139994/EA/AMD/1863T/15

PATENT APPLICATION FORM

COMMONWEALTH OF AUSTRALIA

Regulation 9

Patents Act 1952

608832

LODGED AT SUB-OFFICE
21 APR 1988
Sydney

We, MILES INC.

of FOURTH AND PARKER STREETS, P.O. BOX 1986, BERKELEY, CALIFORNIA, 94701, U.S.A.

hereby apply for the grant of a Standard Patent for an invention entitled ~~IMPROVED~~ METHOD OF PREPARING HIGHLY PURIFIED ALPHA-1- PROTEINASE INHIBITOR which is described in the accompanying complete specification.

For a Convention application - details of basic application-

Number	Country	Date of Application
042,925	U.S.A.	27th April, 1987

Our address for service is ARTHUR S. CAVE & CO., Patent and Trade Mark Attorneys, Level 10, 10 Barrack Street, Sydney, New South Wales, Australia 2000.

Dated this 18th day of March, 1988.

MILES INC.
By Its Patent Attorneys,
ARTHUR S. CAVE & CO.

P.R. TAYLOR F.I.P.A.A.

To:
Commissioner of Patents

ARTHUR S. CAVE & CO.
PATENT AND TRADE MARK ATTORNEYS
SYDNEY

ASC 1

APPLICATION ACCEPTED AND AMENDMENTS ALLOWED 21-1-91

P139994/GHG

Regulation 12(2)

PATENT DECLARATION FORM (CONVENTION)

COMMONWEALTH OF AUSTRALIA

Patents Act 1952

DECLARATION IN SUPPORT OF A CONVENTION APPLICATION FOR A PATENT

In support of the Convention application made for a patent for an invention entitled: METHOD OF PREPARING HIGHLY PURIFIED ALPHA-1-PROTEINASE INHIBITOR

I, JAMES A. GIBLIN of 4th & Parker Streets, Berkeley, California 94710 U.S.A. do solemnly and sincerely declare as follows:-

1. I am authorised by MILES INC. the applicant for the patent to make this declaration on its behalf.

2. The basic Application as defined by Section 141 of the Act was made in the following country on the following date namely:-
in the United States of America on 27 April 1987 by MICHAEL A. SHEARER

3. MICHAEL A. SHEARER of 4927 Silverado Drive, Suisun, California 94585, United States of America is the actual inventor of the invention and the facts upon which the applicant are entitled to make the application are as follows:

The Applicant is the Assignee of the said invention from the actual inventor.

4. The basic application referred to in paragraph 2 of this Declaration was the first application made in a Convention country in respect of the invention the subject of the application.

Declared at Berkeley, CA, U.S.A. this 27th day of November, 1990.

To:
The Commissioner of Patents

ARTHUR S. CAVE & CO.

James A. Giblin
Signature of Declarant

JAMES A. GIBLIN
Assistant Patent Counsel
Miles Inc.

PATENT AND TRADE MARK ATTORNEYS
SYDNEY

2081F/RAP

(12) PATENT ABRIDGMENT (11) Document No. AU-B-15169/88
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 608832

(54) Title
METHOD OF PREPARING HIGHLY PURIFIED ALPHA-1-PROTEINASE INHIBITOR

International Patent Classification(s)
(51)[4] C07K 003/20 C07K 015/06 C12N 009/64

(21) Application No. : 15169/88 (22) Application Date : 21.04.88

(30) Priority Data

(31) Number (32) Date (33) Country
042925 27.04.87 US UNITED STATES OF AMERICA

(43) Publication Date : 27.10.88

(44) Publication Date of Accepted Application : 18.04.91

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(56) Prior Art Documents
EP 0067293
EP 0097274
US 4470968

(57) Field of the Invention: This invention relates to and has among its objects a method of preparing a high purity alpha-1-proteinase inhibitor (PI) concentrate from blood plasma, blood plasma fractions and concentrates. Further objects of the invention will be evident from the following description wherein parts and percentages are by weight unless otherwise specified.

CLAIM

1. A method of preparing highly purified alpha-1-proteinase inhibitor from a mixture of blood plasma proteins comprising

(a) adding a mild reducing agent to a mixture of plasma proteins containing alpha-1-proteinase inhibitor, said reducing agent being in a concentration sufficient to reduce disulfide bonds without reducing alpha-1-proteinase inhibitor;

(b) raising the temperature of the solution of step (a) to above 35°C to denature the reduced protein; and

.../2

(c) recovering alpha-1-proteinase inhibitor from the solution.

23. A method according to any one of claims 1 to 22 further including the steps of

(i) providing for use in step (a) an aqueous solution of the one of the blood plasma and blood plasma fraction dissolved in from 20 to 100 volumes of buffer-solution per weight of the plasma or plasma fraction used, and

(ii) prior to use in step (a), adjusting one of the buffer solution and the resulting aqueous solution of the blood plasma and blood plasma fraction from step (i) to render the pH of the resulting solution at from 9.0 to 11.0.

AUSTRALIA

PATENTS ACT 1952

608832

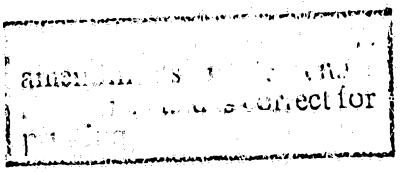
COMPLETE SPECIFICATION

(ORIGINAL)

FOR OFFICE USE

Application Number:
Lodged:

Complete Specification Lodged:
Accepted:
Published:



Priority:
Related Art:

TO BE COMPLETED BY APPLICANT

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Complete Specification for the invention entitled ~~IMPROVED METHOD.~~ METHOD OF PREPARING HIGHLY PURIFIED ALPHA-1- PROTEINASE INHIBITOR

The following statement is a full description of this invention including the best method of performing it known to me:-

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Background of the Invention

Field of the Invention: This invention relates to and has among its objects a method of preparing a high purity alpha-1-proteinase inhibitor (PI) concentrate from blood plasma, blood plasma fractions and concentrates. Further objects of the invention will be evident from the following description wherein parts and percentages are by weight unless otherwise specified.

Description of the Prior Art Insert: Alpha-1-proteinase inhibitor is a glycoprotein having molecular weight of 54,000. The protein consists of a single polypeptide chain to which several oligosaccharide units are covalently bound. Human PI has a role in controlling tissue destruction by endogenous serine proteinases. A genetic deficiency of PI, which accounts for 90% of the trypsin inhibitory capacity in blood plasma, has been shown to be associated with premature development of pulmonary emphysema. The degradation of elastin associated with emphysema probably results from a local imbalance of elastolytic enzymes and the naturally occurring tissue and plasma proteinase inhibitors. PI rapidly inhibits human pancreatic and leukocyte elastases (Biochem. Biophys. Res. Comm., Vol. 72, No. 1, pages 33-39, 1976; ibid., Vol. 88, No. 2, pages 346-350, 1979).

A number of methods employed to isolate PI from blood plasma are set forth in U.S. Patents 4,439,358, 4,379,087, and 4,656,254 and are incorporated herein by reference.

Pannell et al., Biochemistry, Vol. 13, pages 5439-5445, (1974), teaches a method of depleting albumin from plasma

using Sepharose-Blue Dextran adsorption are then pooled and fractionated with solid ammonium sulfate.

Glaser et al., EP 82103473, teach the use of reducing agents, such as dithiothreitol to break disulfide bonds and precipitation by salting out with 50% $(NH_4)_2SO_4$, to remove unwanted proteins.

Summary of the Invention

The invention described herein is a method for preparing a highly purified PI concentrate by depleting the albumin from a solution containing PI by adding a mild reducing agent sufficient to break the disulfide bonds in the albumin and heating the solution containing the PI and mild reducing agent to prevent the destabilized proteins from recombining.

In its broadest form the present invention concerns a method of preparing highly purified alpha-1-proteinase inhibitor from a mixture of blood plasma proteins comprising

(a) adding a mild reducing agent to a mixture of plasma proteins containing alpha-1-proteinase inhibitor, said reducing agent being in a concentration sufficient to reduce disulfide bonds without reducing alpha-1-proteinase inhibitor;

(b) raising the temperature of the solution of step (a) to above 35°C to denature the reduced protein; and

(c) recoverin alpha-1-proteinase inhibitor from the solution.

Another preferred form of the invention concerns a method for

separating alpha-1-proteinase inhibitor from an aqueous solution of plasma proteins containing alpha-1-proteinase inhibitor which comprises the steps of:

(a) contacting an aqueous solution of one of the group of blood plasma and a blood fraction containing alpha-1-proteinase inhibitor with from about 8% to about 23% (w/v), based on volume of the aqueous solution, of a polycondensed polyalkylene glycol selected from polyethylene glycol and polypropylene glycol, at a temperature of from about 2°C to about 50°C for a period of about 0.2 - 24 hours to selectively precipitate unwanted proteins from the solution without precipitating alpha-1-proteinase inhibitor to obtain a mixture containing alpha-1-proteinase inhibitor free of unwanted proteins,

(b) recovering the mixture from step (a), and

(c) separating alpha-1-proteinase inhibitor from the mixture recovered in step (b), by column chromatography providing an eluate containing alpha-1-proteinase inhibitor, with the improvement which comprises the steps of:

(i) adding a mild reducing agent to a mixture of plasma proteins containing alpha-1-proteinase inhibitor, said reducing agent being in a concentration sufficient to reduce disulfide bonds without reducing alpha-1-proteinase inhibitor;

(ii) raising the temperature of the solution of step (a) to above 35°C; and

(iii) recovering alpha-1-proteinase inhibitor from the solution.

In preferred embodiments, PI is separated from blood plasma or blood plasma fractions according to the Coan methods, U.S.

Patents 4,379,087 and 4,439,358 and U.S. Serial No. 793,807. As well as the improvements set forth is U.S. Patent 4,656,254, Improved Methods of Preparing alpha-1-proteinase inhibitor.

The reducing agent can be added to a solution of separated PI to break any protein disulfide bonds without reducing the PI, since PI does not have disulfide bonds. Reducing agents which can be utilized are dithiothreitol, dithioerythritol, 2-mercaptoethanol, mercaptoacetic acid, sodium borohydride and cysteine. The reducing agents are present in a concentration of about 2mM to 8mM. The concentration of reducing agent will vary depending on the type of solution containing PI and the choice of reducing agent. The amount of reducing agent should be sufficient to reduce the disulfide bonds without denaturing or damaging PI or any other protein to be recovered. The pH of the solution containing PI and the reducing agent can be adjusted to optimize the rate of

reaction and also to stop the reaction. Heat denaturation of the reduced protein can be accomplished by raising the temperature to about 35°C or higher.

Heat denaturation can also be accomplished in combination with a heat treating viral inactivation step. In U.S. Patent 4,470,968, there is disclosed a method of stabilizing coagulation Factors II, VIII, IX, and X against heat during pasteurization or heating at a temperature of about 60°-100°C by mixing with heat-stabilizing or pasteurization-stabilizing, amounts of a polyol and a source of citrate ions. Pasteurized compositions of these coagulation Factors were obtained by heating a mixture of unpasteurized protein composition, a polyol, and a source of citrate ions suspended or solubilized usually in an aqueous medium at a temperature and for a time sufficient to pasteurize the protein composition. Following pasteurization or heat treatment, the polyol and citrate ions are removed totally, or in part, as desired, from the protein composition by conventional techniques, and the pasteurized protein composition is processed according to conventional procedures for its ultimate therapeutic use. By way of illustration and not limitation, one may employ the method of U.S. Patent No. 4,470,968 (herein incorporated by reference) or any of the methods of the prior art described therein for heat inactivation.

One advantage of the invention is the availability of an essentially pure PI preparation that does not require the addition of ammonium sulfate to remove unwanted protein.

Another advantage of the invention is the incorporation of a step to remove unwanted protein with a heat inactivation step. The mild reducing agent can be added to the aqueous solution any time prior to the heat inactivation step and unwanted proteins removed when the pasteurized protein

composition is processed according to conventional procedures for ultimate therapeutic use.

The reducing agent and denatured proteins can be removed by ultrafiltration and anion exchange methods without the addition of a salting out step.

Description of the Preferred Embodiments

Current methods for obtaining PI from blood plasma use as a starting material: Cohn Fraction IV and IV-1 and includes reworks of these fractions wherein other proteins are first removed, Cohn Effluent II + III and Cohn Effluent I and cryosupernatant solution. Blood plasma is fractionated according to the Cohn ethanol fractionation technique or its modifications [see for example, Cohn et al., J.Am. Chem. Soc., 68,459, (1946); Oncley et al., ibid., 71,541 (1949); U.S. Patent 2,390,074; "The Plasma Proteins", second edition, Vol. III, pages 548-550, Academic Press, New York, N.Y.] to give a Cohn fraction containing PI. This method for removing proteins containing disulfide bonds could have application in genetically engineered PI, in those situations where the solution containing PI, such a tissue culture medium or other eluates, also contained disulfide proteins, such as albumin.

PI was produced following the method outlined by Coan et al., Vox Sang. 48:333-342, 1985; U.S. Patents 4,379,087 and 4,439,358, and improvements set forth by Shearer et al., U.S. Patent 4,656,254.

Dithiothreitol was added to Coan's purified column eluate at a concentration of 5mM, pH was adjusted to 8.0-8.2. The solution was allowed to mix for twenty minutes and the reaction was stopped by adjusting the pH to 6.5. The PI solution containing the mild reducing agent was heat treated at 60°C for 10 hours in the presence of sucrose and

citrate as indicated by Coan and Mitra, Vox Sang. 46:142-148 (1984) to render the solution free of virus. The denatured proteins were removed by applying the heat-treated solution to a DEAE Sepharose column equilibrated with 0.025M sodium phosphate at pH 6.5. The PI was removed by using a phosphate gradient to 0.1M at pH 6.5. The PI was found to be approximately 100% pure by Specific Activity (mg PI/A_{280}), CAE and HPLC. In contrast, the prior art method of Coan/Shearer provided a PI purity of about 60%.

The above method of removing unwanted proteins from a mixture of plasma proteins containing alpha-1-proteinase inhibitor can be accomplished by the steps comprising

(a) adding a mild reducing agent to a mixture of plasma proteins containing alpha-1-proteinase inhibitor, said reducing agent being in a concentration sufficient to reduce disulfide bonds without reducing alpha-1-proteinase inhibitor;

(b) mixing the composition of Step (a) with a polyol selected from sucrose or reduced sugars and a source of citrate ions in an aqueous medium, said polyol and citrate ions being present in an amount sufficient to stabilize the protein during pasteurization;

(c) and heating the mixture at a temperature and for a time sufficient to pasteurize the composition.

Generally the amount of polyol and citrate ions in the mixture of Step (b) is about 20% to saturation of polyol. On a weight to volume basis and about 0.1-1.0 moles of citrate ion per liter of solution.

Heat denaturation can be accomplished independently of a viral inactivation step by raising the temperature of the solution containing PI and the mild reducing agent sufficient to reduce the disulfide bonds to a level above 35°C. The reducing agent can be removed by ultrafiltration. PI can be recovered from the heat treated solution by column chromatography using an appropriate anion exchange medium.

The claims defining the invention are as follows

1. A method of preparing highly purified alpha-1-proteinase inhibitor from a mixture of blood plasma proteins comprising

(a) adding a mild reducing agent to a mixture of plasma proteins containing alpha-1-proteinase inhibitor, said reducing agent being in a concentration sufficient to reduce disulfide bonds without reducing alpha-1-proteinase inhibitor;

(b) raising the temperature of the solution of step (a) to above 35°C to denature the reduced protein; and

(c) recovering alpha-1-proteinase inhibitor from the solution.

2. The method of claim 1 wherein said mild reducing agent is selected from the group consisting of dithiothreitol, dithioerythritol, sodium borohydride, beta-mercaptoethanol, and cysteine.

3. The method of claim 2 wherein the concentration of said reducing agent is in the range of 2mM to 8mM.

4. The method of claim 3 wherein said reducing agent is 5mM dithiothreitol.

5. The method of claim 2 wherein said mixture in step (a) is mixed from 5 to 95 minutes at a pH range of 7.9 to 8.5.

6. The method of claim 5 wherein the temperature range of the mixture in step (a) is above 35°C.

7. The method of claim 4 wherein the mixture in step (a) is mixed for a period of about 20 minutes at a pH range of 8.0 to 8.2 at a temperature of about 25°C.

8. The method of claim 2 wherein said reaction in step (a) is stopped by adjusting the pH to below 7.8.

9. The method of claim 8 wherein the pH is adjusted to about 6.5.

10. A method according to claim 1 including the further step of treating said aqueous solution of alpha-1-proteinase inhibitor obtained from step (c) to inactivate, and to reduce the infectivity of, infectious microorganisms so as to render the alpha-1-proteinase inhibitor noninfectious to such infectious microorganisms and thereby render the alpha-1-proteinase inhibitor useful for therapeutic and prophylactic purposes.

11. The method of claim 1 which in step (b) further comprises mixing the composition of step (a) with a polyol selected from sucrose or reduced sugars and a source of citrate ions in an aqueous medium, said polyol and citrate ions being present in an amount sufficient to stabilize the protein during pasteurization; and heating the mixture at a temperature and for a time sufficient to pasteurize the composition.

12. The method of claim 11 wherein the amount of polyol and citrate ions in the mixture of step (b) is respectively about 20% to saturation of polyol, on a weight to volume basis, and 0.1-1.0 moles of citrate ion per litre of solution.

13. The method of claim 11 wherein the mixture is heated in step (b) at a temperature of 60°-100°C.

14. The method of claim 11 wherein the mixture is heated for a period of 1-10 hours.

15. The method of claim 11 which further includes the step of removing the polyol and citrate ions from the mixture.

16. The method of claim 15 wherein the polyol and citrate

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ions are removed from the mixture by subjecting the mixture to diafiltration.

17. The method of claim 15 wherein the polyol and citrate ions are removed from the mixture by subjecting the mixture to dialysis or ion exchange chromatography.

18. The method of claim 11 wherein the concentration of citrate ions is 0.3M to 0.5M.

19. The method of claim 11 wherein the concentration of sucrose is 37% (w/v) and the concentration of citrate ions is 0.38M.

20. The method according to any one of claims 1 to 19 wherein the aqueous solution of plasma proteins containing alpha-1-proteinase inhibitor is selected from the group of plasma fractions consisting of Cohn Fraction IV-1, Cohn Fraction IV, reworks of Cohn Fraction IV and IV-1, Cohn Effluent II + III, Cohn Effluent I, and cryosupernatant solution.

21. The method according to claim 20 wherein the aqueous solution of plasma proteins containing alpha-1-proteinase inhibitor is selected from the group consisting of Cohn Fraction IV-1, Cohn Fraction IV, Cohn Effluent II + III and Cohn Effluent I.

22. The method according to claim 21 wherein the aqueous solution of plasma proteins containing alpha-1-proteinase inhibitor is Cohn Fraction IV-1.

23. A method according to any one of claims 1 to 22 further including the steps of

(i) providing for use in step (a) an aqueous solution of the

one of the blood plasma and blood plasma fraction dissolved in from 20 to 100 volumes of buffer-solution per weight of the plasma or plasma fraction used, and

(ii) prior to use in step (a), adjusting one of the buffer solution and the resulting aqueous solution of the blood plasma and blood plasma fraction from step (i) to render the pH of the resulting solution at from 9.0 to 11.0.

24. A method according to claim 23 wherein in step (i) there is used from 20 to 50 volumes of buffer solution per weight of the one of the plasma and plasma fraction.

25. A method according to claim 24 wherein in step (i) there is used from 20 to 30 volumes of buffer solution per weight of the one of the plasma and plasma fraction.

26. A method according to claim 25 wherein in step (i) there is used 24 volumes of buffer solution per weight of the one of the plasma and plasma fraction.

27. A method according to any one of claims 23 to 26 wherein the pH of the one of the buffer solution and the resulting aqueious solution is adjusted to render the pH of the resulting solution from 9.0 to 10.5.

28. A pharmaceutical composition comprising a protease inhibitory effective amount of alpha-1-proteinase inhibitor produced by the method according to any one of claims 1 to 27 and a pharmaceutically acceptable carrier.

DATED this 18th day of October, 1990.

MILES INC.
By Its Patent Attorneys
ARTHUR S. CAVE & CO.