# THE SYSTEMATIC SEPARATION AND PURIFICATION OF USEFUL PROTEINS FROM LIVESTOCK BY-PRODUCTS

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## SUMMARY

The operations as follows were suitable for the large scale application on the systematic separation and purification of useful proteins (Scheme 1). At the first step, prothrombin was isolated from plasma by the method of barium citrate adsorption. After activation of prothrombin, the yield of thrombin was around 70% from total thrombin in the plasma. In the case of using supernatant of barium citrate to separate useful proteins, the supernatant must be neutralized by the sodium sulfate and desalted by the ultra filtration several times. Then this solution was to use for starting material on chromatography. Fifty percentage (w/v) PEG 4000 solution was added to this material to final concentration 6%. After centrifugation, fibrinogen fraction was isolated as the precipitant. Fibrinogen was purified from this precipitant by the method of fractionation with ammonium sulfate and then dialyzed. The yield of fibrinogen by this method was around 50%. Albumin and transferrin were purified from 6% PEG 4000 supernatant by the ion exchange chromatography (DEAE- Sepharose FF), and so on. Each yield of albumin and transferrin were around 60% (from total albumin in the plasma) and around 70%, respectively. Vitronectin and fibronectin were purified from 6% PEG 4000 supernatant by the methods of Affinity chromatography. Each yield of vitronectin and fibrinogen were around 25%, and around 28%, respectively.

## Introduction

In the last few years, we were taking part in the production of thrombin for the clinical bulks from bovine plasma. At the first step, prothrombin was prepared by the method of barium citrate adsorption from plasma. This supernatant had high ionic strength and barium citrate. The barium citrate was removed easily by centrifugation. This precipitation broke down the chromatography media. At the first operation, we could not use this supernatant as a material of another useful proteins. Because our main purpose was to separate only thrombin on this procedure, so we have not received as much attention and threw the supernatant away until recently. But there were various useful proteins in the supernatant. Until now, we separated many proteins for reagent from another frozen bovine plasma. But it was not effcient. Then we had embarked on research attempting to purify these proteins.The methods described here was used for the systematic purification and separation of useful proteins from livestock by-products.

## Material and Methods

### Thrombin (for use clinical bulks)

Frozen bovine plasma was thawed overnight at room temperature. Lipid and fibrin clots in this plasma were removed by centrifugation. Barium chloride was slowly added to the supernatant with stirring to final concentration 0.1 M. After standing 30 minutes, most prothrombin was adsorbed to barium citrate. This precipitant was collected by continuous-flow centrifugation. The supernatant was used for the separation of another products (fibrinogen, albumin, transferrin, vitronectin, and fibronectin) as the starting material. The precipitant was suspended in 40% saturated ammonium sulfate solution. This mixture was centrifugated, and solid ammonium sulfate was added to the resulted supernatant to final concentration 65%. After centrifugation, the precipitant was dissolved with 50 mM phosphate buffer (pH 7.2). The crude prothrombin in this buffer was activated with extract of bovine brains and calcium chloride. After centrifugation, the activated thrombin-rich supernatant was applied to a CM-Sepharose CL-6B column equilibrated with 50 mM phosphate buffer (pH

6.5). Thrombin was bound to the gel. Next, another residual proteins were eluted with the same buffer containing 0.12 M sodium chloride. Thrombin was eluted with starting buffer containing 0.3M sodium chloride.After sterilizating and final concentrating around 50,000 U/ml, thrombin solution was lyophilized under sterile conditions.

Fibrinogen

The starting material was the supernatant of 0.1 M barium chloride treatment described at "Thrombin". Fifty percentage PEG 4000 solution was added to it for final concentration 6%. After centrifugation, the precipitant was washed twice with deionized water, the precipitant was dissolved in 0.1 M phosphate buffer (pH 5.6) and brought to 33% by the slow addition of ammonium sulfate. It was centrifuged again. The precipitant was dissolved in 55 mM sodium citrate buffer (pH 6.0), and saturated ammonium sulfate solution was added to the solution to final concentration 25%. After centrifugation, the precipitant was dissolved in 20 mM sodium citrate buffer, dialyzed overnight, and then lyophilized.

Albumin

The starting material was the supernatant of 6% PEG 4000 treatment described at "Fibrinogen". Its pH and ionic strength is adjusted to 7.0 and 0.02 with acetic acid.It was applied to a DEAE-Sepharose FF column equilibrated with 10 mM phosphate buffer containing 30 mM sodium chloride (pH 7.0). IgG and fibrinogen (these protein were major components in plasma) were eluted from the column at this condition. After washing with the same buffer,the column was washed with the same buffer containing 40 mM sodium chloride. This eluted fraction, contains transferrin, was used for the separation of transferrin as the starting material. Albumin was eluted from the column with the same buffer containing 0.12 M sodium chloride. This fraction was concentrated till ten times with ultrafilter. Its pH was adjusted to 3.0 with 2 M sulfuric acid. Saturated ammonium sulfate solution was added to it to final concentration 2 M and was agitated for 1 hr. This solution was diluted for final concentration of ammonium sulfate 0.8 M and its pH was adjusted to 6.2 with sodium hydroxide. It was applied to Phenyl-TOYOPEARL 650M column equilibrated with 30 mM phosphate buffer (pH 6.2) containing 0.8 M ammonium solufate. Albumin was eluted from the column at this condition.Bilirubin and lipoproteins, bound in the column. Bilirubin and lipoproteins were eluted from the column with deionized water. This eluted albumin fraction was concentrated to approximately 25% (w/v) and applied to a Sephadex G-25 column for desalting. After concentration and sterilization, this albumin fraction was lyophilized.

Transferrin

The starting material was eluted fraction from DEAE Sepharose FF column with phosphate buffer (pH 7.0) containing 40 mM sodium chloride described at "Albumin".It was concentrated by 10 times with ultrafiltration. And its pH was adjusted to 8.6 with Tris.It was applied to a DEAE-Sepharose FF column equilibrated with 25 mM Tris-HCl buffer (pH8.6). After washing 1 column volume of starting buffer, transferrin was eluted from the column with the same buffer containing 70 mM sodium chloride. The eluted fraction was concentrated by 10 times with ultrafiltration and saturated ammonium sulfate solution was added to it to final concentration 1.5 M. After stirring for 30 minutes and standing for 30 minutes, its pH was adjusted to 7.5 with 1 M Tris and was centrifuged for 25 minutes at 4oC. The supernatant was applied to a Phenyl-TOYOPEARL 650M column equilibrated with 25 mM Tris-HCl buffer (pH 7.5) containing 1.5 M ammonium sulfate. After washing 1 column volume starting buffer, transferrin was eluted slowly from the column. This eluted fraction was concentrated to approximatery 25% (w/v), and was saturated iron. After desalting and sterilizing, transferrin fraction was lyophilized.

Vitronectin

The plasma containing 0.1 M barium chloride was clotted in glassware by the addtion of calcium chloride for 1hr at room temperatureand then for 2 hrs at 4oC. After centrifugation, phenyl-methane sulfonyl fluoride in ethanol and ethylene diamino tetra acetic acid (EDTA) were added to the supernatant. This solution was applied to a Heparin-Sepharose pre-column which was equilibrated with 5 mM EDTA and 10 mM phosphate buffer (pH 7.7) containing 0.13 M sodium chloride.The passed fraction was collected and added to urea for final concentration 8 M.After standing for 2 hrs at room temperature, it was applied to a Heparin-Sepharose column equilibrated with 5 mM EDTA and 8 M urea and 10 mM phosphate buffer (pH 7.7). After applying the

sample containing urea, the column was washed with the same buffer, and then with the same buffer containing 0.13 M sodium chloride. After passing 1 column volume of the starting buffer containing 10 mM 2-mercaptoethanol and 0.13 M sodium chloride through,it was held. After 2 hr, the column was opened, and Vitronectin was eluted from the column with the same buffer containing 0.5 M sodium chloride. After sterilizing and refrigeration, vitronectin was stored at -40°C.

Fibronectin

The starting material at this operation was gained by the same treatment on the method of vitronectin. At the first step,this sample solution was applied to a Sepharose 4B pre-column, which was equilibrated with 13 mM sodium chloride. The through fraction was collected and applied to a Gelatin Sepharose 4B, which was equilibrated with 13 mM sodium chloride. And the column was washed with the buffer containing 1M sodium chloride and 0.5 M urea. fibronectin was eluted from the column with the buffer containing 4 M urea. At the last step, fibronectin solution was applied to a Sephadex G-25 equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 M sodium citrate. After sterilizing, fibronectin fraction was lyophilized.

Results and discussion

Thrombin and fibrinogen

Lyophilized thrombin was white or whitegray and specific activity was approximately 600~700 I.U/mg. It was soluble in 0.7% sodium chloride within 1 minute for 0.1%. It was comformable to the pharmacopoeia of Japan. Average yield was around 70%, protein content was above 30% and purity was above 95% on electrophoresis. In a few cases prothrombin was activated less than 60%. It was caused by using extract of frozen brains for activation.Extract of fresh brains should be used for activation.In case of fibrinogen average yield was around 50%,protein content was above 80% and purity was above 90% on electrophoresis.It contains coagulate proteins more than 80%.

Albumin and Transferrin

Average yield of albumin was around 60% and the purity of final product was above 90% on electrophoresis. In case of transferrin average yield was around 50% and purity was above 95%. These values were equal to the value when it was purified from frozen plasma.

Vitronectin and fibronectin

Average yield of vitronectin was around 20% and purity was above 95% on electrophresis. Average yield was much lower than any other protein described here. Because it was hard to purify by this method. These values were equal to the values when it was purified from frozen plasma. Average yield of fibronectin was around 28% and purity was above 95% on electrophoreisis. Average yield of fibronectin from frozen plasma was around 48%. Because fibronectin was adsorped easily to barium citrate in plasma.

Conclusion

From these results except fibronectin (Table 1), there was no change between native plasma and the supernatant of plasma containing 0.1 M barium chloride as starting material for chromatography. The barium citrate precipitate easily in this supernatant after centrifugation. Because of this precipitation broke down the chromatography media, we did not use this supernatant as starting material for chromatography of another useful proteins. When we always produced useful proteins excepting thrombin, we used another plasma. But it was not economically. In conclusion, the presented results suggested that it was no problem to use the supernatant of barium citrate after neutlization and discardation. And It was very economically to do the systematic separation of useful protein on large scale .

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