one of claims 17-25, wherein in step f), the eluate is collected in a pool and the pH of said pool is at least 0.5 pH units higher than the pH of said elution buffer or the pH of said elution buffer gradient at the end of step e).

25

27. The method of any one of claims 17-26, further comprising a step h), optionally performed after step g), wherein the matrix is cleaned by application of an alkaline solution of pH 13 – 14, such as a solution comprising 0.1 M to 0.5 M sodium hydroxide.

5

28. The method of any one of claims 17-26, further comprising a step i) of applying said eluate or pool to a cation exchange resin without any adjustment of the pH of said eluate or pool.

29. The method of claim 27, further comprising before step i) a step of solvent-detergent virus inactivation.

10

30. A method of selecting an elution buffer for a matrix according to any one of claims 10-16 with an adsorbed Fc-containing protein, comprising the steps of:

a) providing a solution comprising an Fc-containing protein and at least one other substance;

15 b) contacting said solution with said separation matrix under conditions where the Fc-containing protein and at least part of the other substance adsorb to the separation matrix;

c) optionally washing said separation matrix with at least one wash buffer;

d) eluting said separation matrix with an elution buffer gradient of decreasing pH, wherein at least part of said elution buffer gradient is within the pH range of 6.0-4.0;

20 e) measuring the content of said Fc-containing protein and said other substance in the eluate obtained in step d) and;

f) selecting an elution buffer having a pH of the buffer gradient where at least 90%, such as at least 95% of the Fc-containing protein is eluted and where less than 25%, such as less than 10% of the adsorbed other substance is eluted.

25

31. The method of claim 30, wherein said other substance comprises host cell proteins, such as CHO cell proteins, or aggregates of said Fc-containing protein.

32. The method of claim 30 or 31, further comprising a step g) of selecting at least one wash
5 buffer having a pH of the buffer gradient where less than 10%, such as less than 5% of the Fc-containing protein is eluted.

Protein A (five domains, EDABC)

(N-terminus)	< Helix 1>	< Helix 2>	< Helix 3>		SEQ ID No:
	QQNAFYQVILNMPNLNADQRN	GFQSLKDDPSQSANVLGEAQKL	ND	SEQ ID No: 1	
ADAQQNNFNK	QQAFAFYEILNMPNLNEAQ	RNGFIQSLKDDPSQSTNVLGEAK	KLNESQAPK (D)	SEQ ID No: 2	
ADNFNKEQ	QQAFAFYEILNMPNLNEEQ	RNGFIQSLKDDPSQSANLLSEAK	KLNESQAPK (A)	SEQ ID No: 3	
ADNKENKEQ	QQAFAFYEILHLPNLNEEQ	RNGFIQSLKDDPSQSANLLAEAK	KLND	SEQ ID No: 4	
ADNKENKEQ	QQAFAFYEILHLPNLTEE	QRNGFIQSLKDDPSVSKEILAEAK	KLND	SEQ ID No: 5	
				(C-terminus)	
	VDNKENKEQ	QQAFAFYEILHLPNLNEEQ	RNAFIQSLKDDPSQSANLLAEAK	KLND	SEQ ID No: 6
VDNKENKEQ	QQAFAFYEILHLPNLTEE	QRNAFIQSLKDDPSQSKEILAEAK	KLND	SEQ ID No: 7	
VD	AKFDKEQ	QQAFAFYEILHLPNLTEE	QRNAFIQSLKDDPSQSKEILAEAK	KLND	SEQ ID No: 8

100

2/4

Z(H18S,P57I)₄

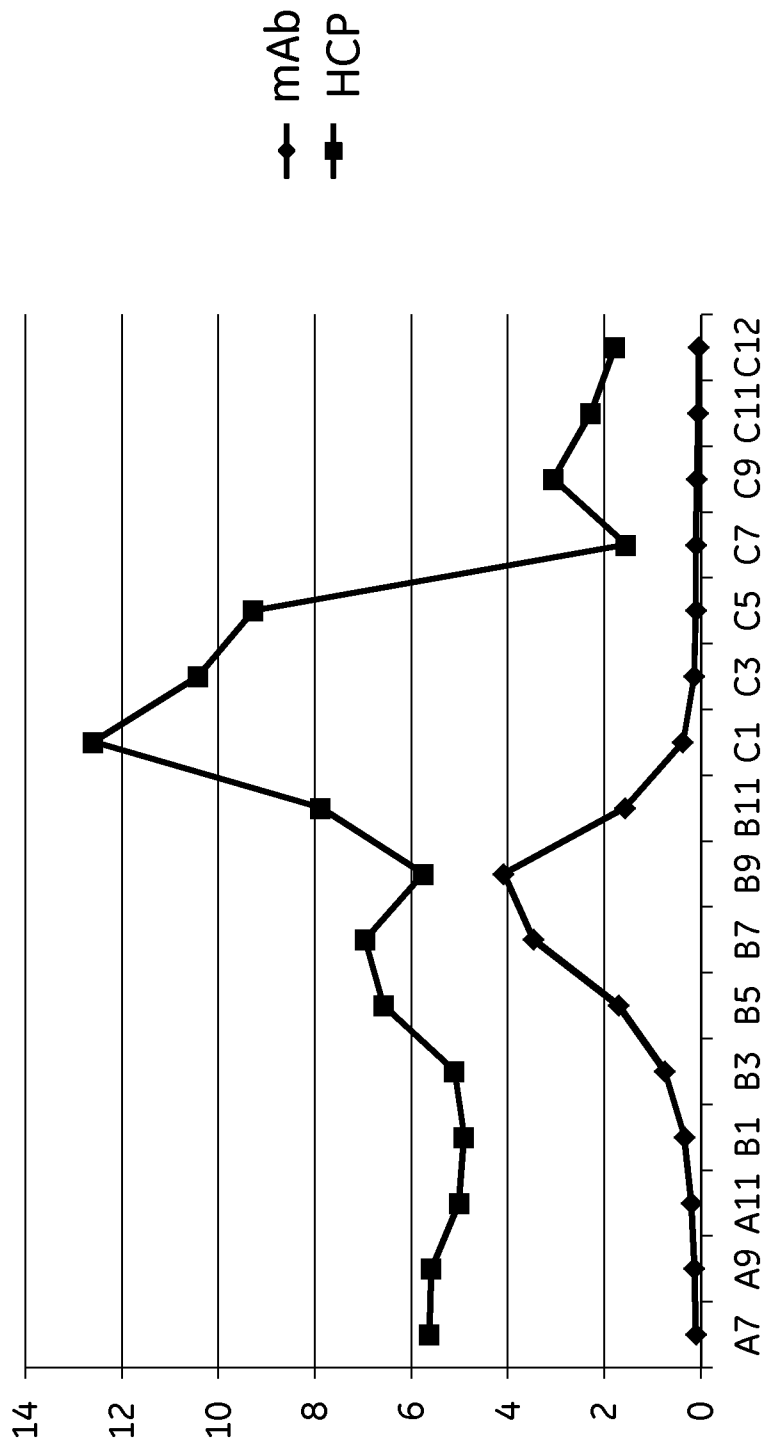


Figure 2

3/4

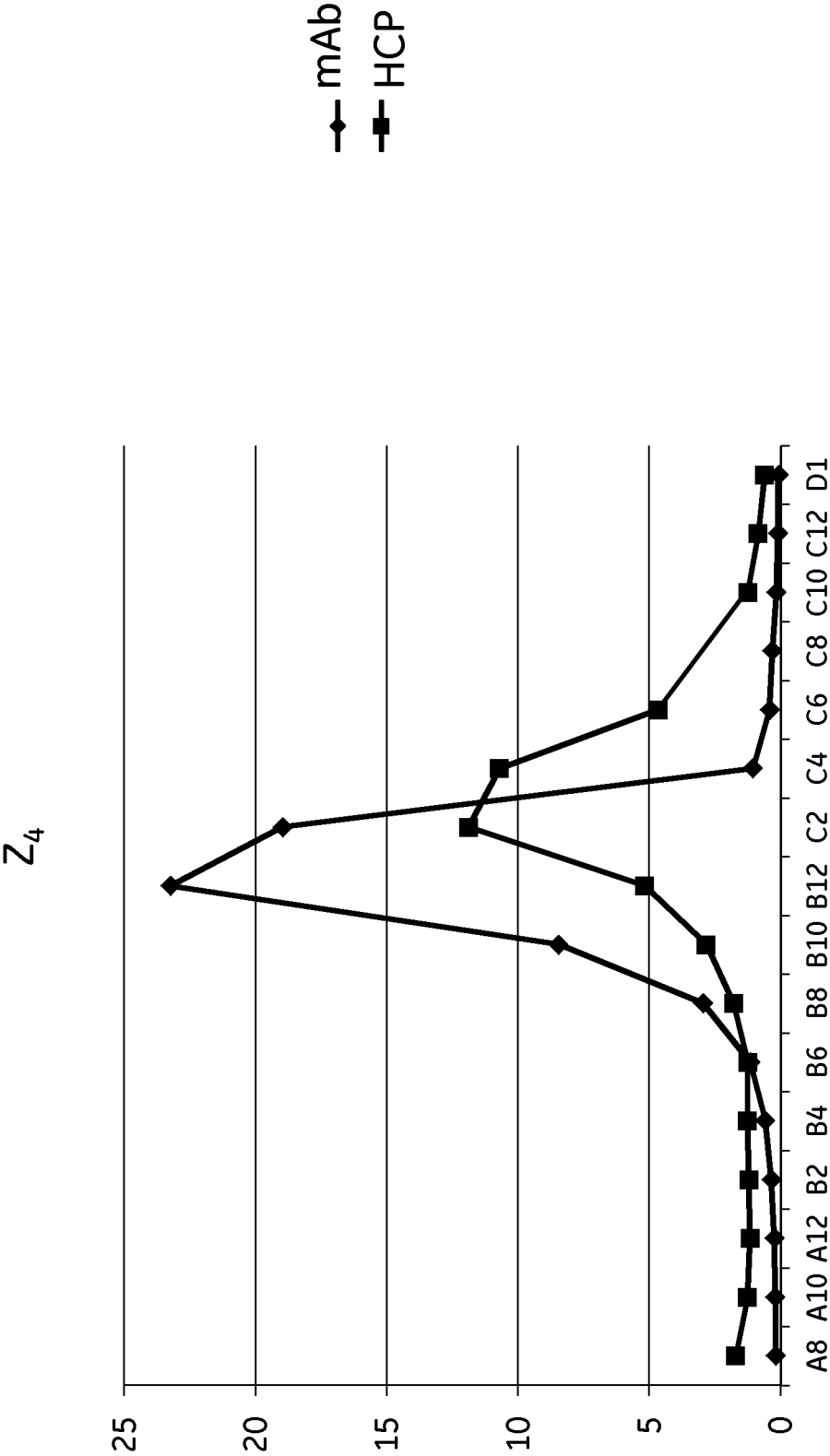


Figure 3

4/4

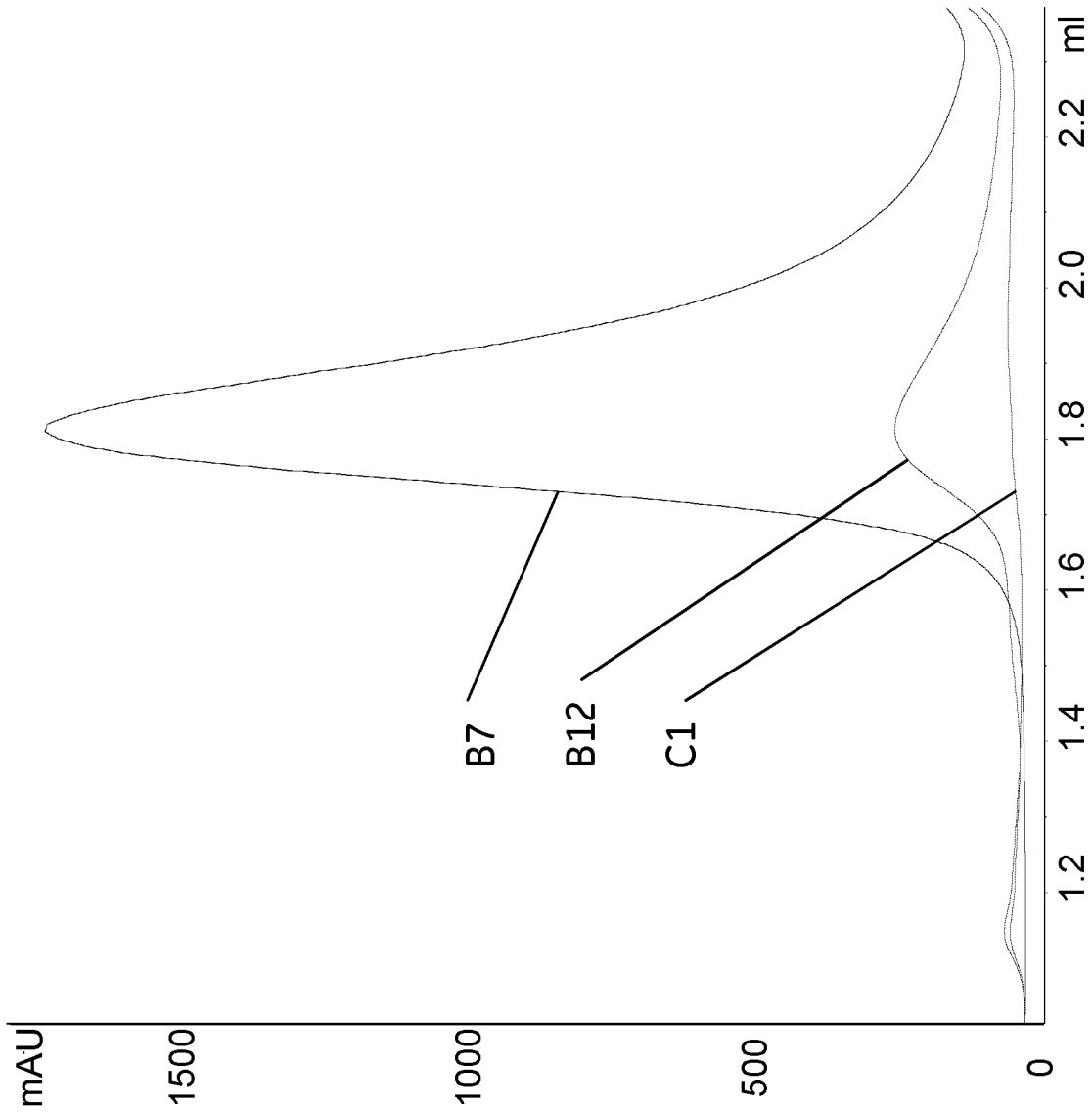


Figure 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2013/050335

A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: B01D, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE, DK, FI, NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, PAJ, WPI data, BIOSIS, COMPENDEX, EMBASE, INSPEC, MEDLINE, XPESP2, XPSRNG, Sequence search (EBI)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005075507 A1 (AFFIBODY AB ET AL), 18 August 2005 (2005-08-18); See abstract; page 14, lines 1-12; page 15, lines 11-18; page 17, line 30 - page 18, line 27; page 28, line 29-31; claims 1, 5, 8, 19-20, 31-32, 35-37, 61-62, SEQ ID NO: 1 --	1-3, 5, 8-32
X	WO 2006092338 A2 (AFFIBODY AB ET AL), 8 September 2006 (2006-09-08); See abstract; claims 1, 5, and 13; SEQ ID NO:1 in figure 1.	1, 10
A	--	2-9, 11-32



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19-07-2013

Date of mailing of the international search report

19-07-2013

Name and mailing address of the ISA/SE

Patent- och registreringsverket
Box 5055
S-102 42 STOCKHOLM
Facsimile No. + 46 8 666 02 86

Authorized officer

Liza Nilsson

Telephone No. + 46 8 782 25 00

INTERNATIONAL SEARCH REPORT

 International application No.
 PCT/SE2013/050335

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EJIMA D. et al., "Effective elution of antibodies by arginine and arginine derivatives in affinity column chromatography", Analytical biochemistry, 2005, Vol. 345, No. 2, pages 250-257; whole document --	25
A	US 6602977 B1 (LJUNGQVIST CHARLOTTA ET AL), 5 August 2003 (2003-08-05); whole document --	1-32
A	WO 2005003156 A1 (AFFIBODY AB ET AL), 13 January 2005 (2005-01-13); whole document --	1-32
A	WO 03080655 A1 (AMERSHAM BIOSCIENCES AB ET AL), 2 October 2003 (2003-10-02); whole document; claim 7 --	1-32
A	WO 2008039141 A1 (GE HEALTHCARE BIO SCIENCES AB ET AL), 3 April 2008 (2008-04-03); abstract; claim 7 --	9, 11
A	WO 2011028753 A1 (GENENTECH INC ET AL), 10 March 2011 (2011-03-10); whole document --	1-32
A	US 20080167450 A1 (PAN HAI), 10 July 2008 (2008-07-10); whole document -- -----	1-32

Continuation of: second sheet

International Patent Classification (IPC)

C07K 14/31 (2006.01)

B01D 15/38 (2006.01)

C07K 1/22 (2006.01)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/SE2013/050335

WO	2005075507 A1	18/08/2005	NONE		
WO	2006092338 A2	08/09/2006	AT	490268 T	15/12/2010
			DE	602006018592 D1	13/01/2011
			DK	1869071 T3	28/02/2011
			US	20090306572 A1	10/12/2009
			US	7993848 B2	09/08/2011
US	6602977 B1	05/08/2003	NONE		
WO	2005003156 A1	13/01/2005	AT	416190 T	15/12/2008
			AU	2004253835 A1	13/01/2005
			CA	2531238 A1	13/01/2005
			DE	602004018141 D1	15/01/2009
			DK	1641818 T3	16/03/2009
			ES	2319426 T3	07/05/2009
			HK	1092481 A1	30/06/2011
			JP	2007537700 A	27/12/2007
			JP	4871126 B2	08/02/2012
			JP	2011229531 A	17/11/2011
			US	7993650 B2	09/08/2011
			US	20100048868 A1	25/02/2010

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/SE2013/050335

WO	03080655 A1	02/10/2003	AT	431360 T	15/05/2009
			AU	2003217119 B2	18/02/2010
			CA	2479896 A1	02/10/2003
			CN	1642976 A	20/07/2005
			CN	102516371 A	27/06/2012
			CN	102558318 A	11/07/2012
			CN	102558317 A	11/07/2012
			CN	102532284 A	04/07/2012
			CN	102516372 A	27/06/2012
			DE	60327606 D1	25/06/2009
			EP	1485407 A1	15/12/2004
			EP	1972689 A3	19/11/2008
			ES	2325362 T5	20/02/2013
			JP	2005538693 A	22/12/2005
			JP	4391830 B2	24/12/2009
			KR	20110004456 A	13/01/2011
			KR	20120126129 A	20/11/2012
			KR	20120125674 A	16/11/2012
			US	7834158 B2	16/11/2010
			US	8354510 B2	15/01/2013
			US	20120238724 A1	20/09/2012
			US	20110112276 A1	12/05/2011
			US	20100022760 A1	28/01/2010
			US	20060194950 A1	31/08/2006
			US	20050143566 A1	30/06/2005
			US	8198404 B2	12/06/2012
WO	2008039141 A1	03/04/2008	AU	2007300751 A1	03/04/2008
			CA	2664530 A1	03/04/2008
			CN	101522278 A	02/09/2009
			EP	2066419 A1	10/06/2009
			JP	2010504754 A	18/02/2010
			US	20100048876 A1	25/02/2010
			US	20120289680 A1	15/11/2012
			US	8329860 B2	11/12/2012
WO	2011028753 A1	10/03/2011	CA	2772653 A1	10/03/2011
			CN	102686738 A	19/09/2012
			EP	2473617 A1	11/07/2012
			JP	2013503877 A	04/02/2013
			KR	20120106719 A	26/09/2012
			MX	2012002565 A	29/05/2012
			US	20130041139 A1	14/02/2013
US	20080167450 A1	10/07/2008	WO	2008085988 A1	17/07/2008