A novel one-step purification of human α -thrombin after direct activation of crude prothrombin enriched from plasma

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Crude prothrombin enriched from human plasma was directly activated to generate α -thrombin without prior exhaustive purification of the proenzyme using a combination of several different types of chromatographic techniques, as in all previously described methodologies. Activated thrombin was separated from other components in a single step by taking advantage of its highly specific affinity to heparin immobilized on a matrix support of Sepharose CL-6B. On the basis of the data presented herein, we have demonstrated the ease with which at least 25 mg of a highly purified enzyme (> 97% homogeneous by laser densitometry) can be obtained per litre of plasma. Our product exhibits a specific activity of at least 4000 National Institutes of Health units/mg and is stable after being freeze-dried for the purpose of long-term storage.

INTRODUCTION

The multi-stage process leading to the coagulation of mammalian blood involves sequential transformation of several proenzymes to enzymes by limited proteolysis (Davie & Fujikawa, 1975). Unique within this sequence of events is the activation of prothrombin by Factor Xa, which also requires Factor V, Ca^{2+} and phospholipid as cofactors (Papahadjopoulos & Hanahan, 1964). The end product of this zymogen activation yields α -thrombin, a serine proteinase that is centrally involved in the final step of the coagulation cascade. Thrombin cleaves the four arginyl-glycyl peptide bonds in fibrinogen to produce fibrin monomers (Magnusson, 1972). These monomers subsequently polymerize to form the basis of a blood clot. Under physiological conditions, the concentration of thrombin is regulated by activation of zymogen and inhibition by antithrombin III, the principal circulating inhibitor of the proteinase (Pepper *et al.*, 1977; Barrowcliffe *et al.*, 1978). However, thrombin is only slowly inactivated by this inhibitor in blood. Heparin, a sulphated glycosaminoglycan, which is also present in the circulation, accelerates the rate of inactivation by 50–500-fold (Rosenberg, 1977).

It is also well known that prothrombin can be activated by some components of snake venom to yield thrombin (Meaume, 1966). Among these include the venom from the mainland tiger snake *Notechis scutatus scutatus* (Jobin & Esnouf, 1966), the taipan snake *Oxyuranus scutellatus scutellatus* (Denson, 1969) and the saw-scaled viper *Echis carinatus* (Schieck *et al.*, 1972). These non-physiological, but specific, activators have been widely used to generate thrombin from its proenzyme. They are also quite commonly used as means to study the later stages of the coagulation cascade *in vitro*.

A milestone for the large-scale preparation of human α -thrombin has been described by Fenton *et al.* (1977), who started with a Cohn fraction III paste processed previously from human plasma. Others have employed large volumes (10–20 litres) of plasma or commercially available concentrates to purify first prothrombin (Mann, 1976). Here we describe a procedure for the isolation of human α -thrombin also starting from human plasma, but on a smaller scale. However, the most outstanding feature of our procedure compared with those previously published resides in the fact that activation, by taipan-snake venom, of the zymogen takes place directly in a crude prothrombin fraction enriched

from plasma without its prior extensive purification (for example, see Bajaj *et al.*, 1981). The only chromatographic step involves heparin-Sepharose CL-6B by taking advantage of the highly specific affinity of thrombin, but not of the other components, for the heparin attached to the column matrix. The entire procedure is rather simple, and thrombin obtained via this method has a specific activity exceeding 4000 National Institutes of Health (NIH) units/mg.

EXPERIMENTAL

Materials

Fresh human plasma was obtained from Blutspendenzentrum SRK Beider, Basel, Switzerland, and was stored at -20°C before use. Snake (*Oxyuranus scutellatus*) venom (V-3129) was purchased from Sigma Chemical Co. Heparin-Sepharose CL-6B and column PD-10 (Sephadex G-25 M) were products of Pharmacia LKB Biotechnology. Human fibrinogen used in the coagulation assay was obtained (as a freeze-dried powder) from KabiVitrum, Stockholm, Sweden. Soybean trypsin inhibitor was a product of Calbiochem and was purchased from Behring Diagnostics, La Jolla, CA, U.S.A. Chromozym-TH was purchased from Boehringer Mannheim. All other chemicals used were of reagent grade or better and were obtained from Merck.

Fractionation of plasma

Prothrombin belongs to the family of vitamin K-dependent blood-coagulation factors. This group of proteins has peculiar affinities for insoluble barium and magnesium salts (Bajaj & Mann, 1973). Therefore, barium adsorption of prothrombin is the initial step most widely employed to separate this zymogen from the other factors in plasma.

Routinely, two litres of fresh frozen plasma were used as starting material. The frozen plasma was thawed rapidly in a 30°C water bath. All subsequent steps were performed at 4°C unless otherwise stated. To the thawed plasma sodium citrate (0.137 g/100 ml) and benzamidine hydrochloride (10 mmol/l) were added with stirring. This was followed by the addition of 160 ml of BaCl_2 (1 mol/l) solution dropwise with slow stirring. The mixture was allowed to continue stirring for 30 min after addition of BaCl_2 was completed. The suspension was centrifuged at 3500 g for 20 min to recover the barium-adsorbed proteins. The collected pellet was washed once, using a Waring blender to

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disperse the pellet vigorously, with 500 ml of Tris/HCl (20 mmol/l) buffer, pH 7.5, containing NaCl (0.15 mol/l), EGTA (1 mmol/l), BaCl_2 (10 mmol/l) and benzamidine hydrochloride (5 mmol/l). The precipitate was recovered by centrifugation as described above.

The pellet obtained from this step was homogenized in a Waring blender using 250 ml of EDTA (0.2 mol/l, pH 7.4). The pH of this suspension was adjusted to 7.4. The resulting solution, viscous and semi-opaque in appearance, was dialysed first against 5 litres of Tris/HCl (20 mmol/l) buffer, pH 7.5, containing NaCl (0.15 mol/l), EGTA (1 mmol/l) and EDTA (0.1 mol/l) for about 5 h with rapid stirring. The dialysis was continued overnight in a fresh buffer having the same composition, but without EDTA.

The overnight-dialysed sample was subjected to $(\text{NH}_4)_2\text{SO}_4$ precipitation: first at 0–35% saturation, from which the supernatant was then subjected to fractionation at 35–70% saturation. The pellet obtained from the 35–70%-satn. fraction was retained and redissolved in a minimum volume (~25 ml) of Tris/HCl (20 mmol/l; pH 7.5)/EGTA (1 mmol/l) (Buffer A) containing benzamidine hydrochloride (1 mmol/l) and soybean trypsin inhibitor (1 mg/ml). This solution was dialysed extensively, first against 10 litres of Buffer A and then against another 10 litres of Buffer A containing NaCl (0.1 mol/l). This step was usually carried out beginning on a Friday afternoon and proceeded throughout the weekend.

The exhaustively dialysed sample was centrifuged (routinely on a Monday morning) at 12 000 g for 40 min to remove materials precipitated during dialysis. An estimation of the prothrombin content was made by measuring the A_{280} of a suitably diluted portion of the clarified sample; an $A_{280}^{10 \text{ mg/ml}}$ value of 14.4 was used (Cox & Hanahan, 1970).

Activation of prothrombin

The activation step was carried out essentially as described previously by Owen & Jackson (1973), who also reported that it was unnecessary to further purify purchased snake venom before use. Stock CaCl_2 solution was added to the clarified solution described above to give a final concentration of 10 mmol/l. Snake venom (at an enzyme/substrate ratio of 1:40, w/w) was then added to the prothrombin-containing solution. Activation was carried out at room temperature for 20 min with gentle stirring. The reaction was terminated by the addition of stock EGTA solution to yield a final concentration of 12 mmol/l. The final solution was placed on ice before proceeding to the next chromatographic step.

Heparin–Sephacrose affinity chromatography

The activated sample was applied to a column (1.6 cm $\times$ 35 cm) of heparin–Sephacrose CL-6B previously equilibrated using Buffer A containing NaCl (0.1 mol/l). After sample application was completed, the column was washed exhaustively, using the same buffer, to remove non-specifically bound proteins. Finally, a gradient consisting 220 ml each of Buffer A containing NaCl at 0.1 and 1.0 mol/l respectively was applied to elute bound protein components. Selected fractions of the eluted material were analysed by SDS/PAGE in a discontinuous buffer system (Laemmli, 1970). Similar fractions were also assayed for the amidolytic activity of thrombin using Chromozym-TH as substrate; small portions (routinely 5 μ l) from each fraction were added to 1 ml of appropriately diluted substrate solution; ΔA_{405} was measured after 15 min incubation at room temperature.

Quantification and storage

Eluted fractions containing pure thrombin were pooled ac-

cording to the gel-electrophoretic pattern and the activity profile. The pooled material was divided into portions, which were frozen immediately on solid CO_2 before storage at -80°C . Before subsequent usage, each portion was desalted on a PD-10 column, NH_4HCO_3 at 50 mmol/l being used as eluent. The protein concentration of the desalted material was determined by measuring its A_{280} ($A_{280}^{10 \text{ mg/ml}} = 17.5$; Fish & Björk, 1979). Finally, the pooled material was concentrated by freeze-drying portions in Eppendorf tubes such that each contained 0.5–1.0 mg of protein. The final product was stored at -20°C .

The salt-free end product was subjected to the following analysis: SDS/PAGE for M_r determination, quantitative N-terminal analysis (Chang, 1988), amino acid analysis (Knecht & Chang, 1986) and amino acid sequence (Chang, 1983; Hunkapiller *et al.*, 1983). The clotting activities of the resulting thrombin preparations were measured using fibrinogen as substrate with a Coagulometer (KC-1) manufactured by Heinrich Amelung G.m.b.H., Lemgo, Germany. The procedure described by the manufacturer was followed, and human α -thrombin (3421 NIH units/mg), purchased from the Centre for Diagnostic Products (Boston, MA, U.S.A.), was used as standard.

RESULTS AND DISCUSSION

Prothrombin can be efficiently fractionated from other plasma coagulation factors by using barium citrate adsorption. As Fig. 1, lane 4, shows, prothrombin (M_r 70 000) constituted one of the two major proteins in this fraction. This fraction was obtained by solubilizing proteins that were co-precipitated with the insoluble barium complex with EDTA (0.2 mol/l), pH 7.4. This was followed by dialysis of the mixture in EDTA-containing buffer to ensure complete release of proteins from the adsorbed complex. This is the most crucial step in the successful recovery of prothrombin from plasma. However, the significance of the EDTA-extraction step has not been overly emphasized in the

Fig. 1. SDS/10%-PAGE summarizing steps of purification

Key to lanes: 1, molecular-mass markers (Bio-Rad) [myosin, 200 kDa (top); β -galactosidase, 116.5 kDa; phosphorylase *b*, 97.4 kDa; BSA, 66.2 kDa; ovalbumin, 42.7 kDa (bottom)]; 2, unfractionated plasma; 3, supernatant from BaCl_2 adsorption; 4, EDTA desorption of BaCl_2 pellet; 5, 35–70%-satd.-(NH_4) $_2\text{SO}_4$ enrichment of prothrombin (Pro-T); 6, activation step showing conversion of prothrombin into thrombin (T); 7, product after heparin–Sephacrose affinity chromatography and desalting using column PD-10 (~5 μ g of protein was applied); 8, molecular-mass markers (Bio-Rad) [phosphorylase *b*, 97.4 kDa (top); BSA, 66.2 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa (bottom)].

Fig. 2. Elution profile for the heparin-Sepharose affinity column

Peaks A and B represented components eluted during sample application and wash; C and D represented those eluted after application of gradient. For column size and elution conditions, see the description in the text. ○, Protein (A_{280}); ▲, amidolytic activity (A_{405}).

more recently described procedures (for example, see Miletich *et al.*, 1980) compared with those published earlier (Ingwall & Scheraga, 1969). As a result we experienced very low yields during our initial attempts.

Another innovation we have introduced in our procedure described here was the direct activation of crude prothrombin after its enrichment by $(\text{NH}_4)_2\text{SO}_4$ fractionation. As Fig. 1, lanes 5 and 6, show, more than 95% of the prothrombin was converted into thrombin as judged by laser densitometry. The mixture after activation with snake venom (Fig. 1, lane 6) was subjected directly to heparin-Sepharose affinity chromatography. A typical elution profile (Fig. 2) showed four major peaks. SDS/PAGE revealed that peak D, eluted at a NaCl concentration between 0.5 and 0.6 mol/l, contained all the thrombin generated during activation (results not shown). Results obtained from activity assays (▲, Fig. 2) using Chromozym TH as substrate coincided with this peak of eluted protein (○, Fig. 2).

The M_r of our prepared thrombin was estimated to be 34800 by SDS/PAGE, and it was more than 97% homogeneous (Fig. 1, lane 7) on the basis of laser densitometry. In addition, its purity was assured by quantitative *N*-terminal analysis, which yielded equimolar quantities of threonine and isoleucine, but no other residues. The amino acid composition obtained using two different preparations were identical with the published data (Table 1), and sequence analyses up to the first 10 amino acid residues were also identical with the established sequence of human α -thrombin.

Although other coagulation factors, such as Factor IX (M_r 57000) and Factor X (M_r 59000), have also been shown to bind to heparin-Sepharose, the probability of their being present as trace contaminants in our preparation was unlikely, since they are eluted at lower ionic strength than is α -thrombin (Fujikawa *et al.*, 1973) from such a column. If they were to be present, additional *N*-termini would have appeared other than those stated above by quantitative *N*-terminal analysis. Furthermore, on an overloaded SDS/PAGE gel, not a trace of either component was detected.

Although large number of procedures exist in the literature describing the isolation of prothrombin and its subsequent activation to yield thrombin, the majority of these methods required a pre-processed plasma fraction (Fenton *et al.*, 1977), commercial concentrate (Blatt *et al.*, 1974) or somewhat complex and time-consuming prior synthesis of a special chromatographic

Table 1. Amino acid composition of human α -thrombin

Amino acid	Found	Expected*
Asp	30.5	30
Glu	31.4	29
Ser	17.4	17
Thr	12.3	12
Gly	29.2	27
Ala	13.1	13
Arg	20.8	20
Pro	15.6	15
Val	15.8	17
Met	7.0	7
Ile	15.4	17
Leu	23.0	25
Trp	n.d.†	9
Phe	11.2	11
Cys‡	3.8	4
Cys	22.0	22
His	4.7	5
Tyr	10.1	11

* Deduced from the cDNA sequence published by Degen *et al.* (1983).

† Abbreviation: n.d., not determined

‡ Evaluated as the number of disulphides (cystine).

matrix (Miletich *et al.*, 1980) to obtain first the pure form of the zymogen. We have demonstrated here the use of heparin-Sepharose affinity chromatography to isolate thrombin directly from plasma in a single chromatographic step. Although there have been previous mentions of the usage of such an affinity-chromatographic procedure (Miller-Andersson *et al.*, 1980; Nordenmann & Björk, 1978), it was only employed in those instances as a final step of further purification.

In conclusion, the procedure described here can be completed within 6 working days, or even 4 days if the second dialysis step is carried out over a weekend. The yield of purified thrombin, based on the average of seven experiments, was 28.5 mg/litre of starting plasma. Direct comparison of yield with existing data cannot be easily made, since the individual methods employed different starting materials (Lundblad *et al.*, 1976; Fenton *et al.*, 1977; Miletich *et al.*, 1980), and most reports quoted yields in terms of activity rather than in quantitative units. Finally, the specific activity, as compared with a standard preparation having

3421 NIH units/mg (from the Centre for Diagnostic Products) was found to be 4150 NIH units/mg of protein on the basis of an average of five preparations. It is noteworthy that this activity was not altered significantly after the preparation was freeze-dried and stored at -80°C for up to 6 months.

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