

SELECTIVE ENRICHMENT OF ANTIBODIES

5

BACKGROUND TO THE INVENTION

TECHNICAL FIELD

The present invention relates to purification processes for proteins, particularly
10 immunoglobulins or other proteins that have an Fc domain.

STATE OF THE ART

Biomolecules such as proteins, polynucleotides, polysaccharides and the like are increas-
15 ingly gaining commercial importance as medicines, as diagnostic agents, as additives in
foods, detergents and the like, as research reagents and for many other applications. The
need for such biomolecules can no longer normally be met - for example in the case of
proteins - by isolating molecules from natural sources, but requires the use of biotechno-
logical production methods.

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The biotechnological preparation of proteins typically begins with the cloning of a DNA
fragment into a suitable expression vector. After transfection of the expression vector into
suitable prokaryotic or eukaryotic expression cells and subsequent selection of trans-
fected cells the latter are cultivated in bioreactors and the desired protein is expressed.
25 Then the cells or the culture supernatant is or are harvested and the protein contained
therein is worked up and purified.

In the case of eukaryotic expression systems, i.e. when using mammalian cell cultures
such as CHO or NSO cells, for example, there has been a significant increase in the last
30 15 years in the concentration of the desired protein in the cell cultures or cell culture
supernatants that can be achieved in the expression step. Over the same period, the
binding capacity of chromatographic materials that are used in the subsequent purification
of the proteins increased only slightly, by comparison. For this reason there is a pressing

need for improved, optimised purification methods for biomolecules, particularly proteins, that can be carried out on a large industrial scale..

- In the case of biopharmaceuticals, such as for example proteins used as drugs, e.g.
- 5 therapeutic antibodies, the separation of impurities is of major importance, in addition to the product yield. A distinction can be drawn between process- and product-dependent impurities. The process-dependent impurities contain components of the host cells such as proteins (host cell proteins, HCP) and nucleic acids and originate from the cell culture (such as components of the medium) or from the working up (such as for example salts or
- 10 dissolved chromatography ligands). Product-dependent impurities are molecular variants of the product with different properties. These include shortened forms such as precursors and hydrolytic breakdown products, but also modified forms, produced for example by deaminations, incorrect glycosylations or wrongly linked disulphide bridges. Also included among the product-dependent variants are polymers and aggregates,
- 15 Other impurities are contaminants. This term covers all other materials of a chemical, biochemical or microbiological nature that do not belong directly to the production process. Examples of contaminants include viruses that may occur in undesirable manner in cell cultures.
- 20 In the case of biopharmaceuticals, impurities give rise to safety concerns. These concerns are intensified if, as very often happens with biopharmaceuticals, the therapeutic proteins are administered directly into the bloodstream by injection or infusion. Thus, host cell components may lead to allergic reactions or immunopathological effects. In addition, impurities may also lead to an undesirable immunogenicity of the protein administered, i.e.
- 25 they may trigger an undesirable immune reaction to the therapeutic agent in the patient, possibly leading to life-threatening anaphylactic shock. Therefore, there is a need for suitable purification processes by means of which all unwanted substances can be reduced to a harmless level.
- 30 On the other hand, even in the case of biopharmaceuticals, economic considerations cannot be ignored. Thus, the production and purification methods used should not compromise the economic viability of the biopharmaceutical product thus produced.

As working up and processing steps for proteins, (column) chromatographic processes as well as filtration and precipitation processes are of crucial importance. Thus, the precise operation of concentrating antibodies contains a step of purification by affinity chromatography. Accordingly, numerous methods of column chromatography and the chromatography materials that can be used in these processes are currently known.

Affinity chromatography matrices are used as the stationary phase in the industrial purification of various substances. Immobilised ligands can be used for specifically concentrating and purifying substances that have a certain affinity for the particular ligand used. For the industrial purification of antibodies (immunoglobulins), particularly the purification of monoclonal antibodies, immobilised protein A is often used as the initial purification step. Protein A is a protein with about 41 kDa of *Staphylococcus aureus* that binds with high affinity (10^{-8} M – 10^{-12} M of human IgG) to the CH₂/CH₃ domain of the Fc region of immunoglobulins. In protein A chromatography, immunoglobulins or fusion proteins that have a protein A-binding Fc region from the mobile phase bind specifically to the protein A ligand, which is covalently coupled to a carrier (e.g. Sepharose). Protein A from *Staphylococcus aureus* (wild-type protein A) and genetically modified recombinant protein A (rec. protein A) interacts through non-covalent interactions with the constant region (Fc fragment) of the antibodies. This specific interaction can be used to separate impurities efficiently from the antibody. By altering the pH, the interaction between the antibody and the protein A ligand can be deliberately stopped and the antibody released or eluted from the stationary phase.

Apart from protein A as affinity ligand, there are many other molecules currently known that bind to the Fc fragment. Thus, individual domains of protein A are used instead of the complete protein (8). Protein variants are known, which differ precisely from the B domain of the protein A, which are suitable for binding Fc-fragment-containing molecules (16, 17). These different variants differ essentially in the mutations that have been inserted in order to increase the stability or binding affinity. These mutants of the B domain are usually known as Z-domain or protein Z. Besides protein A or protein G, various peptides are also suitable for selective binding to the Fc fragment (14). The present great interest in affinity ligands for the Fc fragment lead one to suppose that still more affinity ligands are being found.

Affinity chromatography and particularly the frequently used protein A chromatography are expensive and precisely when there are increasing product concentrations in the fermenter and large quantities of product, there are limits on the chromatographic purification processes that can be carried out. The critical points are: loading capacities,
5 number of cycles, process times, pool volumes and quantities of buffer. In the future, therefore, alternative purification processes will be essential. A general overview of conventional purification strategies, including affinity chromatography and alternative methods of affinity chromatography, can be found in the following articles (7, 10).

10 A more recent method of affinity chromatography uses not a constantly immobilised affinity ligand but, to begin with, a solubilised affinity ligand that is mixed with the target protein (11). The affinity ligand carries a fusion tag or a fusion protein which makes it possible to carry out the immobilisation of the affinity ligand on a solid phase that takes place in the second step. In the next step, as in conventional affinity chromatography, the
15 target protein is separated from the ligand under suitable conditions and thus eluted from the column.

The invention described here uses affinity precipitation instead of affinity chromatography to purify biomolecules. This method appears to have great potential precisely when used
20 on a larger scale (1, 2).

Affinity precipitation is the most effective method of protein precipitation (2). The precipitation of a protein from a solution in general is a well known process that is frequently used. Thus, many proteins have already been separated from a solution by
25 ammonium sulphate precipitation from a protein mixture. During this precipitation, macromolecules (e.g. proteins) are removed from the solution and converted into particles. Depending on the difference in density between the particle and the solution and the particle diameter, this step leads to precipitation. However, this precipitation usually takes place non-specifically.

30 For more selective concentration of a molecule, e.g. a protein, therefore, affinity precipitation was developed. Affinity precipitation makes use of the selective binding of an affinity molecule to a target molecule. Affinity molecules may be for example proteins, peptides, oligonucleotides or small chemical molecules. A distinction is drawn in affinity

precipitation between two principles, first and second order affinity precipitation (7). In first order affinity precipitation, both the affinity molecule and the target molecule have two binding sites, so that it is possible for a network to form between the two molecules and an affinity complex is formed that sediments at a specific size. In second order affinity precipitation, affinity macroligands (AML) are used, for example. In this type of precipitation, the affinity molecule is bound to a stimutable substance, usually a polymer. The stimutable substance changes its solubility characteristics as a result of a change in the ambient conditions, such as e.g. a shift in the pH or temperature, and precipitation occurs. Unlike first order affinity precipitation, there is no need for a bifunctional affinity molecule or for a bifunctional target molecule either.

In the affinity precipitation that is mostly used, the affinity ligands are at present coupled to polymers or other mediators (15).

The affinity precipitation of molecules containing Fc fragment has already been described in a number of studies. The most common application at present is characterised by the use of so-called "smart polymers". "Smart polymers" (or stimuli-responsive "intelligent" polymers or affinity macroligands) are polymers that can change their properties in response to external influences (physical or chemical stimuli). These stimuli may be for example changes in pH or temperature (12-13). Usually, smart polymers react to their stimuli with precipitation in solution. This precipitation can be reversed, after the desired separation of the supernatant solution, by suitable conditions. Smart polymers can be conjugated with various biomolecules, leading to a large accumulation of polymer/biomolecule systems that can be used for all kinds of applications. Examples of these biomolecules include proteins, oligonucleotides and sugars.

Another form of affinity precipitation is the recently described "affinity sinking" method. In this form of affinity precipitation, a linking molecular scaffold is used to bind a number of affinity ligands to one another. This then makes it possible to form the network required for precipitation. The binding of the affinity ligand to the network former may take place both non-covalently and covalently. This method was recently described in the patent "Compositions and methods for purifying and crystallizing molecules of interest" (6). In this, first of all a solution containing antibodies is mixed with an affinity ligand that is covalently bound to a crosslinker. No precipitation is observed. Only after the

subsequent addition of a coordinating ion or molecule does precipitation take place. In a second similar application the affinity ligand is linked to a biotin-binding protein which forms a network with the mediator avidin (9).

- 5 US patent 7,083,943 describes an affinity precipitation in which a binding domain for the target protein is linked to a scaffold domain the amino acid sequence of which is intended to assist the tendency to precipitation.

Chen and Hoffman (15) describe the affinity precipitation of IgG with poly(N-
10 isopropylacrylamide)-protein A conjugates. The precipitation of antibodies with unmodified protein A, however, has not proved effective.

One disadvantage of the methods described in the prior art is that during first order affinity precipitation, precipitation is possible only via the cross-linking molecule, or in second
15 order affinity precipitation a stimutable substance is required. Other disadvantages of the affinity precipitations that are best known are on the one hand a) steric hindrance in which the binding of the target protein is limited by the binding of high-molecular affinity macroligands, b) the resolubilisation of the precipitated polymer complex is slow, c) the non-specific co-precipitation of impurities, that generally require a second precipitation
20 step, d) the additional step of binding an affinity protein to the polymer or to the cross-linker (2-5).

BRIEF SUMMARY

25 The present disclosure relates to affinity precipitation using a binding protein with two binding sites, which dispenses completely with an additional fusion protein or a linker molecule. The affinity protein on its own is sufficient for the precipitation, and there is no need for a stationary phase. The disclosure used here also makes it easier to recover the affinity protein.

30 The disclosure relates in particular to a process for the selective concentration of immunoglobulins or other proteins that contain an Fc domain (target protein), comprising the following steps:

- a. preparing a solution that contains the target protein;

- b. incorporating an Fc-binding protein with precisely two binding sites under conditions that allow binding to occur;
- c. separating the precipitate from the liquid phase;
- d. undoing the binding of the target protein from the Fc-binding protein.

The disclosure may relate to a process in which the Fc-binding protein is a dimer of an Fc-binding domain of protein A or protein G. Preferably, the two monomers of the dimer are linked to one another via a disulphide bridge.

The disclosure may relate to a process in which the dimer is a homodimer, the monomers of which have the SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 or SEQ ID NO: 7 or a sequence that differs from SEQ ID NO. 1 in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids.

In one aspect, there is provided a process for the selective concentration of immunoglobulins or other proteins that contain an Fc domain (target protein), comprising the following steps: a. preparing a solution that contains the target protein; b. incorporating an Fc-binding protein with precisely two binding sites, wherein the Fc-binding protein is a homodimer, the monomers of which have the sequence SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 7 or a sequence that differs from SEQ ID NO. 1 in 1, 2, 3, 4, 5, 6, 7, 8, 9, 20, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids, under conditions that allow binding to occur; c. separating the precipitate from the liquid phase; d. undoing the binding of the target protein from the Fc-binding protein.

In another embodiment the Fc-binding protein is used in a ratio of 0.5 – 20 in relation to the target protein. The solution in step a. preferably has a pH of 5.5 - 8. Preferably, the undoing of the binding in step d. takes place at a pH of 2 - 4.5.

The disclosure also relates to an Fc-binding protein consisting of 2 identical sub-units that have the sequence SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 7 or a sequence that differs from SEQ ID NO. 1 in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids, the two sub-units being linked to each other via a covalent bond. Preferably, the covalent bond is a disulphide bond.

Hence, in another aspect, there is provided a Fc-binding protein that consists of 2 identical sub-units that have the sequence SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 7 or a sequence that differs from SEQ ID NO. 1 in 1, 2, 3, 4, 5, 6, 7, 8, 9, 20, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids, wherein the two-units are linked together via a disulphide bond.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: SDS-PAGE of the Z dimer. To demonstrate the dimerisation via cysteines, iodoacetamide or iodoacetamide with dithiothreitol was added before the application to the SDS-PAGE.

Trace	Sample
1	recombinant Z-dimer mixed with 0.6 μmol iodoacetamide
2	recombinant Z-dimer mixed with 0.3 μmol DTT and iodoacetamide
3	pure recombinant Z-dimer
4	Kombi marker
5	recombinant Z-dimer mixed with 0.2 μmol iodoacetamide
6	recombinant Z-dimer mixed with 0.1 μmol DTT and 0.2 μmol iodoacetamide
7	pure recombinant Z-dimer

Figure 2: Affinity precipitation using Z-dimer. As the first step, protein Z is oxidised. In the second step the Z-dimer is added to a solution containing antibody (mAB), which leads to the selective precipitation of the antibody.

Figure 3: UV diagram of ion exchange chromatography under acid conditions for separating the Z dimer from the antibody (Experiment 2). The UV chromatogram at 220 nm is shown in red, the UV chromatogram at 280 nm is shown in blue and the increasing content of buffer B (20 mM phosphate, 3 M NaCl) is shown in green.

Figure 4: SDS-PAGE for Experiment 2.

Trace	Sample
1	purified antibody
2	protein Z dimer
3	supernatant after precipitation
4	supernatant after an optional washing step
5	resuspended pellet (antibody with Z-dimer)
6	peak 1 of the ion exchange chromatography at an acid pH (antibody)
7	peak 2 of the acid ion exchange chromatography at an acid pH (Z-dimer) ¹⁰
8	Kombi marker

DETAILED DESCRIPTION OF THE INVENTION

15 The present invention relates to methods of depleting impurities, particularly host cell protein (HCP) and DNA, from protein compositions of the kind obtained from cell cultures in which proteins are expressed recombinantly or endogenously. In particular, the invention relates to methods of purifying or concentrating a protein (target protein) by binding an Fc-binding protein or multimer thereof with at least two binding sites. In further
20 steps the precipitate is separated off and then the binding of the Fc-binding protein to the target protein is removed using suitable conditions.

The present invention relates to a process for the selective concentration of immunoglobulins or other proteins that contain an Fc domain (target protein), comprising
25 the following steps:

- a. preparing a solution that contains the target protein;
- b. incorporating an Fc-binding protein with precisely two binding sites under conditions that allow binding to occur;
- c. separating the precipitate from the liquid phase;
- 30 d. undoing the binding of the target protein from the Fc-binding protein.

The target protein may be in particular an immunoglobulin or a protein that contains the Fc domain of an immunoglobulin and can bind to protein A or fragments of protein A. Immunoglobulins consist of two heavy and two light chains. The heavy chains each have

one variable and three to four constant domains depending on the immunoglobulin. These are referred to analogously as VH and CH1, CH2, CH3. The variable domains of a light and a heavy chain form the antigen binding site. The domain CH2 contains a carbohydrate chain which forms a binding site for the complement system. The CH3 domain contains the Fc-receptor binding site. Target proteins to which the process according to the invention can be applied are all proteins that have an Fc domain. Examples of proteins that contain CH2/CH3 regions are antibodies, immunoadhesins and fusion proteins in which the protein of interest is connected to a CH2/CH3 region. In one embodiment of the invention, the target protein is for example an antibody that has a CH2/CH3 region and is thus capable of binding to protein A. The term CH2/CH3 region refers to the amino acids in the Fc region of an antibody that interact with protein A.

The Fc-binding protein comprises according to the invention precisely two binding sites for one Fc domain.

In another aspect the invention relates to a process wherein the Fc-binding protein is a dimer of an Fc-binding domain of protein A or protein G. The two monomers of the dimer are preferably linked together by a disulphide bridge.

By an Fc-binding protein are meant proteins or peptides which are capable of binding to the Fc region. Preferably, Fc-binding proteins bind with a dissociation constant (K_D value) in the range from 10^{-2} - 10^{-13} M.

In preferred embodiments of the invention the Fc-binding protein is a homo- or heterodimer of Fc-binding domains which comprise or contain the sequences listed in Table 1:

SEQ ID NO:	Sequence	Description
1	MVDNKFNKEQ QNAFYELHL PNLNEEQRNA FIQSLKDDPS QSANLLAEAK KLNDAPAKS SACRRRRRRR RP	Z-domain Cys tag
2	MVDNKFNKEQ QNAFYELHL PNLNEEQRNA FIQSLKDDPS QSANLLAEAK KLNDAPAKS SAC	Z-domain Cys
3	DNKFNKEQQN AFYEILHLPN LNEEQRNAFI QSLKDDPSQS ANLLAEAKKL NDAQAPK	Z-domain
4	QQNAFYQVLN MPNLNADQRN GFQSLKDDP SQSANVLGEA QKLNDSQAPK	E domain of protein A (Swissprot P02967)
5	QNNFNKDQQS AFYEILNMPN LNEAQRNGFI QSLKDDPSQS TNVLGEAKKL NESQAPK	D domain of protein A (Swissprot P02967)
6	DNNFNKEQQN AFYEILNMPN LNEEQRNGFI QSLKDDPSQS ANLLSEAKKL NESQAPK	C domain of protein A (Swissprot P02967)
7	DNKFNKEQQN AFYEILHLPN LNEEQRNGFI QSLKDDPSQS ANLLAEAKKL NDAQAPK	B domain of protein A (Swissprot P02967)
8	DNKFNKEQQN AFYEILHLPN LTEEQRNGFI QSLKDDPSVS KEILAEAKKL NDAQAPK	A domain of protein A (Swissprot P02967)
9	TTYKLIVINGK TLKGETTTKT VDAETAEKAF KQYANDNGVD GVWTYDDATK TFTVT	protein G Fc- binding domain from Streptococcus Sp. (Uniprot Q53337)
10	TTYKLIVINGK TLKGETTTKA VDAETAEKAF KQYANDNGVD GVWTYDDATK TFTVT	protein G Fc- binding domain from Streptococcus dysgalactiae (YP_002997067)
11	TTYRLVIKGV TFSGETATKA VDAATAEQTF RQYANDNGIT GEWAYDTATK TFTVTE	IgG binding domain from Streptococcus equi (YP_002123072)

By a homodimer is meant, in this context, an Fc-binding protein that is made up of two
5 sub-units of the same sequence.

By a heterodimer is meant, in this context, an Fc-binding protein that is made up of two sub-units of different sequences, each of which has a binding site for an Fc domain. Preferably, the sub-units contain sequences that are selected from the sequences in Table 1.

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In another aspect, the invention relates to a process in which the dimer is a homodimer the monomers of which have SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO. 7 or a sequence that differs from SEQ ID NO. 1 in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids. The monomers that differ from SEQ ID NO. 1 in 1, 2,
10 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids have the property of binding Fc-domains with a K_D value in the range from 10^{-2} - 10^{-13} M. Methods for determining the K_D value are known to the skilled man.

Binding conditions are conditions in which binding to the target protein by the Fc-binding
15 proteins takes place, preferably in a pH range of pH 5.5 - 9, preferably 6 - 8.

The precipitation occurs spontaneously under binding conditions, such as those found for example in cell-free eukaryotic culture supernatant. There is no need to link the dimers according to the invention to polymers, e.g. polyethyleneglycols, in order to promote
20 precipitation with polymers.

In another aspect the Fc-binding protein is used in a molar ratio of 0.5-20 relative to the target protein.

25 The separation of the precipitate may be carried out by centrifugation and subsequent removal of the supernatant, but also by filtration techniques.

The undoing of the binding to the target protein is carried out under conditions that enable the Fc-binding protein to be separated from the target protein. Preferably, this can be
30 done by adjusting the pH to a range between pH 2 and 4.5.

In another aspect the invention relates to an Fc-binding protein that consists of 2 identical sub-units that have the sequence SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO. 7 or a sequence that differs from SEQ ID NO. 1 in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12,

13, 14, 15, 16, 17, 18, 19 or 20 amino acids, wherein the two sub-units are linked together by a covalent bond. Preferably, the covalent bond is a disulphide bond.

5 EXAMPLES

Equipment and Methods:

Preparation of the dimerised protein Z

- 10 Protein Z was obtained as a recombinant protein from E. coli. The removal of impurities was carried out after separation of the cell debris by ion exchange chromatography.

Protein sequence of the Z domain used:

- Met-Val-Asp-Asn-Lys-Phe-Asn-Lys-Glu-Gln-Gln-Asn-Ala-Phe-Tyr-Glu-Ile-Leu-His-Leu-
15 Pro-Asn-Leu-Asn-Glu-Glu-Gln-Arg-Asn-Ala-Phe-Ile-Gln-Ser-Leu-Lys-Asp-Asp-Pro-Ser-
Gln-Ser-Ala-Asn-Leu-Leu-Ala-Glu-Ala-Lys-Lys-Leu-Asn-Asp-Ala-Gln-Ala-Pro-Lys-Ser-
Ser-Ala-Cys-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Pro

- By inserting a non-native cysteine into the peptide chain of the Z domain it is possible to
20 deliberately connect two protein Z molecules via a disulphide bridge. Directly after the purification of the recombinant protein Z the latter is obtained by oxidation as a dimerised protein.

Cell-free eukaryotic culture supernatant

- 25 The cell culture supernatant of CHO cells optimised to secretory production was obtained by filtration or centrifugation after several days' culture.

SEC Analysis

The analysis of protein impurities and the antibody content of the samples from

- 30 Experiment 1 was carried out by analytical size exclusion chromatography (SEC). A TSK-GEL SW 3000 was used with the precolumn recommended by the manufacturer (TOSOH Bioscience). Analysis was carried out on a Dionex Ultimate apparatus with monitoring of the UV signal at 280 and 220 nm.

Ion exchange chromatography for separating the Z-dimer from the antibody

The ion exchange chromatography was carried out on an ÄKTA Explorer 10 apparatus (GE Healthcare) with observation of the UV absorption at 220 and 280 nm. The column material used was SP Sepharose FF (19 mL gel bed volume). After the application of the pellet resuspended at an acid pH, the column was equilibrated with 20 mM phosphate buffer at pH 3.3 and then the antibody and the Z-dimer were separated from one another over a 25 column volume long gradient. A 20 mM phosphate buffer with 3 M NaCl at pH 3.3 was used for the elution.

Protein A HPLC

Protein A HPLC on a Waters or Dionex apparatus (injector pumps and column oven W2790/5, UV detector W2489) was used to determine the antibody content of cell culture-free supernatant and purified antibody. The antibody content of the solutions was determined using the UV signal of the acidically eluting peak.

Concentration

The solutions obtained in the tests were partly concentrated for the analytical methods (Amicon Ultra, exclusion size 3 kD).

SDS-PAGE:

A 20% homogeneous SDS gel was used to test the individual fractions and supernatants. The protein bands were detected by silver staining according to Heukeshoven.

Host cell protein analysis by ELISA assay

In Experiment 3 the host cell protein analysis was carried out using an ELISA assay.

DNA analysis

The DNA analysis was carried out after single strand production by an enzymatically catalysed detecting reaction.

UF/DF

A 50 kDa Centramate Sheet PES (polyethersulphone) made by Messrs Pall with a surface area of 180 cm² was used for the UF/DF tests in Experiment 3. The UF/DF was carried out so that the antibody was initially concentrated six-fold and then diafiltered (exchanged

for 6 volumes of the buffer) before being circulated for 10 min with the retentate valve fully open. The diafiltration was repeated twice. The total UF/DF was carried out with 50 mM acetate buffer + 100 mM arginine + 150 mM NaCl pH 3.0.

5 Hydrophobic Interaction Chromatography (HIC)

The hydrophobic interaction chromatography (Experiment 3) was carried out with a 27 mL column on an ÄKTA apparatus. The column material used was Toyopearl Phenyl 650 M made by Tosoh. For this purpose, after the UF/DF, 3.5 M ammonium sulphate buffer was added to the retentate until a conductivity of 165 mS/cm was obtained. The column
10 equilibrated with buffer (50 mM acetate + 1.2 M ammonium sulphate pH 4) was charged with the retentate from the UF/DF and then a gradient was run over 40 bed volumes to the buffer 50 mM acetate, pH 4.

Experiments:

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Detecting the dimerisation of protein Z by cysteine

Detection of the dimerisation of protein Z by cysteine was carried out by SDS-PAGE analysis (Figure 1). To ensure that the working up does not induce any oxidation of the
20 protein Z, first of all the free cysteine groups were alkylated with iodoacetamide. For detecting the monomer, additionally reduction was carried out with dithiothreitol (DTT). 2.6 nmol of Z-dimer were mixed with DTT or a corresponding volume of buffer and incubated for five minutes at 95°C. Then the mixture was cooled to ambient temperature and iodoacetamide or a corresponding volume of buffer was added and the mixture was
25 incubated for a further 20 min in the dark. After five minutes' incubation with SDS-PAGE buffer the preparation was applied to the SDS-PAGE.

The SDS-PAGE analysis shows that the Z monomer is formed by the addition of the reducing agent DTT and the gel band of the Z dimer disappears.

30 Selective precipitation using a Z-dimer

The tests described below show that the selective precipitation of an antibody can be achieved with a Z-dimer (Figure 2, Experiment 1). The Z-dimer is the dimerised B-domain of protein A from *Staphylococcus aureus* linked via two non-native cysteines, which

carries a mutation compared with the wild-type (cf. Equipment and Methods). Moreover, Experiment 2 demonstrates that the pellet obtained can be put back into solution by resuspension in the acid pH range and then an antibody can be removed again from the Z-dimer by ion exchange chromatography at an acid pH.

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Experiment 1:

Different ratios of protein Z to antibody that was present in cell-free eukaryotic culture supernatant were tested. In addition, the volume of the individual batches was varied. In test 1, 0.13 μ mol of Z-dimer were incubated, with shaking, with 0.065 μ mol of IgG4 contained in cell-free eukaryotic culture supernatant in a total volume of 5.47 mL for 2 h. In test 2, 16.25 nmol of Z-dimer were incubated, with shaking, with 16.25 nmol IgG1 contained in cell-free eukaryotic culture supernatant in a total volume of 5.2 mL, again for 2 h. Then the mixture was centrifuged for 10 min at 4000 rpm and the supernatant was separated from the pellet. The antibody content of the supernatant was determined by means of the UV signal of analytical SEC and by protein A chromatography (Equipment and Methods). The content of antibody in the pellet was determined by subtracting the antibody content of the supernatant from the total content of antibody used. A precipitation of up to 99% antibody was observed (test 1: 99%/ test 2: 76%). Moreover, the culture supernatant was examined for protein impurities before and after the precipitation by analytical SEC. The chromatogram from the cell-free eukaryotic culture supernatant was compared directly with the supernatant after precipitation. Depletions of protein impurities ranging from 90-99% were observed (test 1 90%/ test 2 99%). This means that 1% or 10% of the protein impurities had been co-precipitated. Protein impurities here may be both host cell proteins and fragments or aggregates of the target protein.

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Experiment 2:

A cation exchanger (SP Sepharose FF) was used to separate the Z-dimer from the antibody. 0.2 μ mol of purified antibody were incubated with 0.2 μ mol of Z-dimer in a volume of 10.1 mL for 36 min. After subsequent centrifugation (4000 rpm, 10 min) the supernatant was removed. Then the pellet was dissolved batchwise in 10 mL phosphate buffer at pH 7.4 and centrifuged again (4000 rpm, 10 min). The pellet thus obtained was resuspended in 20 mL phosphate buffer (20 mM phosphate, pH 3.3) and purified using an

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ion exchanger. The Z-dimer could be separated from the antibody through a gradient over 25 bed volumes (Figures 3 and 4).

Experiment 3:

- 5 A multi-step antibody purification process was carried out with affinity precipitation as the capture step.

500 mg of antibody (3.3 μ mol) were used in a ratio of 1:2 to Z-dimer (6.6 μ mol) in the precipitation from cell-free culture supernatant. After one hour's incubation with gentle agitation, the entire suspension was added to a 0.22 μ m filter (Millipore) and in this way
10 the supernatant of the precipitate was separated by filtration. The precipitate was then washed with buffer (231 mL of 50 mM phosphate buffer, pH 7.4) and in the next step resuspended in the filter with acetate buffer (179 mL 50 mM acetate buffer + 100 mM arginine + 150 mM NaCl pH 3.0). To separate the Z-dimer from the antibody, UF/ DF was carried out with a 50 kDa membrane. The next step was HIC to separate off the Z-dimer
15 and further impurities.

The total yield of the antibody was 98% after the precipitation and decreased to 87% after UF/DF and to 64% after HIC. In addition to the good yields obtained with the selective precipitation of the antibody, DNA and host cell protein analysis showed that the DNA
20 could be reduced by 99% by the precipitation step followed by UF/DF and the host cell protein content could be reduced by 99.9% compared with the initial value (cell-free culture supernatant).

Literature

- 1) Labrou N, Clonis YD. (1994) *J Biotechnol.* 36(2), 95-119.
- 2) Hilbrig F, Freitag R. (2003) *J Chromatogr B Analyt Technol Biomed Life Sci.* 790(1-
5 2),79-90.
- 3) Mattiasson B, Kumar A, Galaev IYu. (1998) *J Mol Recognit.* 11(1-6),211-6.
- 4) Vaidya AA, Lele BS, Kulkarni MG, Mashelkar RA. (1999) *Biotechnol Bioeng.* 64(4),418-
25.
- 5) Garret-Flaudy F, Freitag R. (2000-2001) *Biotechnol Bioeng.* 71(3),223-34.
- 10 6) US 2006/ 0121519 A1 "Compositions and Methods for purifying and crystallizing
molecules of interest"
- 7) H. Chmiel, Bioprozesstechnik, Spektrum Akademischer Verlag, 2nd edition 2006
- 8) EP1179014B1
- 9) Patchornik, G. and Albeck A. (2005), *Bioconjugate Chem.* 16, 1310-1315.
- 15 10) Wilchek, M. and Gorecki, M. (1973), *FEBS Letters.* 31 , 1 , 149-152.
- 11) WO2009062942 KYHSE-ANDERSEN JAN [DK] "DUAL AFFINITY POLYPEPTIDES
FOR PURIFICATION"
- 12) Hoffman, Controlled release (1987) 6, 297-305.
- 13) Hoffmann (1997) Intelligent polymers. In: Park K, ed. Controlled drug delivery.
20 Washington: ACS Publications 485-98.
- 14) Haiou Yang, Patrick V. Gurgel and Ruben G. Carbonell (2009) *Journal of
Chromatography A* 1216, 910–918.
- 15) Chen and Hoffman (1990), *Biomaterials* 11 (9), 631-634.
- 16) Nilsson et al 1987. B. Nilsson, T. Moks, B. Jansson, L. Abrahmsen, A. Elmlblad, E.
25 Holmgren, C. Henrichson, T.A. Jones and M. Uhlén, A synthetic IgG-binding domain
based on staphylococcal protein A. *protein Eng.* 1 (1987), pp. 107–113
- 17) Tashiro, M., Tejero, R., Zimmerman, D.E., Celda, B., Nilsson, B., and Montelione,
G.T. 1997. High-resolution solution NMR structure of the Z domain of staphylococcal
protein A. *J. Mol. Biol.* 272: 573–590.

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PATENT CLAIMS

1. Process for the selective concentration of immunoglobulins or other proteins that contain an Fc domain (target protein), comprising the following steps:
 - a. preparing a solution that contains the target protein;
 - b. incorporating an Fc-binding protein with precisely two binding sites, wherein the Fc-binding protein is a homodimer, the monomers of which have the sequence SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 7 or a sequence that differs from SEQ ID NO. 1 in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids, under conditions that allow binding to occur;
 - c. separating the precipitate from the liquid phase;
 - d. undoing the binding of the target protein from the Fc-binding protein.
2. Process according to claim 1, characterised in that the two monomers of the dimer are linked together by a disulphide bridge
3. Process according to one of the preceding claims, characterised in that the Fc-binding protein is used in a molar ratio of 0.5-20 in relation to the target protein.
4. Process according to one of the preceding claims, characterised in that the solution in step a. has a pH of 5.5-9, preferably 6-8.
5. Process according to one of the preceding claims, characterised in that the undoing of the binding in step d. takes place at a pH of 2-4.5.

6. Fc-binding protein that consists of 2 identical sub-units that have the sequence SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO. 7 or a sequence that differs from SEQ ID NO. 1 in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids, wherein the two sub-units are linked together via a disulphide bond.