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(54) Title: PROTEIN PURIFICATION

(57) Abstract: A therapeutic protein, particularly an immunoglobulin, is recovered in high yield from a proteinaceous mixture (e.g. blood plasma) by adding a 'polyethylene glycol to produce a precipitate, separating the precipitate and resuspending in aqueous liquid. Zinc ions are added to the liquid resuspension to produce a zinc protein precipitate, and the precipitate is separated off and treated to recover the therapeutic protein.

PROTEIN PURIFICATION

The present invention relates to a process for the purification of a therapeutic protein, particularly a blood plasma protein, from a proteinaceous mixture of proteins by employing polyethylene glycol and zinc as precipitation agents.

Differences in solubility behaviour have been used to separate proteins from one another for many years. This process is commonly known as protein precipitation and has been applied to the separation of proteins from natural feedstocks such as blood plasma, bacterial extracts and plant extracts.

Protein precipitation uses the difference in solubility of proteins in a mixture to separate the proteins. The solubility behaviour of a protein is a unique property determined by its composition and conformation and the environment surrounding the molecule. The distribution and degree of hydrophilic and hydrophobic regions determines the degree to which a protein is soluble in an aqueous environment. Proteins are least soluble at their iso-electric point. The availability of solvent (e.g. water) is paramount in influencing the solubility of proteins. Interactions involved in solvation at the protein surface are susceptible to changes in pH, dielectric constant and ionic strength, temperature and surface tension increment, as well as interactions with specific substances which cause the solubility of a protein to change, e.g. by changing the net charge or conformation of the protein.

Many differential precipitation methods are known for the separation of proteins and these are reviewed, for example, in P.R. Foster, Engineering Processes for Bioseparations (1994), Lawrence R. Weatherley, Chapt. 4, "Protein Precipitation" pages 73-109. In his article, the Cohn process is set out in Figure 4.1. Possible precipitation agents include the use of neutral salts (such as ammonium sulphate, sodium sulphate), precipitation with water-miscible organic solvents (such as

ethanol), non-ionic polymers (such as polyethylene glycol, polyvinyl-pyrrolidone, dextran, hydroxyethyl-starch), polyelectrolytes (such as polyethyleneimmine and carboxy-methycellulose), metal ions (including zinc, copper, barium, manganese and calcium) and other precipitants such as fatty acids (e.g. caprylic acid), amino acids (e.g. glycine), ionic polysaccharides (e.g. heparin) and dyes (e.g. Rivanol).

Blood plasma is a source of therapeutic proteins and these are conventionally separated using the Cohn cold ethanol fractionation method (Cohn E.J. et al, 1946 J. Am. Chem. Soc.68, 459-475) or similar procedures. This involves a series of precipitation steps which enable the mixture to be separated into its principal constituents, typically coagulation factors, albumin and immunoglobulins, (the latter two of which constitute about 46% and 13% by weight respectively of the total protein present in the plasma). Whilst the quality and purity of proteins produced by this method is excellent, the yield is often poor, typically being around 50% or less for immunoglobulins. This may be a disadvantage when valuable and possibly rare immunoglobulins are to be recovered from donor plasma. For example, there may be a need to recover immunoglobulins in high yield from people having high levels of certain specific immunoglobulins, such as anti-tetanus, anti-hepatitis, anti-D, (anti-rhesus), anti-rabies, anti-zoster etc.

A number of proposals have been made to use polyethylene glycols for the purification of blood plasma proteins, such as Paul W. Chun et al. Analytical Biochemistry 19, 481-497 (1967) and Cheesebro & Svehag Clin. Chem. Acta. 20 (1968) 527-529. However, as acknowledged in Y.L. Hao et al., "Fractional Precipitation of Proteins with Polyethylene Glycol", Methods of Plasma Protein Fractionation, Academic Press (1980), 57-73, there is a need to remove traces of residual polyethylene glycol from the final purified protein. This has proved to be

problematical. The removal of residual polyethylene glycol (PEG) has been attempted by adsorption of the protein mixture onto an ion exchange or affinity support; but such chromatographic methods tend to be slow, expensive and low yielding, since only relatively dilute solutions may be treated in this way as ion exchange chromatography is inefficient at handling high protein concentrations. Other methods which have been suggested include ultrafiltration and the use of salts to induce a separation of polyethylene glycol solutions into two aqueous phases. However, none of these methods appear to have found use in commercial practice.

Zinc has also been suggested as a means for separating the protein components of human plasma. For example, albumin was precipitated using zinc acetate in R.A. Kekwick et al. "The Separation of Protein Fractions from Human Plasma with Ether", Medical Research Council Special Report Series No.286, p23-29. However, this report showed that a concentration of 20mM zinc acetate was optimal. In addition an ion exchange resin was required to remove the residual zinc. It is also reported in Robert B. Pennell, "Fractionation and Isolation of Purified Components by Precipitation Methods", The Plasma Proteins Academic Press 1960, Chapter 2, page 9-49, that immunoglobulins may be precipitated using 20mM zinc at pH7.2 and 0°C. The precipitation of urokinase and tissue-type plasminogen activator analog has been achieved with zinc concentrations as low as 2mM (P.G. Zaworski and G.S. Gill, Analytical Biochemistry 173, 440-444 (1988)). Whilst the presence of residual zinc ions in therapeutic proteins is believed to be innocuous, large concentrations are clearly to be avoided and may even be toxic.

Hitherto the art has not provided a solution to the problem of separation of therapeutic proteins from proteinaceous mixtures in good purity and high yield. It is an object of the present invention to address this issue.

The present invention provides a process for the purification of the therapeutic protein from a proteinaceous mixture thereof, which comprises;

- adding a polyethylene glycol to an aqueous liquid containing the proteinaceous mixture to produce a precipitate;
- separating the precipitate;
- resuspending the precipitate in aqueous liquid;
- adding zinc ions to the liquid resuspension to produce a zinc protein precipitate; and
- separating the zinc protein precipitate.

In essence, the present invention relies on one or more polyethylene glycol precipitation steps (in which the desired protein may be present in the precipitate or the supernatant), with a final step which generally involves dilution at low pH, followed by precipitation of the desired protein using zinc ions. It has been surprisingly found that much lower amounts of zinc are required to produce the zinc protein precipitate than in the absence of residual polyethylene glycol and that residual polyethylene glycol remains in the supernatant when the zinc precipitate is separated. Thus, the present process is surprisingly effective, in that residual polyethylene glycol is able to be removed employing small amounts of zinc ions, leading to a product which is very low in both residual polyethylene glycol and zinc. On the other hand, the purity of plasma proteins (such as albumin and immunoglobulin) separated by the process is comparable to that produced by the conventional Cohn ethanol fractionation process, but the yield is significantly greater. As described herein, immunoglobulin yields of about 75-95% are possible using the process of the present invention compared to yields of around 50% using the conventional Cohn ethanol fractionation process.

The mechanism of polyethylene glycol (PEG) precipitation is believed to be based on volume exclusion, whereby the PEG molecule, in a random coil configuration, sterically excludes protein from the aqueous solvent, with solubility in PEG being strongly influenced by the molecular size of the protein (ie the larger the protein, the lower the concentration of PEG required for precipitation). The mechanism of metal ion precipitation is different to that of PEG and is due to the ability of metal ions to change the iso-electric point of a protein by binding to specific sites on the protein molecule (e.g. zinc binding to the imadazole group of histidine residues). The present invention demonstrates that effects of PEG and zinc on protein solubility can be combined to exploit these abilities in a synergistic manner.

PEG is generally regarded as non-denaturing and may stabilise protein formulations. Nonetheless, the presence of extraneous ingredients in pharmaceutical preparations is generally undesirable. Polyethylene glycol has proved to be more difficult than other precipitants to remove from the final protein solution because of its molecular conformation. Chromatography, Ultrafiltration and liquid-liquid extraction have typically been employed for this purpose prior to the present invention.

The polyethylene glycol is generally PEG 4000 or 6000.

The polyethylene glycol is generally added at the various polyethylene glycol precipitation stages to achieve a concentration in the proteinaceous liquid of 1-25%, particularly 2-20% by volume. Typically solutions of 4%, 12% and 15% are used at different stages. The polyethylene glycol percentages are given on a v/v volume basis.

A typical fractionation process involves the following steps.

A first step in which 10-20% polyethylene glycol (typically 15% polyethylene glycol) at pH7 results in the production of a protein-containing precipitate. In the case of blood plasma, the precipitate generally contains alpha-, beta- or gamma-globulins. Much of the albumin is left in the supernatant waste.

A second step involving a 2-7% polyethylene glycol (typically 4% or 6% polyethylene glycol) solution at pH4.7 results in a supernatant containing the desired protein (gamma-globulins). In the case of blood plasma, most of the remaining albumin is in the precipitate, and the immunoglobulins are in the supernatant.

In a third step, a 6-18% polyethylene glycol (typically 12% polyethylene glycol) solution at pH8.0 leads to a protein-containing precipitate. For blood plasma, the precipitate purifies the immunoglobulin to greater than 90%, and the final traces of albumin are in the supernatant waste.

In the final step, zinc ions are added to concentrate the product protein into the state of a zinc protein precipitate. Zinc ions are present in a concentration of typically 1-10mM, especially 1-5mM and typically 2-3 mM zinc. The pH is generally in the region 8-9 (especially 8.5).

The proteinaceous mixture is generally dissolved in a buffer, such as sodium acetate, and the pH is typically adjusted by addition of NaOH solution. The precipitate is generally separated by centrifugation or filtration.

The zinc ions may be added in the form of any pharmaceutically acceptable zinc salt or complex which is soluble in the protein solution concerned under the chosen conditions. Zinc chloride and zinc acetate are preferred zinc compounds.

The process can be carried out at room temperature, but is usually carried out at 2-8°C for clinical material to minimise the level of bioburden.

Using the purification process of the present invention, it is possible to achieve polyethylene glycol residual values of less than 0.1% and residual zinc values of less than 1.0ppm.

The invention is particularly applicable to the preparation of therapeutic proteins from natural or biosynthetic sources, including immunoglobulins, monoclonal antibodies, albumin etc and their derivatives. This is also applicable to the separation of synthetic peptides from cell culture and other production mixtures. The invention is especially applicable where the concentration of total protein in the starting material is high (e.g. greater than about 10 g/L) where efficient technology with a greater capacity than chromatography for concentration and purification of proteins is needed to achieve cost effective production. It is also possible to separate immunoglobulins of different specificities. The immunoglobulins may be from human, animal (e.g. cattle, horse, sheep) or a biosynthetic source.

Generally speaking, the low levels of residual zinc in the final product are physiologically tolerated but may be further reduced by ultrafiltration. Viruses may be eliminated by conventional techniques such as solvent/detergent treatment, acid treatment, heat treatment or virus filtration.

Examples of the present invention will now be described by way of example only with reference to the following examples and figures, wherein:

Figure 1 shows a PEG fractionation process according to the invention and used for the production of human IgG; and

Figure 2 shows the process used for the production of sheep IgG.

Example 1 (PEG fractionation of human plasma)

Introduction

Immunoglobulin G is traditionally purified from human plasma by cold ethanol fractionation using a variation of the method described by Cohn referenced above. The quality and purity of IgG obtained by this method is excellent, however the yield is poor, typically around 5g of IgG per litre of starting plasma (from an average initial IgG content of about 10g/L). The PEG fractionation scheme of the present invention can be applied to the preparation of IgG from human plasma and offers a marked increase in yield compared with cold ethanol fractionation. PEG4000 was used.

PEG fractionation of human plasma

The process is set out schematically in Figure 1. Frozen Human plasma was crushed then rapidly thawed at 0.5 ± 1.5°C then transferred to a refrigerated (0.5 ±1.5°C) continuous discharge centrifuge. The supernatant from this thawing process (cryosupernatant) was retained (the cryoprecipitate containing clotting factors was removed). 385mL of cryosupernatant was adjusted to pH 7.0 using 0.25M HCl. The required volume of a solution of PEG (buffered to pH 7.0 with 10mM Tris) was added to the cryosupernatant to give 15% (v/v) PEG. The PEG / protein mixture was stirred for a minimum of 40 minutes then centrifuged. The supernatant was discarded then the precipitate (ca. 45g) was resuspended (1:10) in 20mM sodium acetate buffer at pH 4.7.

A PEG solution (buffered to pH 4.7 with 20mM sodium acetate) was added to the resuspended supernatant to give a final concentration of 6% PEG (v/v). This mixture was equilibrated for 40 minutes then centrifuged. The supernatant (ca. 540mL) was retained, then adjusted to pH 8.0 using 0.25M NaOH.

The required volume of a PEG solution (buffered to pH 8.0 with 10mM Tris) was added to give a concentration of 12% (v/v). After 40 minutes equilibration, the mixture was centrifuged and the precipitate was retained.

The precipitate (30g) was resuspended in 15 volumes of 20mM sodium acetate / 20mM salt buffer at pH 4.0 and the resuspended precipitate adjusted to pH 8.5 with 0.25M NaOH. 20mL of a 50mM zinc acetate solution was added to produce a 2mM concentration. After equilibration for 40 minutes the mixture was centrifuged and the precipitate (26g) retained. The IgG was resolubilised using 20mM acetate / 20mM salt buffer at pH 4.0 at a ratio of 10:1 (v/w) for analysis.

Results

The recovery of IgG (calculated by a Bicinchoninic Acid (BCA) total protein assay and Cellulose Acetate Electrophoresis, (CAE)) and the quality of the product assessed by CAE are shown in the following table. CAE is the European Pharmacopaeia recommended method for testing the purity of blood plasma products, such as IgG.

Table 1: Comparison of yield and purity for PEG / Zinc and ethanol fractionated IgG from human plasma (assuming 10g/L IgG in starting plasma)

	IgG Yield (g/L of starting cryosupernatant)	Recovery IgG (%)	% γ globulin (CAE)	% Albumin (CAE)
PEG / Zinc fractionated IgG	9.7	96	96.5	3.5
Ethanol Fractionation Method 1	5.63±0.41 (n=34)	~56	96.7 ±2.1	<0.1
Ethanol Fractionation Method 2	4.77±0.46 (n=127)	~48	99.4 ±0.17	<0.1

The IgG may be further purified and any residual albumin and aggregates removed using the following protein purification methods:

- Depth filtration
- Ion-exchange chromatography
- Incubation with trace amount of pepsin at pH 4 and other viral inactivation procedures.

Example 2 (Fractionation of sheep plasma to produce anti-toxin IgG: Method for 65Kg starting plasma)

The process is set out schematically in Figure 2. Immune plasma was obtained from sheep which had been immunised with toxoid. The plasma (65kg) was thawed at ambient temperature (15-25°C). The plasma was adjusted to pH 7.0 using 0.25M HCl. The following fractional precipitation, including equilibration, steps were all carried out at 4-8°C and centrifugation steps were carried out using a continuous flow centrifuge at an average of 7000x *g*.

The required volume of a solution of PEG (buffered to pH 7.0 ±0.1 with 10mM Tris) was added to the cryosupernatant to give 15%(v/v) PEG. The PEG / plasma mixture was stirred for a minimum of 40 minutes then centrifuged. The supernatant was discarded then the precipitate (ca. 6.4kg) was resuspended in 10 volumes of 20mM sodium acetate buffer at pH 4.7.

A PEG solution (buffered to pH 4.7 ±0.1 with 20mM sodium acetate) was added to the resuspended supernatant to give a final concentration of 4% PEG (v/v). This mixture was equilibrated for 40 minutes then centrifuged. The supernatant (ca. 68L) was retained, then adjusted to pH 8.0 using 0.25M NaOH.

The required volume of a PEG solution buffered to pH 8.0 (with 10mM Tris) was added to give a concentration of 12%(v/v). After 40 minutes equilibration, the mixture was centrifuged and the precipitate (weight ca. 3.3kg) was retained.

The precipitated IgG fraction was resuspended in 15 volumes of 20mM sodium acetate / 20mM NaCl buffer at pH 4.0. This was titrated to pH 8.5 with 0.25M NaOH, then a 50mM zinc acetate solution added to give a final concentration of 2mM zinc acetate. The mixture was equilibrated for 40 minutes then centrifuged. The precipitate (weight ca. 6kg) was retained then resuspended with acetate buffer and formulated with sucrose (2g per gram of protein) and then frozen before further downstream processing.

The IgG fraction (ca. 25L) can then be further purified using ion-exchange chromatography, depth filtration, ultrafiltration / diafiltration followed by sterile and / or virus filtration and formulation. Fragments of IgG can be generated by digestion with pepsin or papain, and then purified using the techniques listed above.

Results

Table 2 shows the yield of IgG at the zinc acetate precipitate stage of the process for 4 batches of anti-toxin and comparative data for Fraction II of human IgG (prepared by 2 different Methods). The purity of the PEG-fractionated IgG is similar to cold ethanol fractionated IgG, but the yield is much higher.

Table 2: Yield and purity of PEG / Zinc Fractionated IgG compared with cold ethanol fractionated IgG

IgG Yield (g/L plasma) determined by Biuret Assay			% γ Globulin by Cellulose Acetate Electrophoresis	% Recovery of IgG from starting plasma *
Sample	Redissolved ZnAc Precipitate Ovine IgG	Resuspended Fraction II Human IgG		
Anti-toxin Batch A	19.0	N/A	95.5	86.4
Anti-toxin Batch B	17.8	N/A	95.0	81.0
Anti-toxin Batch C	16.5	N/A	98.1	75.0
Anti-toxin Batch D	18.9	N/A	96.0	85.9
Human Immunoglobulin Method 1	N/A	5.63±0.41 (n=34)	96.7± 2.1 (n=20)	~56
Human Immunoglobulin Method 2	N/A	4.77 ±0.46 (n=127)	99.4 ± 0.17 (n=20)	~48

* Assuming 10.0g/L in human plasma and 22g/L in sheep plasma

Human Immunoglobulin is prepared by an adaptation of the Kistler and Nitschmann method of cold ethanol fractionation. Method 1 involved precipitation of Fraction I +III at 8% and Method 2 involved precipitation of Fraction I +III at 12% see PR Foster (2005) In Kirk-Othmer Encyclopaedia of Chemical Technology 5th Ed. Volume II, "Plasma Fractionation".

In the final product there is less than 0.1% residual PEG and less than 1.0 ppm residual zinc ions after ultrafiltration.

CLAIMS

1. A process for the purification of a therapeutic protein from a proteinaceous mixture thereof, which comprises:

- adding a polyethylene glycol to an aqueous liquid containing the proteinaceous mixture to produce a precipitate;
- separating the precipitate;
- resuspending the precipitate in aqueous liquid;
- adding zinc ions to the liquid resuspension to produce a zinc protein precipitate; and
- separating the zinc protein precipitate.

2. A process according to the preceding claim, wherein the polyethylene glycol is selected from PEG 4000 and PEG 6000.

3. A process according to any preceding claim, wherein the polyethylene glycol is added to achieve a concentration in the aqueous liquid of 1 to 25% by volume.

4. A process according to claim 3 wherein the polyethylene glycol concentration is 2 to 20% by volume.

5. A process according to any preceding claim wherein the polyethylene glycol is added to the aqueous liquid in a plurality of steps.

6. A process according to claim 5 wherein polyethylene glycol is added in a first step to a concentration of 10 to 20% to produce the precipitate.

7. A process according to claim 6 wherein further polyethylene glycol is added in a second step to a concentration of 2 to 7%.

8. A process according to claim 7 wherein further polyethylene glycol is added in a third step to a concentration of 6 to 18%.

9. A process according to any preceding claim, wherein zinc ions are added to a concentration of 1-10mM.

10. A process according to claim 9 wherein the zinc concentration is 1-5mM.

11. A process according to any preceding claim, wherein the pH is in the region 8-9.

12. A process according to any preceding claim, wherein the proteinaceous mixture is blood plasma.

13. A process according to claim 12 for the sepration of immunoglobulins.

14. A process according to claim 13 for the separation of immunoglobulins of different specificities.

Figure 1

PEG Fractionation scheme for production of IgG from human plasma

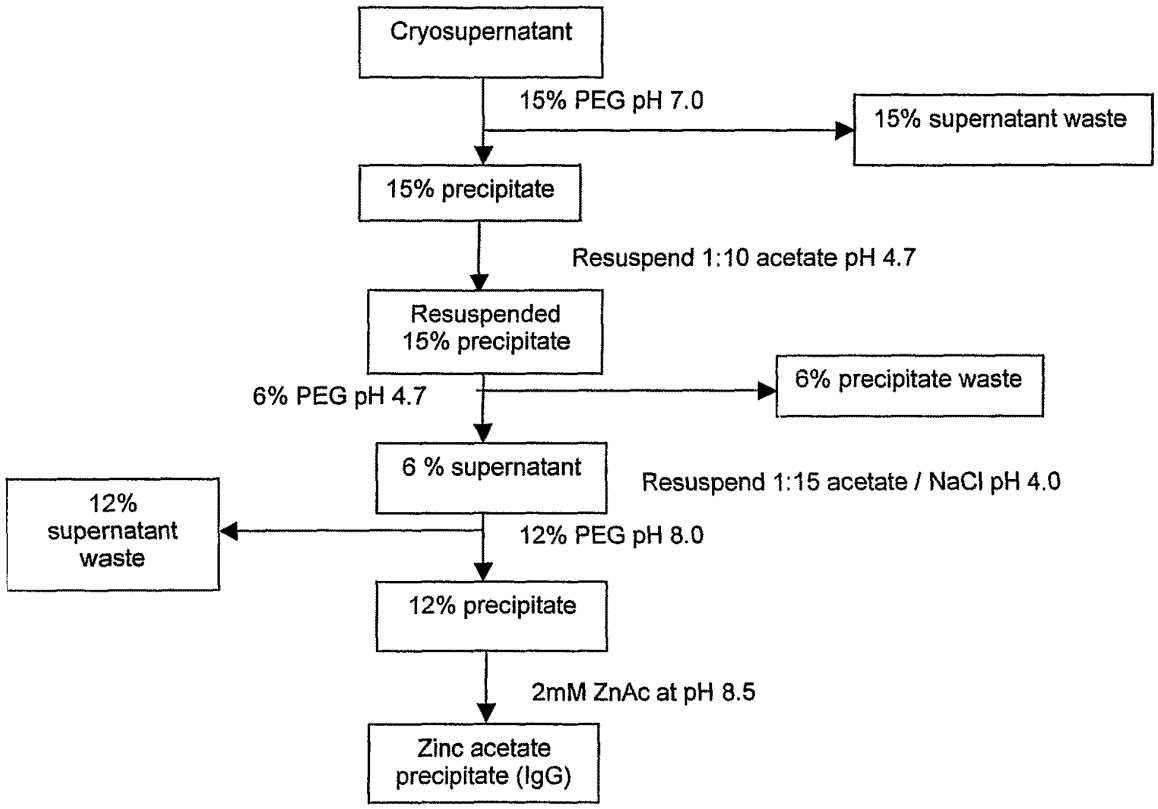
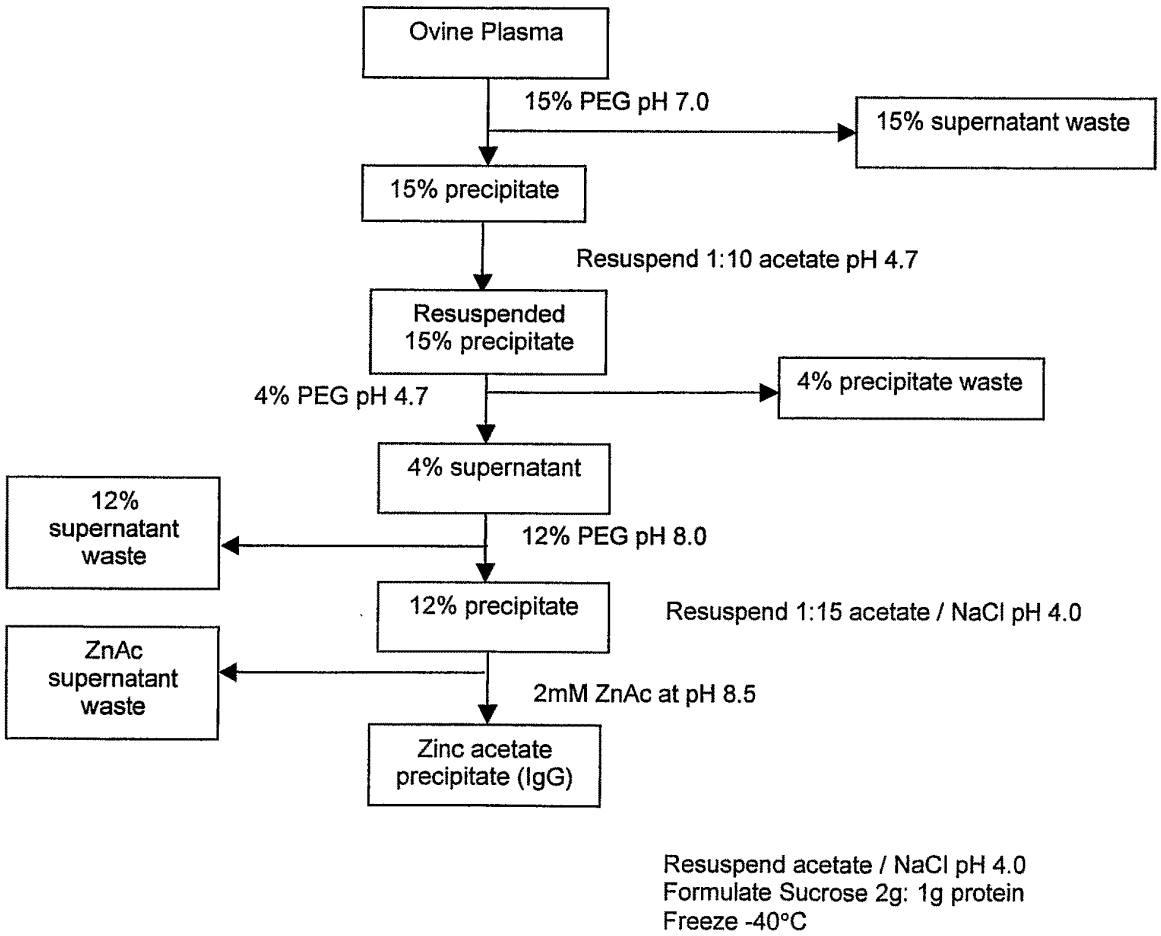


Figure 2

PEG Fractionation scheme for production of Zinc IgG intermediate from sheep plasma



INTERNATIONAL SEARCH REPORT

International application No
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A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/06 G01N33/49 G01N33/50

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)
C07K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/46211 A (AMGEN INC [US]) 22 October 1998 (1998-10-22) claims 1-5,18,21,23	1-14
X	WICKERHAUSER M ET AL: "Large scale preparation of macroglobulins." VOX SANGUINIS 1972 JUL-AUG, vol. 23, no. 1, July 1972 (1972-07), pages 119-125, XP009097174 ISSN: 0042-9007 the whole document	1-14

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	OSADA T ET AL: "Purification and characterization of alpha-macroglobulin and ovomacroglobulin of the green turtle (Chelonia mydas japonica)." JOURNAL OF BIOCHEMISTRY FEB 1988, vol. 103, no. 2, February 1988 (1988-02), pages 212-217, XP009097449 ISSN: 0021-924X page 213, left-hand column, last paragraph - page 214, right-hand column	1-14
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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