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(19) (CA) **CANADIAN PATENT** (12)

(54) Purified Antithrombin-III and Methods of Producing the Same

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ABSTRACT OF THE DISCLOSURE

Antithrombin-III is purified by contacting an aqueous solution of antithrombin-III derived from human plasma with an insoluble carrier containing as a ligand a hydrophobic group and recovering a non-adsorbed fraction. By this purification, there are provided antithrombin-III preparations containing human plasma-derived antithrombin-III, in which virus is inactivated and which are substantially free from contaminants of at least pyrogen and thermally denatured protein produced by the inactivation.

BACKGROUND OF THE INVENTION

1. Field of the Invention:

The present invention relates to antithrombin-III preparations and a method for production thereof and more particularly, to a method for purifying antithrombin-III and human plasma-derived antithrombin-III-containing antithrombin-III preparations obtained by the method, in which virus is inactivated and from which contaminants are substantially removed.

2. Description of the Prior Art:

Antithrombin-III is a sort of glycoprotein belonging to α_2-globulin present in plasma, has a molecular weight of 65,000 to 68,000, has an activity of inhibiting protease and strongly inhibits coagulation activity of thrombin.

Furthermore, antithrombin-III exhibits not only the activity of inhibiting thrombin but also the activity of inhibiting activation factor X, activation factor IX, etc. It is also reported that antithrombin-III inhibits plasmin or trypsin.

It is known that these inhibitory activities generally are more accelerated in the presence of heparin.

Antithrombin-III having such pharmacological activities can be used to correct abnormally accentuated

coagulation, more specifically, disseminated intravascular coagulation (DIC).

In the step of purifying antithrombin-III, contamination of pyrogen or protein impurities is concerned and investigations have been made on various methods for removing these contaminants. An additional new problem is the existence of thermally denatured protein formed by heat treatment at 60°C for 10 hours in a liquid state which is performed to inactivate hepatitis virus, etc. that might be included in plasma protein components. To remove the thermally denatured protein, a method for treating antithrombin-III again using immobilized heparin has recently be proposed [Japanese Patent Application KOKAI (Laid-Open) No. 63-23896 (EP-A-0252392)]. However, it has become clear that this method is not very effective for removal of pyrogen, etc.

SUMMARY OF THE INVENTION

In view of these circumstances, the present inventors have made investigations and as a result, have found that antithrombin-III preparations having more excellent safety can be prepared by treating an antithrombin-III-containing aqueous solution with a hydrophobic carrier for chromatography. The present invention has thus been accomplished.

According to the present invention, there is provided a method for purification of antithrombin-III

which comprises contacting an aqueous solution of antithrombin-III derived from human plasma with an insoluble carrier containing as a ligand a hydrophobic group and recovering a non-adsorbed fraction.

According to the present invention, there is also provided an antithrombin-III preparation comprising human plasma-derived antithrombin-III, in which virus is inactivated and which is substantially free from contaminants of at least pyrogen and thermally denatured protein formed by the inactivation.

The antithrombin-III preparation provided in accordance with the present invention has the following properties.

(1) The preparation contains antithrombin-III derived from human plasma.

(2) Viruses are inactivated.

(3) The preparation is substantially free from impurities or contaminants.

Examples of the contaminants or impurities are pyrogen, thermally denatured protein, plasma protein other than antithrombin-III (for example, ceruloplasmin, transferrin, albumin, haptoglobin, α_2-macroglobulin, α_1-antitrypsin, IgA, IgG, etc.) and the like.

(4) Specific activity of antithrombin-III is approximately 6 to 7 units/mg protein.

BRIEF DESCRIPTION OF THE DRAWING

The appended drawing shows an elution pattern

of antithrombin-III-containing fraction by gel filtration before and after the treatment with a hydrophobic carrier for chromatography.

DETAILED DESCRIPTION OF THE INVENTION

Antithrombin-III used in the present invention is not particularly limited as long as it is derived from human and purified to such an extent that is usable as a drug. Antithrombin-III can be purified from, for example, human whole blood, plasma, sera or sera squeezed from coagulated blood, etc.

As starting materials for preparing antithrombin-III, there are, for example, plasma, fractions IV, IV-1, IV-4 and IV-1 + IV-4 obtained by the Cohn's cold ethanol method; residual fraction remained after blood coagulation factor VIII was recovered from citrate-containing plasma; etc.

For purification of antithrombin-III, there are methods disclosed in, for example, Japanese Patent Application KOKAI (Laid-Open) No. 48-35017 (U.S. Patent 3,842,061) and Japanese Patent Application KOKOKU No. 59-7693 (U.S. Patent 4,340,589).

The purity of antithrombin-III is not particularly limited but one showing higher purity provides better effects.

A protein concentration of the antithrombin-III-containing aqueous solution is generally in the range

of approximately 0.1 to 10 (w/v) %.

It is preferred that the antithrombin-III-containing aqueous solution be subjected to a heat treatment (50 to 70°C, for about 5 to 30 hours) prior to the treatment with hydrophobic carrier for chromatography according to the present invention.

The insoluble carrier containing a hydrophobic group as a ligand that is used in the present invention is as described below.

Examples of the hydrophobic group include an alkyl group (having about 1 to about 10 carbon atoms, preferably about 3 to about 5), a phenyl group which may optionally be substituted, etc.

Examples of the insoluble carrier are cellulose, agarose, dextran, polyacrylamide, amino acid copolymers, polyvinyl type polymers, polystyrene type polymers, etc.

As the insoluble carrier containing the hydrophobic group as a ligand, alkyl type Sepharose* (for example, octyl type Sepharose, etc.), phenyl type Sepharose*, alkyl type polyvinyl (for example, butyl type polyvinyl, etc.) and the like are commercially available. Insoluble carriers other than those described above can also be prepared easily in a manner similar to preparing commercial products.

The purification step in the present invention is generally performed by contacting the antithrombin-III-containing aqueous solution with the insoluble

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carrier containing the hydrophobic group as its ligand. The step may be carried out either by the column process or by the batch process.

The contact may be effected under conditions at pH of approximately 6 to 9 in a salt concentration of approximately 1 to 5 M. The solvent suited for these conditions is exemplified by 20 mM sodium citrate buffer (pH 7.5) containing 3 M sodium chloride, 50 mM phosphate buffer (pH 7.5) containing 2 M sodium chloride, etc.

The purification procedure of the present invention is specifically described below. In the case of the batch process, the antithrombin-III-containing aqueous solution adjusted to the conditions described above is brought into contact with the hydrophobic carrier for chromatography, which has been equilibrated under the same conditions. In more detail, 1 ml of the carrier is mixed with 1 to 100 ml of the aqueous solution at 2 to 20°C for about 30 minutes to about 2 hours. Thereafter, centrifugation is carried out to recover the supernatant.

In the case of the column process, the antithrombin-III-containing aqueous solution adjusted to the conditions described above is developed with the hydrophobic carrier for chromatography packed in a column, which has been equilibrated under the same conditions; a non-adsorbed fraction is recovered.

The thus obtained antithrombin-III may be

formed into preparations in a conventional manner. Preferably, the non-adsorbed fraction described above is further purified by subjecting the adsorbed fraction to the step of recovery by treating with an anion exchanger.

Examples of the anion exchanger include DEAE type (for example, DEAE-agarose, DEAE-dextran, DEAE-cellulose, etc.), QAE type (for example, QAE-agarose, QAE-dextran, etc.) and the like.

The contact is effected under conditions, for example, at pH of approximately 6 to 8 in a salt concentration of approximately 0.01 to 0.1 M. The solvent suited for these conditions is exemplified by 0.01 M citrate buffer (pH 7) containing 0.05 M sodium chloride and the like.

The conditions for elution are, for example, at pH of approximately 6 to 8 in a salt concentration of approximately 0.1 to 0.3 M. The solvent suited for these conditions is exemplified by 0.01 M citrate buffer (pH 7) containing 0.17 M sodium chloride and the like.

The elution may be carried out either by the column process or by the batch process.

In the case of the batch process, the antithrombin-III-containing aqueous solution adjusted to the conditions described above is brought into contact with the anion exchanger, which has been equilibrated under the same conditions. In more detail, 1 ml of the anion exchanger is mixed with 1 to 100 ml of the

aqueous solution at 2 to 20°C for about 30 minutes to about 2 hours. Thereafter, centrifugation is carried out to recover the exchanger. Further, the above-mentioned solvent for elution is added to the exchanger. The conditions for mixing are the same as in the contact. The resulting mixture is centrifuged to recover the supernatant.

On the other hand, in the column process, the antithrombin-III-containing aqueous solution adjusted to the conditions described above is applied to the anion exchanger packed in a column, which has been equilibrated under the same conditions; a non-adsorbed fraction is discarded. If necessary, after the column is washed, the solvent for elution is flown to recover the adsorbed fraction.

If necessary and desired, the thus obtained antithrombin-III may be further purified and formed into preparations in a conventional manner. Examples of the preparation forms include an aqueous solution, a suspension, a freeze dried preparation, etc. To make these preparations, pharmaceutically acceptable additives (a diluent, an isotonic agent, a surface active agent, etc.) are suitably mixed with antithrombin-III in a conventional manner. These preparations can be applied intravenously, subcutaneously, etc. The freeze dried preparations can be used by dissolving them in distilled water for injection upon use.

According to the method for purification of

the present invention, pyrogen, protein impurities, and thermally denatured protein formed by the heat treatment, which might be contained in the antithrombin-III-containing aqueous solution, can be efficiently removed from the aqueous solution. Therefore, the antithrombin-III preparation having excellent safety can be provided.

In addition, according to the method of the present invention, the treatment step is simple and suited for mass production of the antithrombin-III preparation. Thus, the present invention is extremely useful for production in an industrial scale.

The present invention is described in more detail by referring to the examples below but is not deemed to be limited thereto.

Example 1

In 100 liters of physiological saline was suspended 10 kg of paste of fraction IV-1 obtained by Cohn's cold alcohol fractionation. Barium sulfate was added to the suspension in a concentration of 5 (w/v) %. The mixture was agitated at room temperature for 30 minutes to adsorb trace prothrombin present onto barium sulfate and remove the same. A pH of the supernatant was adjusted to 6.5 and polyethylene glycol #4,000 was added to the supernatant in a concentration of 13 (w/v) %. The formed precipitates were removed by centrifugation. Polyethylene glycol #4,000 was further added in a concentration of 30 (w/v) % and the formed precipitates

were recovered by centrifugation. The precipitates were dissolved in about 20 liters of chilled physiological saline. The solution was charged in a heparinized Sepharose* column which had been previously equilibrated with physiological saline to adsorb antithrombin-III onto the column. The column was washed with 0.4 M sodium chloride solution to remove protein impurities. Thereafter 2.0 M sodium chloride solution was run through the column and the eluate was recovered.

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Sodium citrate was added to the aqueous solution of antithrombin-III in a concentration of 0.6 M. After a pH of the mixture was adjusted to 7.8, a heat treatment was performed at 60°C for 10 hours. Subsequently, sodium chloride (final concentration of 3 M) and then sodium citrate (final concentration of 20 mM) were added to the mixture. A pH of the resulting mixture was adjusted to 7.5. On the other hand, the antithrombin-III-containing aqueous solution was brought into contact with butyl type polyvinyl carrier (BUTYL TOYOPEARL* 650, manufactured by Toyo Soda Mfg. Co., Ltd.) which had been equilibrated with 20 mM sodium citrate buffer (pH 7.5) containing 3 M sodium chloride and then developed with the buffer described above to recover the non-adsorbed fraction. Subsequently, while dialyzing to 0.9% sodium chloride solution, the non-adsorbed fraction was concentrated to give 1 (w/v) % aqueous solution of antithrombin-III. If necessary and desired, centrifugation was carried out to make the solution

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transparent.

To the 1 (w/v) % aqueous solution of antithrombin-III were added 2 (w/v) % of ~~manitol~~ mannitol and 0.2 (w/v) % sodium citrate. The mixture was diluted with a small quantity of cold distilled water in such a way that the concentration of sodium chloride became 0.5%. After its pH was adjusted to 7.6 with 1N sodium hydroxide, germ-free filtration was performed through a sterilized ~~millipore~~ Millipore* filter. By separately charging 500 units each, freeze drying was conducted to give a dry preparation.

Example 2

An antithrombin-III preparation similar to Example 1 was prepared in a manner similar to Example 1 except that fraction IV-1 + IV-4 was used instead of the fraction IV-1 paste.

Example 3

An antithrombin-III preparation similar to Example 1 was prepared in a manner similar to Example 1 except that the fraction remained after recovering blood coagulation factor VIII through a treatment of citrate-containing plasma at low temperature was used instead of the fraction IV-1 paste.

Example 4

After hydrophobic chromatography treatment was

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carried out in a manner similar to Example 1, a treatment with DEAE-dextran followed.

That is, the antithrombin-III-containing aqueous solution obtained in Example 1 through the treatment by hydrophobic chromatography was adjusted to have concentrations of 0.05 M of sodium chloride and 0.01 M of sodium citrate and to show a pH of 7. Then, the aqueous solution was passed through DEAE-dextran (DEAE-Sephadex* A-50, manufactured by Pharmacia Inc.), which had been equilibrated with 0.05 M sodium chloride-containing 0.01 M citrate buffer (pH 7). After the column was further rinsed with the same solvent, elution was carried out with 0.17 M sodium chloride-containing 0.01 M citrate buffer (pH 7) and the thus eluted fraction was recovered. Subsequently, the procedure for forming into a preparation was performed in a manner similar to Example 1 to give the antithrombin-III preparation.

Example 5

Antithrombin-III dry powders obtained in Example 4	500 units
Sodium chloride	50 mg
Sodium citrate	52 mg

The preparation having the above composition is dissolved upon use in 20 ml of distilled water for injection. The solution is intravenously administered or intravenously dripped. One unit corresponds to the antithrombin-III level contained in 1 ml of plasma from

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healthy volunteer.

Experiment 1

(1) Removal of pyrogen

After the hydrophobic chromatography treatment of antithrombin-III was carried out in a manner similar to Example 1, a test for checking pyrogens contained in the antithrombin-III-containing fraction was conducted twice before and after the treatment (referred to as the first sample and the second sample, respectively). The test was carried out in accordance with the Pyrogen Test Method defined in the Japanese Pharmacopeia. The results are shown in Table 1.

Table 1

	Total Temperature (°C)	
	Before Treatment	After Treatment
First Sample	3.2	0.1
Second Sample	2.4	0.1

As shown in Table 1, it was noted that the total temperature was lowered through the treatment by hydrophobic chromatography and pyrogen was substantially removed.

(2) Removal of protein contaminants

(a) Specific activity

After the hydrophobic chromatography treatment was carried out in a manner similar to Example 1, the

total protein amount and antithrombin-III activity in the antithrombin-III-containing fraction were determined before and after the treatment to calculate its specific activity. Total protein was determined by the Kjeldahl method. The antithrombin-III activity (AT-III activity) is determined as follows. The sample was reacted with thrombin at 28°C for 5 minutes and fibrin was added to the reaction mixture. In this case, a degree of prolongation in its coagulation time was measured. By comparing with a calibration curve previously prepared, its titer was determined. One unit corresponds to the antithrombin-III level contained in 1 ml of plasma from healthy volunteer. The results obtained are shown in Table 2.

Table 2

	Before Treatment	After Treatment
Total protein amount (mg/ml)	5.78	4.77
AT-III activity (unit/ml)	28.4	30.4
Specific activity (unit/mg protein)	4.9	6.4

AT-III : antithrombin-III

As shown in Table 2, it was noted that the antithrombin-III activity and specific activity increased through the treatment by hydrophobic chromatography. In particular, the specific activity increased from 4.9

(units/mg protein) to 6.4 (units/mg protein).

(b) Removal of plasma protein impurities

After hydrophobic chromatography treatment of antithrombin-III was carried out in a manner similar to Example 1, the presence or absence of plasma protein impurities in the antithrombin-III-containing fraction was examined by the Ouchterlony's test before and after the treatment. The results are shown in Table 3.

Table 3

	Before Treatment	After Treatment
Albumin	+	-
Haptoglobin	+	-
α_2-Macroglobulin	+	-
α_1-Antitrypsin	+	-
IgA	+	-

(c) Gel filtration

After hydrophobic chromatography treatment of antithrombin-III was carried out in a manner similar to Example 1, the antithrombin-III-containing fraction before and after the treatment was applied to gel filtration. Its elution patterns were then compared. The results are shown in the figure. Conditions for the gel filtration are as follows.

Carrier: TSK Gel* G3000 SW-XL (manufactured by Toso Co., Ltd.)

Eluent: 0.2 M sodium chloride-containing

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0.1 M phosphate buffer (pH 6.8)

Detection: absorbancy at 280 nm

As shown in Fig. 1, the fraction before the treatment shows two peaks. In these peaks, the high molecular fraction is considered to be based on thermally denatured protein, in view of the elution pattern prior to the heat treatment. It was noted that this high molecular fraction disappeared and gave one peak through the treatment by hydrophobic chromatography.

(d) Removal of plasma protein impurities

The hydrophobic chromatography treatment and treatment with the anion exchanger were carried out in a manner similar to Example 4. With respect to the thus obtained antithrombin-III-containing fraction and the antithrombin-III-containing aqueous solution prior to the treatment with hydrophobic chromatography described above, the presence or absence of plasma protein impurities was examined by the Ouchterlony's test. The test was performed in a protein concentration of 100 mg/ml. The results are shown in Table 4.

Table 4

	Prior to Treatment	After Hydrophobic Chromatography Treatment	After Treatment with Anion Exchanger
Prealbumin	+	+	-
Albumin	+	-	-
α_1-Acid-Glycoprotein	-	-	-
α_1-Antitrypsin	+	-	-
Prothrombin	-	-	-
Retinol-Binding Protein	+	-	-
C9	-	-	-
Ceruloplasmin	-	-	-
α_2-HS-Glycoprotein	-	-	-
Haptoglobin	+	-	-
α_2-PA-Glycoprotein	-	-	-
α_2-Macrogloblin	+	-	-
Factor IX	-	-	-

- to be continued -

Factor VIII	-	-	-
C3c	-	-	-
C5	-	-	-
Transferrin	+	+	-
β_1-SPI-Glycoprotein	-	-	-
Factor XIII-A	-	-	-
Factor XIII-S	-	-	-
Plasminogen	-	-	-
Fibrinogen	-	-	-
Fibronectin	-	-	-
β-Lipoprotein	-	-	-
IgG	+	+	-
IgA	+	-	-
IgM	-	-	-
Lysozym	-	-	-
α_1-Lipoprotein	-	-	-

Notes: +: Precipitation line was noted.

-: No precipitation line was noted.

Experiment 2

The antithrombin-III freeze dried preparation obtained in Example 1 was dissolved in distilled water for injection. The solution was administered to 5 mice through the tail vein in a dose corresponding to 4,000 units/kg. Observation was continued for 7 days but no abnormality was found. Furthermore, the same solution was administered to rabbits in a dose of 5,000 units/kg. Observation was made for 24 hours but no abnormality in the body temperature was noted.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method for purification of antithrombin-III which comprises:

contacting an aqueous solution of antithrombin-III derived from human plasma with an insoluble carrier containing as a ligand a hydrophobic group, and

recovering a non-adsorbed fraction thereof.

2. A method for purification of antithrombin-III according to Claim 1, wherein the contact is effected at a pH value of 6 to 9 at a salt concentration of 1 to 5M.

3. A method for purification of antithrombin-III according to Claim 1, wherein the hydrophobic group is an alkyl group.

4. A method for purification of antithrombin-III according to Claim 1, wherein the aqueous solution of antithrombin-III is heat treated to inactivate virus prior to the contact with the insoluble carrier.

5. A method for purification of antithrombin-III according to Claim 1, which further comprises bringing the non-adsorbed fraction recovered into contact with an anion exchanger and eluting and recovering a fraction adsorbed by the anion exchanger.

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6. A method for production of antithrombin-III which:

(1) is derived from human plasma,

(2) is free from virus, and

(3) is free from impurities that include pyrogen, thermally denatured protein and plasma protein other than antithrombin-III, which method comprises:

contacting an aqueous solution of antithrombin-III derived from human plasma, the said aqueous solution being free from virus and being suspected of containing at least one of the impurities, with an insoluble chromatography carrier containing as a ligand an alkyl group having 1 to 10 carbon atoms or a phenyl group at a pH value of from about 6 to about 9 at a salt concentration of about 1 to about 5M.

7. A method according to claim 6, wherein the aqueous solution of antithrombin-III that is contacted with the insoluble chromatography carrier has been subjected to a heat treatment at a temperature of 50 to 70°C for a period of time of from about 5 to about 30 hours prior to being contacted with the insoluble chromatography carrier, and the contact of the aqueous solution of antithrombin-III with the insoluble chromatography carrier is conducted at room temperature.

8. A method according to claim 6 or 7, wherein the insoluble chromatography carrier is based on cellulose, agarose, dextran, polyacrylamide, amino acid copolymer, polyvinyl type polymer or polystyrene type polymer.

9. A method according to Claim 6 or 7, wherein the insoluble chromatography carrier is polyvinyl carrier having an alkyl group having 3 to 5 carbon atoms.

10. A pharmaceutical preparation for treating abnormally accentuated coagulation comprising, in admixture with at least one pharmaceutically acceptable additive, an effective amount of antithrombin-III derived from human plasma, in which virus is inactivated and which is substantially free from contaminants of at least pyrogen and thermally denatured protein formed by the inactivation.

11. A pharmaceutical preparation according to Claim 10, wherein the antithrombin-III is substantially free from plasma proteins, as impurities, other than pyrogen a thermally denatured protein and antithrombin-III.

12. A pharmaceutical preparation according to Claim 10, wherein the antithrombin-III is substantially free from pyrogen, a thermally denatured protein, albumin, haptoglobin, α_2-macroglobulin, α_1-antitrypsin and IgA, as impurities.

13. A pharmaceutical preparation according to any one of Claims 10 to 12, wherein the antithrombin-III has a specific activity of antithrombin-III of from about 6 to about 7 units/mg protein.

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14. An antithrombin-III aqueous solution comprising antithrombin-III derived from human plasma, in which virus is inactivated and which is substantially free from contaminants of at least pyrogen and thermally denatured protein formed by the inactivation.

15. An antithrombin-III aqueous solution according to claim 14, which is substantially free from plasma proteins, as impurities, other than pyrogen a thermally denatured protein and antithrombin-III.

16. An antithrombin-III aqueous solution according to claim 14, which is substantially free from pyrogen, a thermally denatured protein, albumin, haptoglobin, α_2-macroglobulin, α_1-antitrypsin and IgA, as impurities.

17. A pharmaceutical preparation according to claim 10, 11, 12 or 13, wherein the antithrombin-III is obtained by contacting an aqueous solution of antithrombin-III derived from human plasma and free from virus with an insoluble chromatography carrier containing as a ligand a hydrophobic group and recovering a non-adsorbed fraction thereof.

18. A method according to claim 6, 7, 8, or 9, which further comprises recovering a non-adsorbed fraction.

19. A method according to claim 18, which further comprises further purifying the non-adsorbed fraction with an anion exchanger.

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PATENT AGENTS

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FIG. 1