

(19) **DANMARK**

(10) **DK/EP 2427486 T3**



(12)

Oversættelse af europæisk patentskrift

Patent- og
Varemærkestyrelsen

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- (51) Int.Cl.: **C 07 K 14/765 (2006.01)**
- (45) Oversættelsen bekendtgjort den: **2015-05-26**
- (80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2015-02-25**
- (86) Europæisk ansøgning nr.: **10718977.1**
- (86) Europæisk indleveringsdag: **2010-05-07**
- (87) Den europæiske ansøgnings publiceringsdag: **2012-03-14**
- (86) International ansøgning nr.: **EP2010056262**
- (87) Internationalt publikationsnr.: **WO2010128142**
- (30) Prioritet: **2009-05-07 EP 09159642**
- (84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO SE SI SK SM TR**
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- (74) Fuldmægtig i Danmark: **Novozymes A/S Patents, Krogshøjvej 36, 2880 Bagsværd, Danmark**
- (54) Benævnelse: **FREMGANGSMÅDE TIL OPRENSNING af ALBUMIN**
- (56) Fremdragne publikationer:
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WO-A2-02/101021
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DESCRIPTION

FIELD OF THE INVENTION

[0001] The present invention relates to a method for purifying albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof. The albumin may be a serum albumin, such as human serum albumin, obtained from an animal or from a microorganism such as a yeast.

BACKGROUND OF THE INVENTION

[0002] Albumin is used to treat patients with severe burns, shock or blood loss. It is also used to supplement media used for growing higher eukaryotic cells and as an excipient for pharmacologically active compounds, many of which need to be stabilised. Albumin fusion proteins are a fusion of a protein to albumin, or to a variant or fragment thereof, and increases the half life of the protein, for example increased *in vivo* half life. At present albumin is obtained from blood products, such as serum, or produced recombinantly in microorganisms such as yeast or from transgenic plants or animals. The albumin must be purified from the production source in order to provide a product which is sufficiently pure to meet the user's needs and/or to achieve a high yield of product.

[0003] A problem with current albumin products is the purification process required. High purity can be achieved but this requires multiple chromatographic purification steps which can be time consuming and/or expensive. For example, the purification process described in WO 2000/044772 comprises a three-step process: cation exchange chromatography followed by anion exchange chromatography followed by dye-binding (affinity) chromatography. Therefore, what is required is a simpler purification process.

SUMMARY OF THE INVENTION

[0004] The invention provides a simpler purification process for albumin. Thus the invention relates to a process for purifying albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof, the process comprising:

1. (i) loading a solid matrix comprising 2-chloro-4,6-di(2'-sulphoanilino)-S-triazine bound to a solid support with an aqueous solution comprising albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof;
2. (ii) washing the matrix to remove at least some impurities; and
3. (iii) eluting the albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof from the matrix to provide a purified albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof

wherein:

the fragment is selected from domain I, domain II and domain III, as well as combinations of two of these; and

the variant comprises a sequence having at least 60% sequence identity to the sequence of HSA (SEQ ID NO: 1).

[0005] The inventors have identified that well known separation steps, previously used in purification of albumins, such as cation exchange and dye binding processes; can be replaced by a single affinity process which increases clearance of undesired proteins relative to such steps and/or increases the yield.

[0006] Throughout this specification, the term 'albumin' includes naturally occurring albumin, albumin-related proteins and variants thereof such as natural and engineered variants. Variants include polymorphisms, fragments such as domains and sub-domains, fragments and/or fusion proteins. The albumin may have at least 40, 50, 60, 70, 80, 90, 95, 96, 97, 98, 99 % similarity or identity to SEQ ID No. 1.

BRIEF DESCRIPTION OF DRAWINGS

[0007]

Figure 1. SDS-PAGE of Prosaptide albumin fusion purification, see example 2.

Lane	Sample	Load
1.	rHA	1µg
2.	Load	1/100
3.	Load	1/1000
4.	Flow Through	nt.
5.	Wash 1	nt.
6.	Wash 2	nt.
7.	Wash 3	nt.
8.	Wash 4	nt.
9.	Eluate	1/1000
10.	rHA	1µg

Figure 2 shows SDS-PAGE of T20 albumin fusion purification, see example 2

Lane	Sample	Load
1.	Load	1/100
2.	Load	1/1000
3.	Flow Through	Nt
4.	Wash 1	Nt
5.	Wash 2	Nt
6.	Wash 3	Nt
7.	Wash 4	Nt
8.	Eluate	1/100
9.	Eluate	1/1000
10.	rHA	1µg

Figure 3 shows SDS-PAGE of IL1RA Albumin fusion purification, see example 2.

Lane	Sample	Load
1.	Load	1/100
2.	Load	1/1000
3.	Flow Through	nt
4.	Wash 1	nt
5.	Wash 2	nt
6.	Wash 3	nt
7.	Wash 4	nt
8.	Eluate	1/100
9.	Eluate	1/1000
10.	rHA	1µg

Figure 4 shows SDS-PAGE of AlbuPure™ Purified Animal Albumins, see example 8:

Lane	Sample	Load
1	SeeBlue®Plus2 MW standard	5µL

Lane	Sample	Load
2	Rabbit Serum Albumin	2µg
3	Mouse Serum Albumin	2µg
4	Rat Serum Albumin	2µg
5	Dog Serum Albumin	2µg
6	Human Serum Albumin	2µg
7	SeeBlue®Plus2 MW standard	5µL

Figure 5 shows. SDS-PAGE of AlbuPure™ Purified Albumin Fragments, see example 8:

Lane	Sample	Load
1	HSA	1µg
2	Human Serum Albumin Domain 1+2	1µg
3	Human Serum Albumin Domain 2+3	1µg
4	Human Serum Albumin Domain 1+3	1µg
5	Human Serum Albumin Domain 3	1µg
6	SeeBlue® Plus2 MW standard	5µL

DETAILED DESCRIPTION OF THE INVENTION

[0008] The present invention relates to a process for purifying albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof. The term purifying is in this application and claims intended to mean a procedure by which at least one undesired compound, such as cell debris, other plasma or host cell proteins, salts, lipids carbohydrates etc. is reduced relative to the desired compound. Depending on the starting material for the process; the undesired compound is completely or partially removed from the desired compound, albumin, a variant or fragment thereof or a fusion protein comprising albumin, a variant or fragment thereof.

[0009] Albumin is known in the area as the most abundant protein of plasma and it has been described and characterized from a large number of mammals and birds, where it is believed to have a role in keeping the correct osmotic pressure and it also has a role in transport of various compounds in the blood stream.

[0010] The process of the invention may in principle be used for purifying any known albumin such as albumin derived from human beings, dog, sheep, goat, bovine, cow, donkey, rabbit, mouse, rat, hamster, guinea pig and chicken. A preferred albumin is human serum albumin in particular human serum albumin having the sequence disclosed in SEQ ID NO: 1.

[0011] Variants of albumin is according to the invention intended to mean compounds having the overall structure of albumin but which has been altered in at least one amino acid residue compared with the parent albumin. In this connection the parent albumin is understood as the natural not altered albumin compound. The variant may differ in more than one position from the parent albumin, and in principle there is no well defined upper limit for the number of alterations, including substitutions, deletions or insertions of amino acid residues as well as chemical modifications; as long as the variant maintains the overall structure of albumin. In a preferred embodiment the albumin variant comprises one alteration, preferably at least 2 alterations, more preferred at least 5 alterations, more preferred at least 10 alterations, even more preferred at least 20 alterations and most preferred at least 25 alterations compared with the parent albumin.

[0012] The variant albumin has preferably at least 60% sequence identity to the parent albumin, preferably at least 70% sequence identity, more preferred at least 80% sequence identity, even more preferred at least 90% sequence identity, even more preferred at least 95% sequence identity and most preferred at least 98% sequence identity to the parent albumin.

[0013] In a preferred embodiment the albumin, fragment or variant thereof has at least 40, 50, 60, 70, 80, 90, 95, 96, 97, 98, 99 % sequence identity to SEQ ID No. 1.

[0014] Identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the

parameter "identity".

[0015] For purposes of the present invention, the degree of identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, J. Mol. Biol. 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, Trends in Genetics 16: 276-277), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:
(Identical Residues x 100)/(Length of Alignment – Total Number of Gaps in Alignment).

[0016] In describing the various variants of the present invention, the nomenclature described below is adapted for ease of reference. In all cases, the accepted IUPAC single letter or triple letter amino acid abbreviation is employed.

[0017] Substitutions. For an amino acid substitution, the following nomenclature is used: Original amino acid, position, substituted amino acid. Accordingly, the substitution of threonine with alanine at position 226 is designated as "Thr226Ala" or "T226A". Multiple mutations are separated by addition marks ("+"), e.g., "Gly205Arg + Ser411 Phe" or "G205R + S411 F", representing mutations at positions 205 and 411 substituting glycine (G) with arginine (R), and serine (S) with phenylalanine (F), respectively.

[0018] Deletions. For an amino acid deletion, the following nomenclature is used: Original amino acid, position*. Accordingly, the deletion of glycine at position 195 is designated as "Gly195*" or "G195*". Multiple deletions are separated by addition marks ("+"), e.g., "Gly195* + Ser411*" or "G195* + S411*".

[0019] Insertions. For an amino acid insertion, the following nomenclature is used: Original amino acid, position, original amino acid, new inserted amino acid. Accordingly the insertion of lysine after glycine at position 195 is designated "Gly195GlyLys" or "G195GK". Multiple insertions of amino acids are designated [Original amino acid, position, original amino acid, new inserted amino acid #1, new inserted amino acid #2; etc.]. For example, the insertion of lysine and alanine after glycine at position 195 is indicated as "Gly195GlyLysAla" or "G195GKA".

[0020] In such cases the inserted amino acid residue(s) are numbered by the addition of lower case letters to the position number of the amino acid residue preceding the inserted amino acid residue(s). In the above example the sequences would thus be:

Parent:	Variant:
195	195 195a 195b
G	G - K - A

[0021] In a preferred embodiment the variant albumin is derived from human serum albumin having the sequence shown in SEQ ID NO: 1, by substitution, deletion or insertion of at least 1 amino acid residue, preferably at least 2 amino acid residues, more preferred at least 5 amino acid residues, even more preferred at least 10 amino acid residues.

[0022] In another preferred embodiment the variant albumin is made of hybrid albumin comprising a part derived from human serum albumin and a part derived from another albumin.

[0023] As examples of variant albumins can be mentioned the natural variant having lower plasma half-life disclosed in (Biochim BiophysActa. 1991, 1097:49-54) having the substitution D494N.

[0024] Preferred albumin variants includes variants generated in albumin in order to provide a reactive thiol group on the surface such as the variants of SEQ ID NO: 1: L585C, D1C, A2C, D562C, A364C, A504C, E505C, T79C, E86C, D129C, D549C, A581C, D121C, E82C, S270C, A578C, L595LC, D1 DC, A2AC, D562DC, A364AC, A504AC, E505EC, T79TC, E86EC, D129DC, D549DC, A581AC, A581AC, D121DC, E82EC, S270SC, A579AC, C360*, C316*, C75*, C168*, C558*, C361*, C91*, C124*, C169* and C567*, described in the unpublished PCT application PCT/EP2010/051751, included herein by reference; and the variants of SEQ ID NO: 1 altered in positions 492, 493, 494, 495, and 496, disclosed in the unpublished EP patent application EP09174698.2, included herein by reference.

[0025] A fragment of albumin is intended to be understood as a molecule comprising part of an albumin molecule but wherein at

least another part of the albumin molecule is absent. The fragment may comprise the N- or C-terminal part of albumin or an internal part of albumin and it may even be composed of two or more albumin fragments that are not directly connected in the natural albumin molecule. As example of preferred albumin fragments are the domain I, domain II and domain III; as well as combinations of two of these. The term fragments of albumin encompass also fragments of albumin variants.

[0026] A fragment of albumin may even comprise one or more parts of one albumin and one or more parts of one or more different albumins, e.g. a part of human serum albumin and another part of rabbit serum albumin.

[0027] A fragment of albumin comprises at least of 10 amino acid residues, preferably at least 25 amino acid residues, more preferred at least 50 amino acid residues, even more preferred at least 100 amino acid residues, more preferred at least 200 amino acid residues, more preferred at least 300 amino acid residues, more preferred at least 400 amino acid residues, and most preferred at least 500 amino acid residues.

[0028] The term 'fusion protein' comprising albumin, a variant or fragment thereof is according to the invention intended to mean a polypeptide comprising sequences of albumin, a variant or fragment thereof and further one or more sequences that are distinct from albumin, a variant or fragment thereof. The non-albumin part(s) of the fusion protein may in principle be any polypeptide, however, it is preferred that it has a medical use.

[0029] The fusion protein may or may not have a linker sequence between the albumin part(s) and the non-albumin part(s), which linker may contain a cleavage site for a specific protease.

[0030] In a preferred embodiment the non-albumin part of the fusion protein is a polypeptide having a medical use, e.g. a polypeptide having a therapeutic use.

[0031] As examples of fusion proteins can be mentioned the albumin fusion polypeptides disclosed in the WO 9315199, WO 01079271 and WO 03059934.

[0032] A conjugate comprising albumin, a variant or fragment thereof is according to the invention intended to mean a compound prepared by chemically attaching one or more conjugation partners to comprising albumin, a variant or fragment thereof. The conjugation partner may be a bioactive compound such as a therapeutic or diagnostic compound. The therapeutic compound may be a chemotherapy drug for use in cancer chemotherapy. It may be cytostatic or cytotoxic; it may be a tumor-inhibiting agent.

[0033] The conjugation partner may be attached to the albumin, a variant or fragment thereof using methods known as such in the art.

[0034] Examples of suitable conjugation partners and methods for joining the conjugation partner and the albumin, a variant or fragment thereof can be found in WO 2009019314 and in the unpublished EP patent application EP09174698.2.

[0035] The first step in the claimed method is providing a solid matrix comprising 2-chloro-4,6-di(2'-sulphoanilino)-S-triazine bound to a solid support. The solid matrix comprising 2-chloro-4,6-di(2'-sulphoanilino)-S-triazine bound to a solid support has the ability to specifically bind albumin. Such matrices having the ability to bind a specific protein with a higher affinity than other compounds are known in the art.

[0036] The albumin specific ligand may in principle be any ligand having a high affinity to albumin. A ligand specific for albumin may in principle be found by binding candidate ligands to a solid support, testing the affinity by contacting the candidate ligands with an albumin solution, rinsing the ligands with an albumin free solution and subsequent evaluating the candidate ligands ability to bind albumin by the amount of albumin attached to the candidate ligands after the rinse.

[0037] In the present invention, the albumin specific ligand is 2-chloro-4,6-di-(2'-sulphoanilino)-S-triazine.

[0038] The solid support may in principle be any solid material that is inert under the contemplated conditions. By inert it is meant that the solid support does not take part or only in an insignificant degree take part in chemical interactions with components of the aqueous solution comprising albumin, the washing solutions and the elution solution.

[0039] Examples of solid supports that may be used according to the invention includes polymers such as cellulose or agarose and derivatives thereof, polyethylene, polystyrene, polyacrylate, and silicates.

[0040] Such solid supports and methods for binding the albumin specific ligand to the solid support are known in the art, and the

skilled person will appreciate how to apply such teachings to the present invention. As an example of a preferred method for binding the albumin specific ligand to the solid support can be found in WO 97/10887. (A particular preferred solid matrix comprising the albumin binding ligand 2-chloro-4,6-di-(2'-sulphoanilino)-S-triazine bound to a solid support is the solid matrix available under the tradename 'Albupure TM' from ProMetic BioSciences Ltd, Cambridge, UK.

[0041] The solid matrix comprising the albumin binding ligand is usually packed as a fixed bed in a cartridge, a column or other form of container wherein the solid material can be packed and the aqueous solution comprising the albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof, washing and elution solutions can flow through the fixed bed comprising the solid matrix, however, other well known techniques such as mixing the solid matrix with the appropriate solution in a container followed by a separation step e.g. filtration or centrifugation; and fluid bed technologies may also be applied to the process of the invention. Techniques for providing affinity materials such as the solid matrix comprising an albumin specific ligand is known in the art and it will be within the skills of the average practitioner to select and apply a suitable technique to the present invention.

[0042] The aqueous solution comprising albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof may in principle be any such solution. Examples of suitable aqueous solutions comprising albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof includes: plasma or fractions thereof, culture supernatants from a cell culture of a cell capable of producing such protein, milk of transgenic animals capable of producing such proteins and extracts of transgenic plants capable of producing such proteins. The aqueous solution comprising albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof may be the medium wherein the protein is produced, e.g. a cell culture supernatant, or it may be a partially purified fraction thereof.

[0043] The pH of the aqueous solution comprising albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof to be contacted with the solid matrix should have a pH in the range of 4-9.5 preferably, 4-9, more preferred 4-8, and most preferred 4.5-8. If the aqueous solution does not have such a pH by itself it is generally necessary to adjust the pH using well known pH regulating compounds e.g. sodium hydroxide or potassium hydroxide for increasing the pH and hydrochloric acid, sulphuric acid or acetic acid for reducing the pH.

[0044] The aqueous solution comprising albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof is loaded on to the solid matrix comprising an albumin binding ligand using well known techniques. The skilled person will appreciate that loading conditions may be optimized taking into consideration factors such as loading amount, loading rate, flow velocity, contact time etc., and such optimization is well within the skills of the skilled person.

[0045] During the loading the aqueous solution comprising albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof to the solid matrix the albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof will bind to the albumin binding ligand connected to the solid matrix.

[0046] After loading the solid matrix comprising an albumin binding ligand, now bound to albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof is washed to remove impurities.

[0047] In this connection impurities are intended to mean compounds other than albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof contained in the aqueous solution comprising albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof. The impurities may be compounds such as proteins, carbohydrates, lipids, small organic molecules, salts, chemical reagents etc., and usually a mixture of various different impurities will be present.

[0048] The matrix is washed with aqueous solutions generally composed of water, inorganic salts and buffer systems. Additional compounds such as surfactants, preservatives chelators may also be present. Generally washing solutions are known in the art and can be used according to the invention. In case that the solid matrix is placed in a fixed bed the washing steps are typically performed by perfusing the fixed bed with washing solution in a volume corresponding to at least 1 volume of the fixed bed,

preferably at least 2 volumes, more preferred at least 3 volumes and most preferred at least 5 volumes of the fixed bed.

[0049] The washing solution may have a pH in the range of 4-9.5, more preferred 4-9, even more preferred 4-8, and most preferred 5-8.

[0050] The wash may be performed in one or more steps where the solid matrix in each step is washed with a washing solution.

[0051] It is preferred to use two or more washing steps, that may be performed using washing solutions having same pH or by using washing solutions having different pHs. In one preferred embodiment the pHs of the two or more washing steps are different and the pH of the two or more washing steps are consecutively rising or are consecutively declining, preferably the pH of the two or more washing steps are consecutively rising.

[0052] In the embodiment of two or more washing steps of consecutively rising pH the pH of the first washing step is preferably in the range of pH 4.0 - 8.0, preferably in the range of pH 4.0 - 7.0 more preferred in the range of pH 4.0 - 6.0. The last washing step is preferably in the range of pH 7.0-9.5, preferably in the range of pH 8.0-9.5 and most preferred in the range of pH 8.5-9.5. Preferably there is at least one further step between the first and the last step such as 1, 2, 3, 4 or 5 steps between the first and the last step.

[0053] After the washing steps the albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof is eluted from the solid matrix using an elution solution comprising a salt of a fatty acid, a thiocyanate salt, or by using a elution solution having a pH at 10.0 or higher or any combination thereof.

[0054] The salt of a fatty acid preferably a salt of a fatty acid having a solubility in the elution solution at the elution conditions of at least 10 mM, preferably at least 20 mM more preferred at least 50 mM, even more preferred at least 100 mM and most preferred at least 500 mM. As examples of suitable salts of fatty acids can be mentioned sodium and potassium salts of acetate, propanoate, butyrate, pentanoate, hexanoate, heptanoate, octanoate, nonanoate and decanoate.

[0055] The concentration of the salt of the fatty acid in the elution solution is preferably in the range of 5mM to 0.5 M, preferably in the range of 10 mM to 100 mM.

[0056] In principle any thiocyanate may be used according to the invention, however, it is preferred to use a thiocyanate salt having a solubility in the elution solution at the elution conditions of at least 10 mM, preferably at least 20 mM more preferred at least 50 mM, even more preferred at least 100 mM, even more preferred at least 500 mM and most preferred at least 1 M. Further it is preferred to use a thiocyanate salt have a cation that is acceptable in the final product. Suitable examples of thiocyanate salts that may be used according to the invention includes: sodium thiocyanate and potassium thiocyanate. The thiocyanate salt is preferably used in the elution solution in a concentration in the range of 5 mM to 1 M, preferably in the range of 10 mM to 500 mM.

[0057] The elution solution may further comprise buffer systems, salts and preserving agents, preferably of pharmaceutical grade. It will be within the skills of the average practitioner to compose the elution solution based on the teachings herein and the art of affinity matrices.

[0058] The elution is generally done in a small volume in order to obtain the albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof in a high concentration, but on the other side it is desirable to use a larger volume in order to obtain a larger yield. The skilled person will therefore have to optimize the elution with respect to volume, flow rate etc., using well known methods for optimization.

[0059] According to the process of the invention a recovery of the albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate comprising albumin, a variant or fragment thereof is high, generally more than 25%, preferably more than 40%, more preferred more than 50%, more preferred more than 60%, even more preferred more than 70%, even more preferred more than 80% and most preferred more than 90%.

[0060] The process of the invention may be performed as a part of a longer purification procedure involving one or more additional purification steps before and/or after the process of the invention or it may be performed without additional steps. Generally, in particular where the solid matrix is provided in a fixed bed, it is preferred that a separation step is performed before the process of the invention in order to remove particulate material and/or lipid micelles from the aqueous solution comprising the albumin, a variant or fragment thereof, a fusion protein comprising albumin, a variant or fragment thereof, or a conjugate

comprising albumin, a variant or fragment thereof, which particulate material and/or micelles otherwise might clog the fixed bed and deteriorate the flow properties of the bed.

[0061] The invention is now further described in the following examples which should not be considered limiting in any way.

EXAMPLES

Materials:

[0062] Solid matrix: A solid matrix comprising the ligand 2-chloro-4,6-di-(2'-sulphoanilino)-S-triazine was used. This solid matrix is available under the tradename 'AlbuPure™' from ProMetic BioSciences Ltd, Cambridge, UK

[0063] Recombinant human serum albumin (rHSA): The culture supernatant of a fermentation of a recombinant yeast comprising an expression plasmid encoding human serum albumin was used as a source of rHSA. Generation of the expression plasmid, transforming the yeast strain and fermentation conditions are essentially as disclosed in WO 00/44772 example 1.

Example 1: Comparison of rHSA 2 step AlbuPure™ purification and 3 step conventional purification

[0064] The conventional 3 step purification of cation ion exchange, anion exchange and dye affinity chromatography was performed as described in WO 2000/044772.

[0065] The 2 step AlbuPure™ purification was performed by conditioned the rHSA fermentation supernatant for chromatography using acetic acid and water to achieve a pH of approximately 5.3 and a conductivity of approximately 3mS.cm⁻¹. A column packed with AlbuPure™ matrix was equilibrated with 50mM sodium acetate pH 5.3. A volume of conditioned sample equivalent to 20mg rHSA.mL⁻¹ matrix was loaded. The matrix was washed sequentially with 50mM sodium acetate pH 5.3, 50mM sodium phosphate pH 6.0, 50mM sodium phosphate pH 7.0 and 50mM ammonium acetate pH 8.0 before being eluted with 50mM ammonium acetate, 10mM sodium octanoate pH 7.0 and finally regenerated with 0.5M sodium hydroxide. The AlbuPure™ eluate was conditioned for anion ion exchange chromatography by diluting with water to 2.5mS/cm and adjusting the pH with acetic acid to pH 5.5. The anion exchange chromatography was performed using DE-FF Sepharose as described in WO 2000/044772. The albumin yield was calculated from the concentration measured by GP-HPLC and related back to the total amount at the start. The host cell proteins (HCP) were measured by sandwich ELISA using anti yeast antibodies and measured as a fold clearance relative to the starting material.

Table 1:

Purification Step	3 Step Process (WO 2000/044772)		2 Step Process (AlbuPure™ & DE-FF)	
	Total rHA Yield (%)	HCP Clearance (Fold)	Total rHA Yield (%)	HCP Clearance (Fold)
Start	100		100	
Step 1 Eluate	49	104	70	1394
Step 2 Eluate	53	728	60	95619
Step 3 Eluate	42	99736		

[0066] Consequently, the 2 step process gives 18% more yield (product) while maintaining approximately equivalent HCP levels (within the limits of the assay).

Example 2: rHSA fusions

[0067] Yeast derived culture supernatant containing c-terminal rHSA fusions of prosaptide, T20 (PCT/IB03/00434) and IL1RA were conditioned for chromatography using acetic acid and water to achieve a pH of approximately 5.3 and a conductivity of between 2.6 and 3.3mS.cm⁻¹. A 1.6cm × 11.0cm (22.1 mL) column packed with AlbuPure™ matrix was equilibrated with 50mM

sodium acetate pH 5.3. A volume of conditioned sample equivalent to 20mg fusion protein.mL⁻¹ matrix was loaded. The matrix was washed sequentially with 50mM sodium acetate pH 5.3, 50mM sodium phosphate pH 6.0, 50mM sodium phosphate pH 7.0 and 50mM ammonium acetate pH 8.0 before being eluted with 50mM ammonium acetate, 10mM sodium octanoate pH 7.0 and finally regenerated with 0.5M sodium hydroxide. SDS_PAGE of Prosaptide albumin fusion purification is shown in figure 1.. SDS-PAGE of T20 albumin fusion purification is shown in figure 2. SDS-PAGE of IL1RA Albumin fusion purification is shown in figure 3.

Example 3: anti FITC (scFv(vHvL)-rHSA-FLAG) antibody

[0068] Yeast derived culture supernatant containing the anti FITC (scFv(vHvL)-rHSA-FLAG) (disclosed in EP application No 09 159 642) antibody fusion (N-terminal fusion) was conditioned for chromatography using water to achieve a pH of 6.2 and a conductivity of 7.9mS.cm⁻¹. A 4.4cm × 11.0cm (167.3mL) column packed with AlbuPure™ matrix was equilibrated with 50mM sodium phosphate pH 6.0. A volume of conditioned sample equivalent to 9.5mg protein.mL⁻¹ matrix was loaded. The matrix was washed sequentially with 50mM sodium phosphate pH 6.0, 50mM sodium phosphate pH 7.0 and 50mM ammonium acetate pH 8.0. Bound protein was eluted first with 50mM ammonium acetate, 10mM sodium octanoate pH 7.0 and then with 50mM ammonium acetate, 30mM sodium octanoate, 200mM sodium chloride pH7.0. The matrix was regenerated with 0.5M sodium hydroxide. A total of 84% of the anti FITC (scFv(vHvL)-rHSA-FLAG) was recovered in the 2 eluates combined, as estimated by GP.HPLC.

Example 4: anti AMA (vNAR-rHSA-FLAG) antibody fusion

[0069] Yeast derived culture supernatant containing the anti AMA (vNAR-rHSA-FLAG) antibody fusion (N-terminal fusion) was purified without adjustment at a pH of 5.8 and a conductivity of 47.0mS.cm⁻¹. A 2.6cm × 11.0cm (58.4mL) column packed with AlbuPure™ matrix was equilibrated with 50mM sodium phosphate pH 6.0 and a volume of sample equivalent to 14.25mg protein.mL⁻¹ matrix loaded. The matrix was washed first with 50mM sodium phosphate pH 6.0 then 50mM ammonium acetate pH 8.0. Bound protein was eluted first with 50mM ammonium acetate, 10mM sodium octanoate pH 7.0, then with 50mM ammonium acetate, 30mM sodium octanoate, 200mM sodium chloride pH7.0 and finally with phosphate buffered saline containing 1 M potassium thiocyanate pH 8.6. The matrix was regenerated with 0.5M sodium hydroxide. A total of 53% of the anti AMA (vNAR-rHSA-FLAG) was recovered in the 3 eluates combined, as estimated by GP.HPLC.

Example 5: Effect of loading the AlbuPure matrix in the pH range 4.0-10.0

[0070] The effect on step recovery and matrix capacity of loading at pH 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0 was investigated. Load material was prepared by diluting partially purified N-terminal anti-FITC scFv-rHA fusion to 10mg.mL⁻¹ with a buffer of the appropriate pH increment. The pH of each load sample was then checked and adjusted using sodium hydroxide or glacial acetic acid as necessary. Atoll 5mm dia. x 10mm bed height centrifugally driven Medi-aScout® MiniColumns containing 200µL AlbuPure™ matrix (ProMetic Biosciences) were equilibrated with 1 mL 50mM sodium acetate pH 5.0. Sufficient load material (1 mL) was applied to each column to achieve a 50mg fusion protein.mL⁻¹ matrix loading. Each column was washed first with 1 mL 50mM sodium acetate pH 5.0 (wash 1) and then 1 mL 50mM ammonium acetate pH 8.0 (wash 2). Each column was then eluted with 1mL 50mM ammonium acetate, 50mM sodium octanoate pH 8.0. All chromatographic fractions were collected and the scFv-rHA fusion concentration estimated by gel permeation high performance liquid chromatography (GP.HPLC). Recoveries and capacities were tabulated and are shown in Table 2. The recovery in this instance is calculated from the total amount bound to the column (amount in load minus amount in flow through) rather than the amount loaded to give a more representative result in this experiment. The capacity was calculated from the amount bound (amount in load minus amount in flow through) divided by the matrix volume (200µL).

Table 2: The effect of load pH on scFv-rHA fusion recovery and AlbuPure matrix capacity

Load conditions	Recovery (%)					Capacity (mg/mL matrix)
	Flow through	Wash 1	Wash 2	Eluate	Mass Balance	
pH 4.0	5.3	1.1	14.3	89.3	105.2	52.0
pH 5.0	11.2	1.2	10.9	86.6	100.1	49.5
pH 6.0	18.6	3.6	2.8	92.4	100.2	45.8
pH 6.5	25.3	7.1	1.0	89.8	100.5	41.5

Load conditions	Recovery (%)					Capacity (mg/mL matrix)
	Flow through	Wash 1	Wash 2	Eluate	Mass Balance	
pH 7.0	26.4	8.3	0.3	88.8	100.3	41.3
pH 7.5	30.5	12.5	0.2	86.8	103.4	38.8
pH 8.0	32.7	10.8	0.1	73.4	93	38.3
pH 8.5	37.1	12.7	0.1	75.7	97.5	35.0
pH 9.0	43.0	15.9	0.0	69.5	98.5	31.7
pH 9.5	45.6	17.1	0.0	58.3	94.4	30.0
pH 10.0	72.1	18.7	0.0	31.8	99.7	15.9

[0071] Data indicates that a range of 4.0-9.5 for loading pH is suitable.

Example 6: Effect of different washing pH combinations

[0072] The effect on step recovery and yeast antigen (YA) clearance of washing with combinations of buffers at different pH increments was investigated. Load material was prepared by thawing and filtering (0.8µm) frozen N-terminal anti-FITC scFv-rHA fusion fermentation culture supernatant. No further conditioning was performed. Atoll 5mm dia. x 10mm bed height centrifugally driven MediaScout® MiniColumns containing 200µL AlbuPure™ matrix (ProMetic Biosciences) were equilibrated with 1 mL 50mM sodium acetate pH 5.0. Sufficient load material (1 mL) was applied to each column to achieve a 20mg fusion protein.mL⁻¹ matrix loading. Each column was washed as shown in Table 2. Each column was then eluted with 1mL 50mM ammonium acetate, 50mM sodium octanoate pH 8.0. All chromatographic fractions were collected and the scFv-rHA fusion concentration estimated by gel permeation high performance liquid chromatography (GP.HPLC). YA levels were estimated by enzyme linked immunosorbent assay (ELISA). Recoveries and YA clearance were tabulated and are shown in Table 3. The YA clearances are normalized back to the full wash regime (pH 5, 6, 7 & 8), as used in Example 1.

Table 3: The effect of different combinations of washing pH on scFv-rHA fusion recovery

Wash conditions	Recovery (%)							YA Clearance
	Flow through	Wash 1	Wash 2	Wash 3	Wash 4	Eluate	Mass Balance	
pH 5, 6, 7 & 8	5.2	0.7	0.2	6.0	4.5	87.9	104.5	1.00
pH 5 & 4	9.0	1.0	0.2	N/A	N/A	51.0	61.2	1.36
pH 5 & 5	9.8	1.0	0.2	N/A	N/A	84.3	95.2	0.40
pH 5 & 6	11.0	1.4	0.5	N/A	N/A	88.1	101.0	0.15
pH 5 & 7	7.6	1.0	6.0	N/A	N/A	87.6	102.1	0.40
pH 5 & 8	5.2	0.7	7.1	N/A	N/A	91.0	104.0	0.33
pH 5 & 9	8.1	1.0	32.9	N/A	N/A	63.8	105.7	0.78
pH 5 & 10	11.0	1.0	76.9	N/A	N/A	13.1	101.9	0.67
pH 8, 7, 6 & 5	10.2	10.	2.4	0.0	0.0	81.0	103.6	3.94
pH 4, 7, & 10	10.5	1.0	5.7	66.4	N/A	18.6	102.1	1.29
pH 10, 7, & 4	10.0	89.5	1.4	0.0	N/A	0.0	101.0	N/A
pH 8 & 5	10.0	10.5	2.1	N/A	N/A	73.1	95.7	2.00
pH 4 & 10	9.5	0.1	82.9	N/A	N/A	11.2	103.7	0.76
pH 10 & 4	12.4	87.4	1.7	N/A	N/A	0.0	101.4	N/A
pH 4	9.5	1.0	N/A	N/A	N/A	41.4	51.9	0.37
pH 8	10.2	11.0	N/A	N/A	N/A	88.3	109.5	0.28

[0073] Data indicates that wash conditions between pH 5 and pH 9, to be performed in any order to give recoveries and/or yeast antigen (HCP) clearances better than cation exchanges as per Example 1. At pH 10 material is prematurely eluted, as shown

below pH 4 also works but care is required with the elution if pH 4 is the last wash prior to elution.

Wash conditions	Recovery (%)						
	Flow through	Wash 1	Wash 2	Wash 3	Wash 4	Eluate	Mass Balance
pH 4 & 8	3.0	0.8	8.6	N/A	N/A	82.1	94.5

Example 7: Effect of different elution conditions

[0074] The effect on step recovery for various eluents was investigated. Load material was prepared by diluting partially purified N-terminal anti-FITC scFv-rHA fusion to 4mg.mL⁻¹ with 50mM sodium acetate pH 5.0. Atoll 5mm dia. × 10mm bed height centrifugally driven MediaScout® MiniColumns containing 200µL AlbuPure™ matrix (ProMetic Biosciences) were equilibrated with 1 mL 50mM sodium acetate pH 5.0. Sufficient load material (1 mL) was applied to each column to achieve a 20mg fusion protein.mL⁻¹ matrix loading. Each column was washed first with 1 mL 50mM sodium acetate pH 5.0 (wash 1) and then 1 mL 50mM ammonium acetate pH 8.0 (wash 2). Each column was then eluted with the appropriate buffer (Table 3). All chromatographic fractions were collected and the scFv-rHA fusion concentration estimated by gel permeation high performance liquid chromatography (GP.HPLC). Recoveries were tabulated and are shown in Table 4.

Table 4: The effect of different eluents on scFv-rHA fusion recovery

Elution conditions	Recovery (%)				
	Flow through	Wash 1	Wash 2	Eluate	Mass Balance
50mM ammonium acetate, 50mM sodium octanoate pH 6.0	0.5	0.0	6.0	84.2	90.6
50mM ammonium acetate, 50mM sodium octanoate pH 7.0	2.2	0.0	7.2	88.2	97.6
50mM ammonium acetate, 50mM sodium octanoate pH 8.0	2.6	0.2	6.5	90.4	99.8
2M sodium octanoate pH 8.2	4.8	0.5	7.4	25.7	38.4
100mM sodium octanoate pH 8.0	0.2	0.0	5.0	95.4	100.7
0.5M octanoate pH 10	0.0	0.0	4.0	56.2	60.1
1M octanoate pH 10	0.0	0.0	6.4	26.2	36.3
50mM ammonium acetate 500mM sodium propionate pH 8.0				26	
50mM ammonium acetate 500mM sodium butyrate pH 8.0				56	
50mM ammonium acetate 500mM sodium hexanoate pH 8.0				86	
PBS, 0.5M potassium thiocyanate pH 7.0	0.5	0.0	5.3	104.6	110.3
PBS, 1M potassium thiocyanate pH 7.0	0.2	0.0	6.2	93.0	99.5
PBS, 0.5M sodium thiocyanate pH 7.0	1.9	0.2	7.2	86.1	95.4
PBS, 1M sodium thiocyanate pH 7.0	0.5	0.0	6.0	94.0	100.5
50mM sodium carbonate pH 10	0.0	0.0	2.0	66.3	68.3
50mM glycine pH 10	0.0	0.0	2.9	58.6	61.5
50mM sodium phosphate pH 10	0.0	0.0	2.6	62.6	65.2
50mM ammonium acetate pH 10.0	2.4	0.2	6.7	71.0	80.3
50 mM potassium tetraborate pH 10.0	0.5	0.0	6.5	28.1	35.0

Example 8: Albumin fragments and Animal albumins

[0075] Animal albumins and albumin fragments were purified from shake flask culture supernatant using a single chromatographic step using AlbuPure™ matrix. Culture supernatant (350mL) was applied to a 6cm bed height, 2.0mL packed bed equilibrated with 50mM sodium acetate pH5.3. Following loading, the column was washed with 50mM sodium acetate pH5.3 then 50mM ammonium acetate pH8.0. Product was eluted with either 50mM ammonium acetate 10mM octanoate pH8.0, 50mM Ammonium Acetate 30mM Sodium Octanoate 200mM Sodium Chloride pH7.0 or 200mM Potassium thiocyanate. The column cleaned with 0.5M NaOH. SDS-PAGE of AlbuPure™ Purified Animal Albumins: is shown in figure 4.

SEQUENCE LISTING

[0076]

<110> Novozymes Biopharma UK Ltd.

<120> Purification Process

<130> 11732.204-WO

<160> 1

<170> PatentIn version 3.3

<210> 1

<211> 585

<212> PRT

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<400> 1

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Gln Cys Pro Phe Glu Asp His Val Lys Leu Val Asn Glu Val Thr Glu
35     40     45

Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu Asn Cys Asp Lys
50     55     60

Ser Leu His Thr Leu Phe Gly Asp Lys Leu Cys Thr Val Ala Thr Leu
65     70     75     80

Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro
85     90     95

Glu Arg Asn Glu Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn Leu
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115    120    125

Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg
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Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg
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Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser
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REFERENCES CITED IN THE DESCRIPTION

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- [WO9710887A \[0040\]](#)
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- Biochim BiophysActa, 1991, vol. 1097, 49-54 [\[0023\]](#)

PATENTKRAV

1. Fremgangsmåde til oprensning af albumin, en variant eller et fragment deraf, et fusionsprotein, som omfatter albumin, en variant eller et fragment deraf, eller et konjugat, som omfatter albumin, en variant eller et fragment deraf, hvilken fremgangsmåde omfatter:

5 (i) fyldning af en fast matrix, som omfatter 2-chlor-4,6-di(2'-sulfoanilino)-S-triazin bundet til en fast bærer, med en vandig opløsning, som omfatter albumin, en variant eller et fragment deraf, et fusionsprotein, som omfatter albumin, en variant eller et fragment deraf, eller et konjugat, som omfatter albumin, en variant eller et fragment deraf;

(ii) vask af matrixen for at fjerne mindst nogle urenheder; og

10 (iii) eluering af albuminet, et albumin, en variant eller et fragment deraf, et fusionsprotein, som omfatter albumin, en variant eller et fragment deraf, eller et konjugat, som omfatter albumin, en variant eller et fragment deraf, fra matrixen for at tilvejebringe et oprenset albumin, en variant eller et fragment deraf, et fusionsprotein, som omfatter albumin, en variant eller et fragment deraf, eller et konjugat, som omfatter albumin, en variant eller et fragment deraf;

15

hvor:

fragmentet er valgt blandt domæne I, domæne II og domæne III samt kombinationer af to af disse; og

varianten omfatter en sekvens, som udviser mindst 60% sekvensidentitet med sekvensen for HSA (SEQ ID NO: 1).

20

2. Fremgangsmåde ifølge krav 1, hvor albuminet stammer fra et pattedyr, såsom et menneske, en kanin, en mus, en ged, et får, en ko; eller fra fugle, såsom høns.

3. Fremgangsmåde ifølge krav 1 eller 2, hvor albuminet, en variant eller et fragment deraf, et fusionsprotein, som omfatter albumin, en variant eller et fragment deraf, eller et konjugat, som omfatter albumin, en variant eller et fragment deraf, omfatter en sekvens, som udviser mindst 70% sekvensidentitet med sekvensen for HSA (SEQ ID NO: 1), fortrinsvis mindst 80%, fortrinsvis mindst 85% identitet, mere foretrukket mindst 90% identitet, endnu mere foretrukket mindst 95% identitet, endnu mere foretrukket mindst 97% sekvensidentitet og mest foretrukket mindst 99% sekvensidentitet.

25

30 4. Fremgangsmåde ifølge krav 3, hvor sekvensen, som udviser mindst 80% sekvensidentitet med HSA, omfatter mindst 10 på hinanden følgende aminosyrer, mere

foretrukket mindst 25 aminosyrer, mere foretrukket mindst 50 aminosyrer og mest foretrukket mindst 100 aminosyrer.

5. Fremgangsmåde ifølge krav 1, hvor den vandige opløsning, som omfatter albumin, en variant eller et fragment deraf, et fusionsprotein, som omfatter albumin, en variant eller et fragment deraf, eller et konjugat, som omfatter albumin, en variant eller et fragment deraf, er valgt blandt en fermenteringssupernatant, en delvist oprenset fermenteringssupernatant, serum, en serumfraktion eller et delvist oprenset serum eller en delvist oprenset serumfraktion, mælk fra transgene husdyr eller ekstrakt fra transgene planter.
6. Fremgangsmåde ifølge krav 1 eller krav 5, hvor den vandige opløsning, som omfatter albumin, en variant eller et fragment deraf, et fusionsprotein, som omfatter albumin, en variant eller et fragment deraf, eller et konjugat, som omfatter albumin, en variant eller et fragment deraf, som fyldes på matrixen, har en pH i intervallet 4-9,5, mere foretrukket 4-9, endnu mere foretrukket 4-8 og mest foretrukket 4-7.
7. Fremgangsmåde ifølge krav 1, hvor albuminet, en variant eller et fragment deraf, et fusionsprotein, som omfatter albumin, en variant eller et fragment deraf, eller et konjugat, som omfatter albumin, en variant eller et fragment deraf, elueres fra matrixen ved anvendelse af en vandig opløsning, som omfatter et salt af en fedtsyre eller thiocyanatsalt, eller elueres ved pH 10 eller derover.
8. Fremgangsmåde ifølge krav 7, hvor saltet af fedtsyren har en opløselighed i vand på mere end 10 mM.
9. Fremgangsmåde ifølge krav 8, hvor fedtsyresaltet er valgt blandt salte af butyrat, pentanoat, hexanoat, heptanoat, octanoat, nonanoat og decanoat.
10. Fremgangsmåde ifølge et hvilket som helst af kravene 7-9, hvor koncentrationen af fedtsyresaltet i elueringsbufferen ligger i intervallet fra 5 mM til 0,5M, fortrinsvis i intervallet fra 10 mM til 100 mM.
11. Fremgangsmåde ifølge krav 7, hvor thiocyanatsaltet er valgt blandt natrium-, kalium-, ammonium-, bariumsalte og salte med andre alkalimetaller fra Gruppe 1 eller 2.
12. Fremgangsmåde ifølge krav 7 eller 11, hvor thiocyanatsaltet anvendes i en koncentration i intervallet fra 5 mM til 1M, fortrinsvis i intervallet fra 10 mM til 500 mM.

DRAWINGS

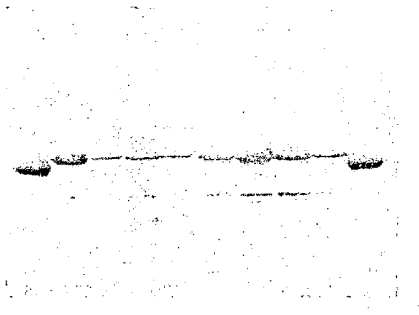


Fig. 1

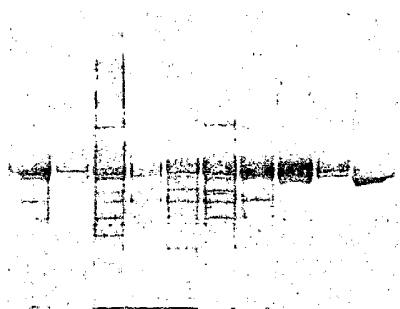


Fig. 2

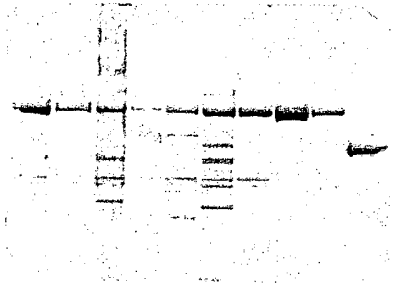


Fig. 3

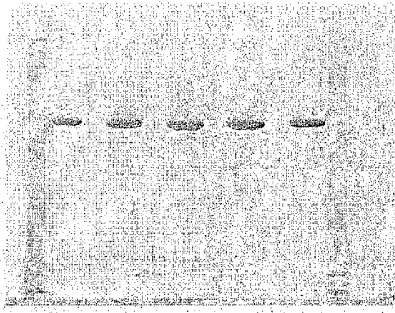


Fig. 4

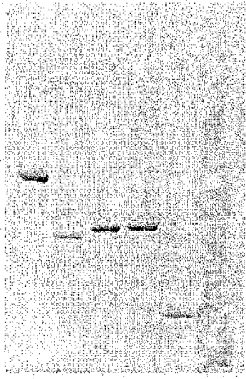


Fig. 5