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Purification of human prothrombin from Nitschmann fraction III using DEAE membrane radial flow chromatography

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Abstract

A DEAE membrane radial column (4×5 cm) was used to purify human prothrombin from Nitschmann fraction III and performances of the column were investigated. Sample flow-rate of 20–30 ml/min (i.e., 24–36 bed volumes/h) and elution velocity up to 40 ml/min (i.e., 48 bed volumes/h) were obtained without compromising the separation efficiency. Its breakthrough capacity was one-third to half that of the commercial DEAE Sepharose Fast Flow media. It was concluded that the novel column is an attractive alternate to traditional axial column.

Keywords: Nitschmann fraction III; Membrane radial flow chromatography; Human prothrombin

1. Introduction

Prothrombin is an extensively studied protein as it plays vital roles in the blood-clotting pathway [1,2]. It is the purpose of this paper to describe a novel chromatographic procedure for the preparation of human prothrombin.

Membrane chromatography and radial column chromatography were designed to bypass the limitation of conventional gel chromatography [3–6]. Convection through thin microporous membranes eliminates diffusion and pressure drop concerns, thus minimizing processing time and maximizing purification efficiency [7]. While radial column configuration allows extremely high flow-rates at low linear velocities under low operating pressure [8,9].

A DEAE membrane radial column (4×5 cm), which combined advantages of both membrane and radial flow chromatography, was used to prepare human prothrombin from Nitschmann Fraction III in this work. The performance of the column was explored and the effects of a variety of variables, including elution velocity and sample flow-rate, were studied. The breakthrough capacity of the improved column was also determined.

2. Experimental

2.1. Nitschmann fraction III

Nitschmann fraction III, derived from fractionation of plasma for albumin and IgG by Nitschmann method (cold ethanol procedure), was a gift from the plasma fractionational group of our laboratory. This material (20–30% total solids by dry weight) was received as frozen paste and stored at −20° until used.

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For general laboratory production, which may be scaled accordingly, 15 g fraction III frozen paste were suspended at room temperature (20–25°) in 1000 ml of 0.04 *M* NaCl and buffered with 0.06 *M* Tris–HCl, pH 7.5. After being mixed with an overhead propeller stirred for 30 min, a uniform suspension was obtained. Insoluble materials were discarded following centrifugation at 1500 rpm for 15 min; recovered fluids were used to purify human prothrombin.

2.2. Chromatographic equipment

An XK 26/20 column, DEAE Sepharose Fast Flow medium and AKTA-explorer-100 system were obtained from Amersham Pharmacia Biotech.

The DEAE membrane radial column (4×5 cm) was purchased from the Institute of Chemical Physics, Chinese Academy of Sciences. The membrane was prepared from cellulose and the covalent linkage of DEAE was made after chemical modification of the membrane. The membrane thickness was 0.3 mm and the mean pore diameter was 2–30 μm. A roll of membrane with total flow accessible membrane volume of 50 ml was sealed in a cylindrical column. The packed column is a weak anion-exchanger with excellent flow properties, which was suitable for the separation of DNA, peptide and protein. Its entire working range is pH 2–14. It can be sterilized with acetic acid, up to 2 *M* NaOH or 5% aqueous formalin solution. Characteristics such as capacity, elution behavior and pressure/flow-rate are normally not significantly changed by cleaning-in-place or sanitation. After the last chromatographic separation has been performed, at least two bed volumes of a compatible bacteriostat can be passed through the column.

2.3. Chemicals

Except where indicated, all reagents were purchased from Gibco-BRL (Life Technologies, USA). All buffer solutions were stored at 4°C until used. The loading buffer (buffer A) was 0.06 *M* Tris–HCl, 0.04 *M* NaCl, pH 7.5. The elution buffer (buffer B) was 0.06 *M* Tris–HCl, 0.25 *M* NaCl, pH 7.5.

2.4. Protein analysis

Total protein was determined by the method of Bradford with slight modification [10]. As protein standard, bovine serum albumin (BSA) was diluted to 0, 0.2, 0.4, 0.5, 0.8, 0.9 and 1 μg/ul in 0.06 *M* Tris–HCl, pH 7.5 buffer. Samples were diluted between 1:1 and 1:100 in the same buffer. The diluted standard or sample (60 μl) was then mixed with Coomassie Blue reagent (3 ml). As a blank, the same buffer (60 μl) and Coomassie Blue reagent (3 ml) were mixed. The absorbance at 595 nm was measured on DU series spectrophotometers (Beckman Instruments) and protein concentrations in the samples were read from the standard curve using a linear regression program.

2.5. Activation of prothrombin

Prothrombin preparations were activated to thrombin in the presence of rabbit brain cephalin (100 μg/ml, 30 ml/100 ml prothrombin) and 0.25 *M* $CaCl_2$ (10 ml/100 ml prothrombin) added dropwise with continuous mixing. The mixture was allowed to react for 15 min at 37°, with brief stirring once every minute, then placed on ice to stop the reaction and filtered through three layers of dry gauze.

2.6. Coagulation assays

Prothrombin was determined as generated thrombin activity, which was expressed as fibrinogen clotting time. The clotting activity of the thrombin-containing solutions was assayed as follows: 200 μl of 5 mg/ml human fibrinogen dissolved in buffered saline (0.85%, w/v, sodium chloride buffered with 0.06 *M* Tris–HCl to pH 7.5) was incubated in polypropylene tube at 37°C. After 20 min incubation, 100 μl of the solution to be tested (diluted between 1:1 and 1:100) were directly added in each tube, and the coagulation time was measured. The prothrombin activity was inversely related to clotting time. Preliminary experiments indicated that the linear range of this method is 5–60 s. All clotting assays were done in triplicate.

2.7. Column chromatographic procedures

The XK 26/20 column (3×2.6 cm) packed with 15 ml DEAE Sepharose Fast Flow media and the DEAE membrane radial column (4×5 cm) were connected to the AKTA explorer-100 system. All solutions were filtered through 0.22-μm filters and degassed prior to use. After equilibrated with buffer A, the columns were fed with samples at optimized flow-rates, respectively, then washed with buffer A until the absorbance at 280 nm of the eluent fell to the baseline. Adsorbed materials were eluted with buffer B and collected. The produced prothrombin was analyzed for total protein and clotting activity. After a chromatographic process, columns were regenerated using buffer A for the next experiment. All runs were performed in duplicate at 25°C.

Purification process was first developed on the XK 26/20 column, which will be reported in detail elsewhere. The achieved process was employed onto the membrane radial column directly in this work. In order to evaluate its performance, the effects of flow velocity, sample flow-rate and breakthrough capacity were investigated.

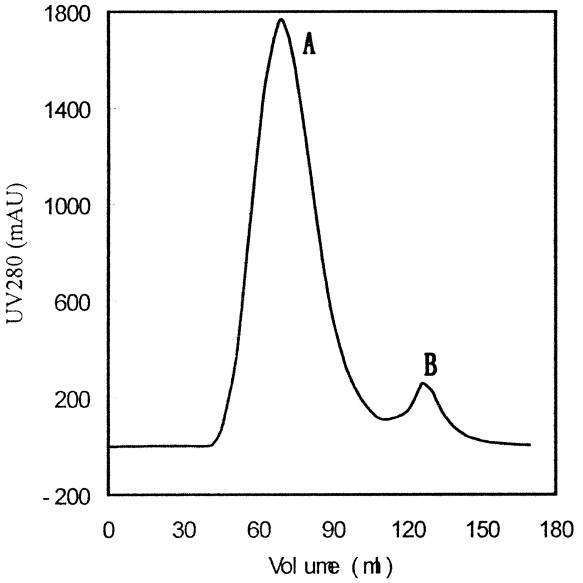


Fig. 1. Chromatography of Nitschmann fraction III on the Sepharose DEAE Fast Flow XK 26/20 column equilibrated and first eluted with 0.04 *M* NaCl–0.06 *M* Tris–HCl buffer. pH 7.5 (buffer A), followed by 0.25 *M* NaCl (buffer B), 0.06 *M* in Tris–HCl and pH 7.5. Bed resin, 3×2.6 cm; sample size, 50 ml; flow-rate, 3 ml/min; temperature, 25°C. Prothrombin is fraction B.

3. Results and discussions

3.1. XK 26/20 fractionation of Nitschmann fraction III using DEAE Sepharose Fast Flow

As part of the present study, purification of prothrombin was achieved using an XK 26/20 column. Experiment results indicated that a sample flow-rate of 3 ml/min (12 bed volumes/h) and elution velocity of 6 ml/min (24 bed volumes/h) would be optimal values with regards to excellent separation. Fig. 1 shows the typical elution profile. Prothrombin was eluted with 0.25 *M* NaCl and concentrated at peak B. Fifty ml Nitschmann fraction III solution (4.3 mg/ml) were fed to the column and approximately 25 ml prothrombin were collected. The protein concentration of the purified prothrombin was 1.1 mg/ml and the coagulation time of the generated thrombin was about 15 s. The value of activity/concentration rate (specific activity) of the produced prothrombin was 15/1.1.

To determine the breakthrough capacity from different sample loading (4.3 mg/ml), column experiments were performed at the flow-rate of 3 ml/min. When the loading size was 300 ml, a detectable amount of prothrombin activity was found in the washing fluid (data not shown). So its breakthrough capacity was approximately 20 ml/ml media, if the sample (3.4 mg/ml) was applied at the flow-rate of 3 ml/min.

3.2. DEAE membrane radial column fractionation of Nitschmann fraction III

After equilibrated with buffer A, the column was applied with 200 ml fraction III suspensions. Fig. 2. shows the elution profile at a flow-rate of 20 ml/min. A single peak (peak B) containing prothrombin was also eluted from the DEAE membrane radial column at almost the same ionic strength as that observed with XK 26/20 column as demonstrated in Fig. 1. Approximately 100 ml prothrombin with almost the

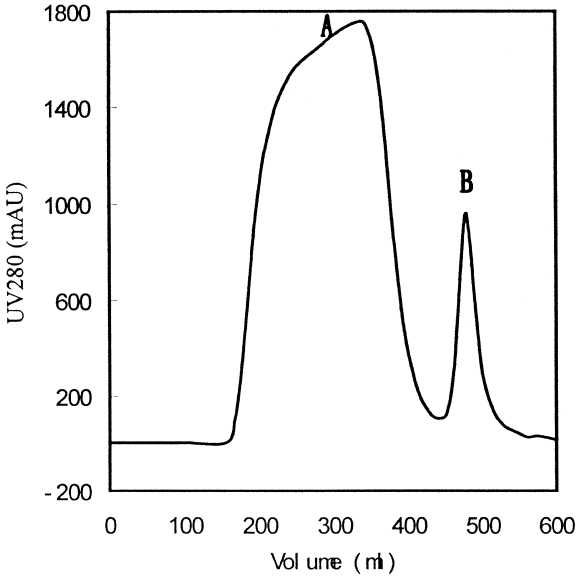
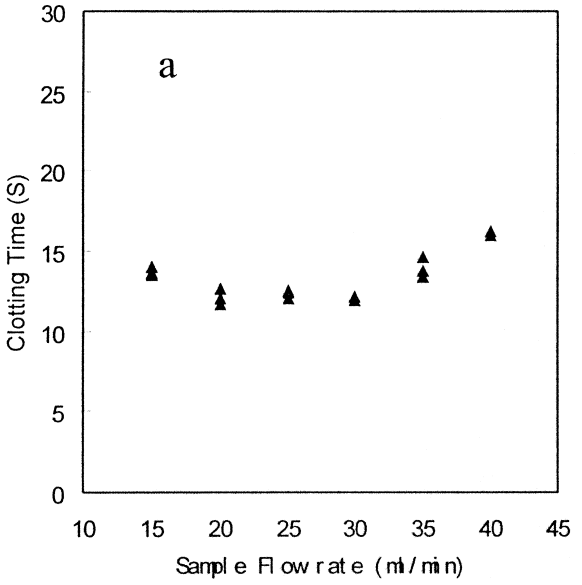


Fig. 2. Chromatography of Nitschmann fraction III on the membrane radial column equilibrated and first eluted with 0.04 *M* NaCl–0.06 *M* Tris–HCl buffer, pH 7.5 (buffer A), followed by 0.25 *M* NaCl (buffer B), 0.06 *M* in Tris–HCl, pH 7.5. Bed resin, 4×5 cm; sample size, 200 ml; flow-rate, 20 ml/min; temperature, 25°C. Prothrombin is fraction B.

same protein concentration and activity were pooled, indicating the same resolution and yield of the improved column as that of the XK column.

3.2.1. Effect of sample flow-rate

Aliquots of 200 ml feedstock were applied to the DEAE membrane radial column at sample flow-rates varying from 15 to 40 ml/min. The elution velocity was defined as 25 ml/min. Experiment results (Fig. 3) demonstrated that increasing sample flow-rate had considerable effects on total protein concentration of peak B. The total protein decreased sharply as the sample velocity increased from 15 to 25 ml/min, but remained constant (approx. 1.2 mg/ml) as the sample velocity increased from 25 to 40 ml/min, while the influence on prothrombin activity was not so obvious. Within the experimental range, flow-rate above 35 ml/min resulted in low activity, while lower velocity (<20 ml/min) caused more unwanted materials and consequently also low prothrombin activity. From the data, we can see that it was slightly high (approx. 12 s) in the range of 20–30 ml/min (i.e., 24–36 bed volumes/h). So the optimal



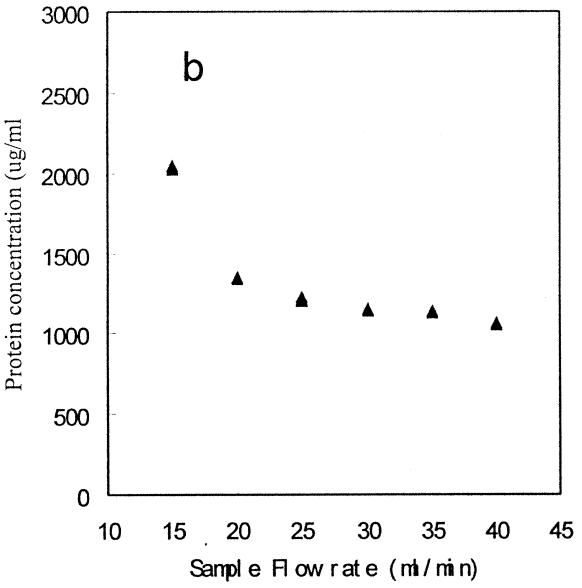


Fig. 3. Correlation between the activity of prothrombin (a) and the amount of protein (b) found in the eluate with increasing sample flow of the membrane radial column chromatography, sample size and elution flow-rate were defined as 200 ml and 25 ml/min, respectively.

sample flow-rate was 20–30 ml/min. The operating pressures of all the chromatographic processes did not exceed 0.2 MPa, indicating low backpressure of the column.

3.2.2. Effect of elution flow-rate

In order to investigate the effect of elution velocity on the membrane radial column, the bound materials were eluted with flow-rates varied from 15 to 40 ml/min, while the sample size and sample flow-rate were defined as 200 ml and 25 ml/min, respectively. Contrast to the effect of sample flow-rate, elution flow-rate had obvious effect on total protein concentration and prothrombin activity of Peak B (Fig. 4). Within the range investigated, increasing amount of flow-rates resulted in decreasing amount of protein and clotting time. The decline trend was sharp as the elution flow-rate was between 10 and 20 ml/min, and smooth within the range of 25–40 ml/min. The results suggested that the column allowed an elution velocity of 25–40 ml/min (i.e., 30–48 bed volumes/h). Higher flow-rate within the range investigated would result in short process time of course.

3.2.3. Effect of sample volume

In a chromatographic process, breakthrough is another important operation parameter, which is defined as the point at which the solute being purified appears in the effluent solution [11]. In order to determine the breakthrough capacity, column experiments were performed under subsaturating conditions. Different volumes (4.3 mg/ml) were applied to the column at fixed flow-rate of 25 ml/min. The total protein concentration and prothrombin activity of peak B were investigated. Fig. 5 shows the effect of a 160-ml stepwise increase in sample volume from 80 to 880 ml. When the sample size was below 560 ml, the prothrombin concentration and activity increased as the sample size increased. As the sample volume was greater than 560 ml, a considerable amount of prothrombin activity was found in the washing fluid (data not shown) and the protein concentration and coagulation time remained almost constant in the eluate. Complimentary experiments indicated that the column was beginning to saturate after approximately 300–600 ml of the feedstock had been loaded onto the bed at a flow-rate of 25 ml/min. This applied amount (approx. 6–10

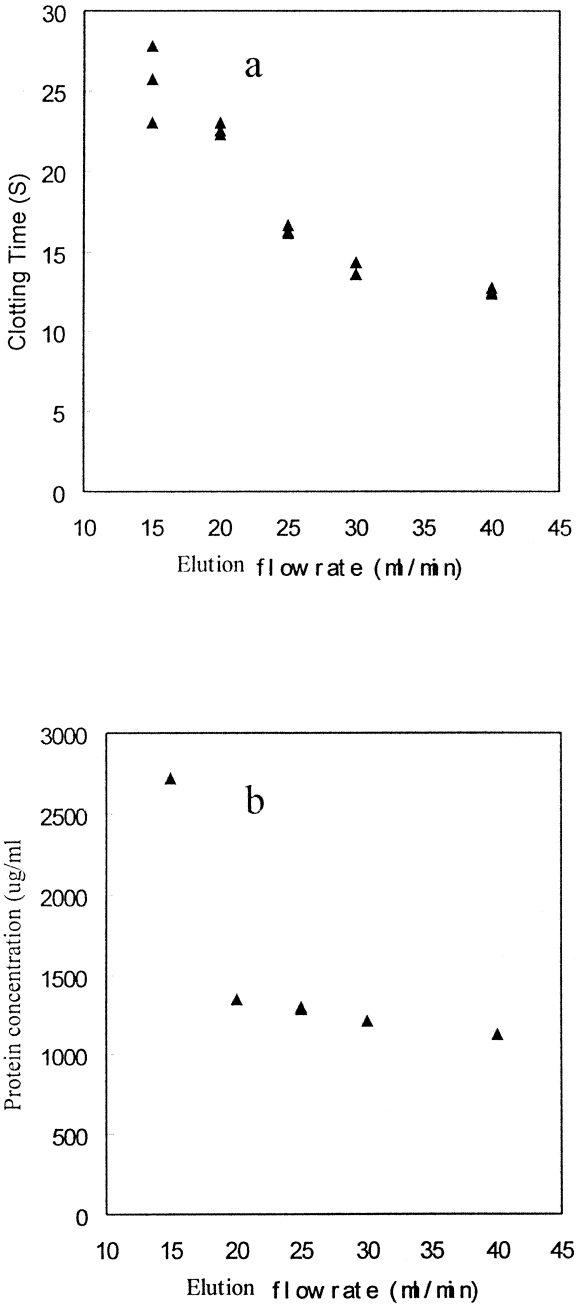


Fig. 4. Correlation between the activity of prothrombin (a) and the amount of protein (b) found in the eluate with increasing elution velocity of the membrane radial column chromatography, sample size and sample flow-rate were defined as 200 ml and 25 ml/min, respectively.

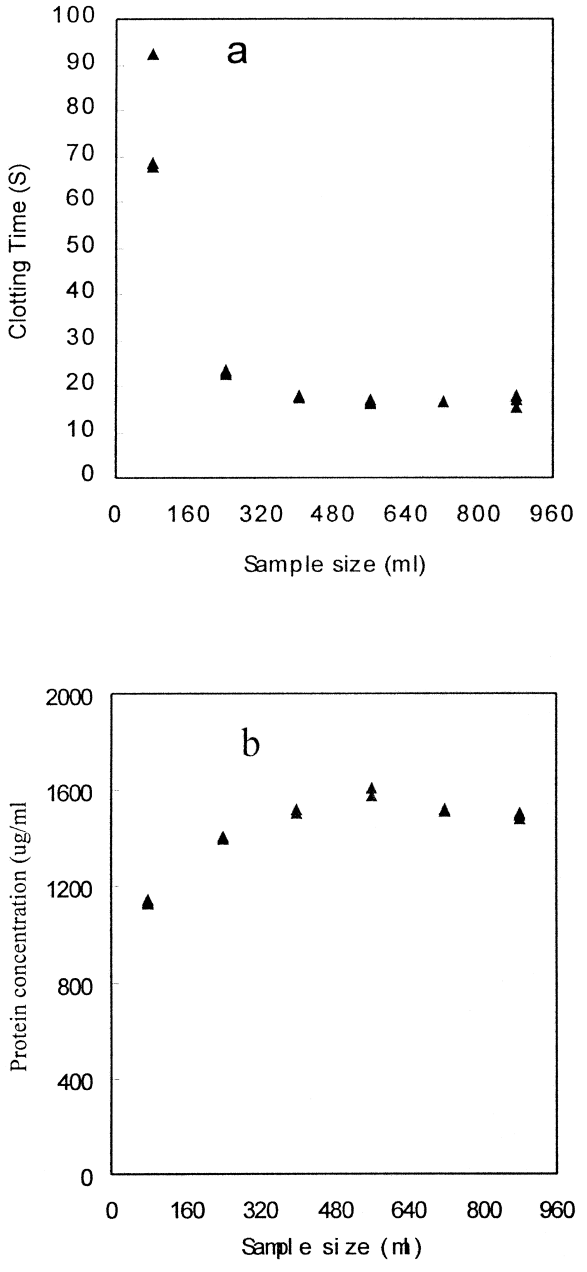


Fig. 5. Correlation between the activity of prothrombin (a) and the amount of protein (b) found in the eluate (Peak B) with increasing sample volume of the membrane radial column chromatography at the flow-rate of 25 ml/min.

ml/ml media) was chosen in order to operate within the range of the column capacity, and thus minimize breakthrough losses. From the result we can see that the capacity of the membrane column was about one-third to half that of commercial DEAE Sepharose Fast Flow media.

4. Conclusions

A DEAE membrane radial column was used to purify human prothrombin from Nitschmann fraction III in this report. The performance of column was evaluated and the effects of a variety of variables including elution velocity and sample flow-rate were studied. Its breakthrough capacity was also determined. The main advantage of the procedure is that it permits quick separation, thus helping us to separate quickly, completing in hours or minutes a separation that might otherwise take longer time at low operation pressure. Since the process time is shorter, the chance of bacterial contamination and the proteolysis of human prothrombin by other components are diminished. For the above-mentioned reasons it was thought to be a suitable method to purify prothrombin from Nitschmann fraction III, though its breakthrough capacity was not high.

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