Bovine Thrombin and Activated Factor X

Separation and Purification*

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SUMMARY

Parke-Davis bovine thrombin was resolved into three distinct protein peaks on a Sephadex G-200 column.

A stepwise chromatographic technique on diethylaminoethyl (DEAE) cellulose was developed for the complete separation and partial purification of thrombin and activated Factor X. Thrombin was quantitatively eluted in the first protein peak with 0.1 M NaCl, pH 7.0. A second peak with residual thrombin activity, containing more than 65% of the applied protein, was eluted with 0.14 M NaCl in 0.05 M sodium citrate, pH 5.8. When this fraction was rechromatographed, to remove the residual thrombin, Factors II, VII, IX, and nonactivated X were detected in this peak. Activated Factor X was eluted from the column with 0.2 M sodium citrate, pH 5.8. This activated Factor X fraction did not clot a standard fibrinogen solution during 24 hours either at 22° or 37°, with or without added calcium, nor after removal of the citrate by dialysis against 0.14 M NaCl.

The DEAE-cellulose-chromatographed thrombin was further purified by Sephadex G-200 chromatography. More than 68% of the resulting thrombin peak had a constant specific activity of approximately 7000 National Institutes of Health units per mg of tyrosine.

At high dilutions, when the thrombin activity in the commercial bovine thrombin preparation was negligible, the activated Factor X activity was still detectable in appreciable quantity. The purified thrombin fraction was free of plasminogen and plasmin.

During blood coagulation in vitro, the activation of prothrombin (Factor I) leads to the formation of a proteolytic enzyme thrombin by the action of prothrombin activator (prothrombinase). Thrombin then initiates a series of well-defined events in the polymerization of its natural soluble substrate fibrinogen to the insoluble product fibrin (1).

Activated Factor X (autoprothrombin C) is an essential component of prothrombin activator (2-8). In the intrinsic pathway of blood coagulation it has been suggested that Factor X is activated by activated Factor VIII (9). Factor X is also activated in the extrinsic pathway by activated Factor VII (2). Activation of purified Factor X can also occur by the direct action of Russell's viper venom (10) and trypsin (3). Spontaneous activation of purified Factor X has been observed (11) and activation with ammonium sulfate can also be achieved (12).

Both thrombin and activated Factor X have been shown to activate Factor V (4, 13). Crude activated Factor X (Product I) has been reported to cause platelet viscous metamorphosis (14); on the other hand, however, thrombin has been claimed to be the responsible agent (15). Thrombin has been shown to possess esterase activity capable of catalyzing the hydrolysis of a variety of synthetic basic amino acid esters (16-18). By means of different methods, activated Factor X has been reported both to possess (17, 19, 20) and to lack (9) esterase activity. However, the reported esterase characteristics of activated Factor X are different from those of thrombin; tosyllysine methyl ester, an exceedingly sensitive synthetic substrate for thrombin, is insensitive to activated Factor X (17). The most striking difference between thrombin and activated Factor X is that only thrombin can convert fibrinogen to fibrin. Thrombin, moreover, has been shown to possess both the esterase and clotting activities that according to some investigators are inseparable (21), whereas others find them to vary independently (22).

Inactivation of the clotting activity of thrombin can be achieved by binding the active site with ß-trypsin-binding macroglobulin. Fractions of thrombin thus treated are still capable of hydrolyzing synthetic substrates such as N-p-tosyl-L-arginine methyl ester and N-carbobenzoxy-L-tyrosine p-nitrophenyl ester (23). Most recently, preparations of bovine thrombin have been reported to activate plasminogen, trypsinogen, and chymotrypsinogen A (24, 25). Other workers have also shown the enhancement effect of thrombin on the glucose oxidation of platelets (26).
Untreated commercial preparations of thrombin have been used by some investigators to study the physicochemical properties of the enzyme and its effects on biological systems. Two commercial thrombin preparations have been previously reported to be contaminated with autoprothrombin C (27). There are as yet no methods available for the detection of other coagulation factors including activated Factor X in the presence of high levels of thrombin. Thrombin can be obtained by citrate activation of purified prothrombin (28), but it has been shown that unless this purified prothrombin is contaminated with Factors V, VII, and X, no thrombin is evolved during the process of citrate activation (29, 30).

Parke-Davis thrombin is prepared commercially by the re-calification of bovine prothrombin in the presence of bovine tissue thromboplastin. The conditions under which prothrombin can be activated in such a system have been shown to require the presence of Factors V, VII, and X (2).

Chromatographic methods for the purification of thrombin have been described (31, 32); but since activated Factor X has not been recognized in these techniques, it is not certain if such thrombin fractions are devoid of activated Factor X. Much effort has been expended in the chemical purification of thrombin in terms of homogeneity by ultracentrifugal studies (33). Such investigations with the use of ultracentrifugal data have not considered the possible contamination with trace amounts of other clotting factors. The presence of these factors cannot be recognized by physical means.

The present report describes a simple and reliable method for the complete separation and partial purification of thrombin and activated Factor X from a commercial preparation of thrombin.

**EXPERIMENTAL PROCEDURE**

**Materials**

Bovine plasma barium sulfate eluate was prepared from fresh oxalated bovine plasma as previously described (11). A crude bovine fibrinogen fraction was prepared by repeated (NH₄)₂SO₄ precipitation on twice BaSO₄-treated plasma (3). The concentration of the fraction employed contained approximately 300 mg of fibrinogen per 100 ml, the pH having been adjusted to 7.35. Tyrosine, utilized in the standard calibration curve of protein determination, and crystalline bovine serum albumin were obtained from Sigma. Cephalin, a mixture of phospholipids, was prepared from human brain by the method of Bell and Alton (34). Thrombin, topical (Bio. 2073, 5000 NIH units) was purchased from Parke, Davis and Company. Six different lots of thrombin were used. Unless otherwise stated, all sodium chloride solutions were freshly adjusted to pH 7.0 with 0.005 M tris(hydroxymethyl)aminomethane just before use. The NaCl-citrate buffer was prepared by dissolving 0.14 M NaCl in 0.05 M sodium citrate at pH 5.8. Citrate buffer was adjusted to pH 5.8 with equimolar citric acid. Barium sulfate (Merck, x-ray diagnostic grade) was used. Diethyylaminoethyl cellulose (Selectadex, type 20, Lot 1338, 1.17 meq per g of exchange capacity) was obtained from Carl Schleicher and Schuell Company, Keene, New Hampshire. Sephadex G-200 (bead form), Lot 10-4522 and Blue Dextran 2000 were obtained from Pharmacia. Polyvinylpyrrolidone, used in a concentration of 50% adjusted to pH 7.0 with 1 M NaOH, was purchased from Aloe Scientific, St. Louis, Missouri.

**Methods**

Factor II (prothrombin) was measured according to the method of Koller, Loeliger, and Duckert (35). Factor X was assayed by the procedure of Bachmann, Duckert, and Koller (36), and Factor VII by the ability to correct the abnormal Quick prothrombin time of a severe congenital Factor VII-deficient plasma. Factor IX concentrations were determined by the following modification of the partial thromboplastin time (37). The following reagents (0.1 ml of each) were mixed at 37°C, (a) kaolin-cephalin mixture (4 g of kaolin per 100 ml of 0.14 M NaCl mixed with an equal volume of cephalin suspension containing approximately 3.5 μg of phosphorus per ml), (b) Factor IX-deficient plasma, and (c) 1:10 dilution of test fraction. The three reagents were incubated at 37°C for 5 min; and then 0.1 ml of 0.025 M CaCl₂ was added and the clotting time was recorded.

One unit of any coagulation factor is defined as the amount present in 1 ml of fresh normal human plasma. A unit of activated Factor X is defined as the activity that would evolve from 1 ml of normal human plasma, when the precursor is fully activated by the venom. Accordingly, it was established that 1 unit of activated Factor X, without venom, gave the same clotting time as 1 unit of plasma Factor X, when the latter was measured in the system containing venom. Thus, an average milliliter of bovine plasma contains less than one-half the quantity of Factor X present in 1 ml of human plasma.

In this study, activated Factor X was determined by means of a system identical with the assay for nonactivated Factor X with the same cephalin concentration except that the venom was omitted (3). In constructing the calibration curve for activated Factor X, however, the Bachmann Factor X assay method with venom was employed. Normal human plasma was employed as a control.

**Definition of Thrombin Activity**—Thrombin activity was determined with bovine fibrinogen as substrate at a concentration of 300 mg/100 ml. Unless otherwise stated, thrombin activity, expressed in NIH units, was derived from a standard calibration curve with NIH bovine thrombin (Lot 3B) as reference. In another set of experiments, thrombin activity was calculated from a standard calibration curve established by dissolving one vial of Parke-Davis thrombin in 0.14 M NaCl to give a fibrinogen clotting time of 15 sec, and the number of units present in that initial dilution was then calculated from the units of activity stated on the label. In the actual test, 0.2 ml of an appropriate dilution of the fraction to be assayed was added to 0.2 ml of fibrinogen at 37°C.

**Sephadex G-200 Filtration**—Sephadex was packed in a Lucite column measuring 2.5 × 63.5 cm as reported previously (11). The 200 to 270 mesh size was used. The Sephadex was left to swell in 0.14 M NaCl for at least 30 days at 4°C before packing. The buffer, temperature, and flow rate used for a particular experiment are mentioned in the appropriate sections.

**Void Volume Determination of Sephadex G-200 Column**—Both blue dextran 2000 and a cephalin preparation were used to determine the void volume of the gel bed. The elution profile of blue dextran 2000 was followed at 630 μm in Beckman model DU spectrophotometer, and that of the cephalin was followed by determining the lipid phosphorus. Phosphorus was determined by the method of King (38).

**Preparation of DEAE-cellulose Column**—The cellulose was sieved to obtain the 60 to 100 mesh for packing the column. The cellulose was first washed successively with 1 M sodium citrate, 1
m citric acid, and 1 m NaCl, and then rinsed with 10 liters of 0.04 sodium citrate, pH 7.0, on a Buchner funnel. The washed cellulose was stored in 0.04 m sodium citrate, pH 7.0, at room temperature until required for chromatography. Prior to packing the column, a portion of the stock cellulose was rinsed with 0.075 m NaCl and finally suspended in the same solution. A glass burette serving as a supporting column for the cellulose bed (25-ml capacity, internal diameter 1.2 cm, height 35 cm) was siliconized with Siliclad (Clay-Adams Inc., New York) according to the method recommended by the manufacturer. A small piece of absorbent cotton wool was placed in the bottom of the column to retain the cellulose. The slurry of cellulose was poured into the column and packed by gravity to a height of 24 cm, followed by hydrostatic pressure packing until the cellulose bed height was between 17 and 20 cm with a flow rate of less than 20 sec per drop. Packing was carried out at room temperature.

Examinations of Protein Concentration—Tyrosine was determined by the method of Folin and Ciocalteau (39), and the results were expressed in protein concentration with the conversion factor being that 1 mg of tyrosine is equivalent to 11.4 mg of total protein (40). In determining the tyrosine concentration of the fractions suspected to have low tyrosine content, as judged previously from a pilot run, a 1-ml aliquot of the test material was used for the hydrolysis.

RESULTS

Staphylococcus Chromatography of Parke-Davis Bovine Thrombin—Untreated Parke-Davis bovine thrombin, when passed through a column of Sephadex G-200 gel, resolved into three distinct protein peaks (Fig. 1). A small thrombin peak coincided with the first protein peak and spread over the entire area of the middle protein peak. The major thrombin activity coincided with the protein peak where albumin is generally eluted.

Fractionation of Parke-Davis Bovine Thrombin on DEAE-cellulose Column—Fig. 2 shows a typical chromatogram of Parke-Davis thrombin on a DEAE-cellulose column. Under the experimental conditions, more than 98% of the thrombin activity was eluted in the first peak with 0.1 m NaCl, pH 7.0. In 12 different runs the recovery of thrombin ranged between 98 and 110%, and the specific activity of the peak fractions ranged between 3000 and 3500 units per mg of tyrosine. The total recovery of protein in this first peak was approximately 25%. When the starting eluting fluid was 0.070 m NaCl, pH 7.0. The dialyzed fraction was centrifuged in a polypropylene centrifuge tube at 5000 X g for 20 min at 4°. The dialysate, containing approximately 87 mg of protein, was passed through a Sephadex G-200 column (previously conditioned with 0.075 m NaCl, pH 7.0) and allowed to soak in by gravity. The absorption time of the 3-ml fraction on the cellulose was usually less than 20 min. When the thrombin fraction was just about completely soaked in, 4.5 ml of the starting eluting fluid were immediately layered on the cellulose to wash down the remaining traces of protein. After the elution fluid had soaked in, the column was filled with the same solution, and the tubing leading from the reservoir containing the buffer was connected. The flow rate was regulated to 30 to 36 ml per hour, by adjusting the height of the reservoir, and a stepwise elution technique was employed. Chromatography was performed at room temperature and fractions of 3 ml each were collected in siliconized tubes and immediately placed in ice before being assayed.

Residual thrombin not eluted in the first peak was eluted with 0.14 m NaCl in 0.05 m sodium citrate, pH 5.8, in the second peak. More than 70% of the total protein applied was confined to this second peak. In the presence of this level of thrombin, it was not possible at this step to assay for any other clotting factors that might be present in trace quantity. This second peak was subsequently subjected to further investigation.

In the presence of high thrombin activity one cannot specifi-
early assay for activated Factor X activity. No activated Factor X was measured in Fractions 10 to 23 and 30 to 49.

Before any attempt was made to elute the activated Factor X, five fractions were allowed to elapse starting from the last tube which had a fibrinogen clotting time of more than 1 hour. This precaution always resulted in activated Factor X being free of thrombin.

If the activated Factor X was eluted too soon, it contained trace amounts of thrombin capable of clotting fibrinogen within 3 to 12 hours. Such contaminated fractions of activated Factor X, however, can be salvaged by the following procedure. Rechromatography of the activated Factor X is carried out first by dialyzing the pooled fraction against 100 times its volume of 0.05 M NaCl, pH 7.0, for 12 hours (concentrating by dialysis against polyvinylpyrrolidone at 4°C) and then further by dialyzing against 100 times its volume of 0.1 M NaCl, pH 7.0. Chromatography on the DEAE-cellulose column is initiated with 0.14 M NaCl in 0.05 M sodium citrate, pH 5.8, to remove traces of thrombin, followed by 0.2 M sodium citrate, pH 5.8, to elute the activated Factor X.

In the third peak, eluted with 0.2 M sodium citrate, pH 5.8, and containing activated Factor X activity, no thrombin was measurable. Thrombin in these fractions was tested for by incubating an equal volume of the undiluted samples containing activated Factor X activity with equal volumes of fibrinogen and 0.25 M CaCl₂ at 37°C. No clots were observed within a 24-hour period. The specific activity of the activated Factor X in this peak ranged from 18,000 to 26,000 units per mg of tyrosine. The peak fraction (No. 51) gave a substrate plasma clotting time of 22.5 sec at a 1:10,000 dilution in the presence of an optimal amount of cephalin, as compared to the substrate buffer time of 323 sec. The amount of activated Factor X protein required to give the 22.5 sec clotting time was 3.65 mg. When the activated Factor X fractions were pooled and dialyzed against 0.1 M NaCl, they were also devoid of thrombin. The protein recovered in this peak (Fractions 50 to 55) was less than 2.5% of the total starting protein.

Studies in our laboratory have also shown that the activated Factor X recovered from the Parke-Davis thrombin, as well as the activated Factor X derived from the activation of highly purified precursor by either venom or trypsin, resist elution with sodium citrate at pH 5.8 up to 0.15 M and that the main bulk of activity comes down with the 0.2 M fraction whereas the nonactivated Factor X is eluted with 0.08 to 0.1 M sodium citrate.

**Gel Filtration of DEAE-cellulose-chromatographed Thrombin Fractions**—Fractions containing thrombin activity in the first peak (Fig. 2) from 12 different runs were pooled and concentrated by lyophilization, and an aliquot was chromatographed on a Sephadex G-200 column at 4°C. Fig. 3 indicates that this step further separated the thrombin peak from another major protein peak devoid of clotting activities. A 2-fold purification over the lyophilized fraction was achieved. The inset in Fig. 3 indicates the specific activities of Fractions 38 through 43, which represented 68% of the total thrombin activity recovered in this run. The thrombin recovery was more than 75%. An identical elution profile was obtained when the filtration was carried out at room temperature and also when the thrombin fraction was obtained from a single pool. However, at room temperature, the recovery of thrombin was usually less than 50% resulting in lower specific activity of the enzyme.

**Gel Filtration of DEAE-cellulose-chromatographed Activated Factor X**—Passage of the activated Factor X fraction, recovered from the DEAE-cellulose column, through Sephadex G-200 resulted in a loss of more than 25% of the starting activity without any improvement in the specific activity of the enzyme.

**Rechromatography of Residual Thrombin Peak on DEAE-cellulose**—When the middle protein peak of Fig. 2 was rechromatographed on a second DEAE-cellulose column under the same conditions to remove the residual thrombin, it was found that the resulting middle protein peak, now completely free of thrombin, contained Factors VII, IX, nonactivated X, and occasionally prothrombin, each of which amounted to less than 1 unit per ml. Occasionally, there was a trace of activated Factor X detected in the third peak, but never in the middle peak.

**DEAE-cellulose Chromatography of Bovine Plasma Barium Sulfate Eluate**—According to the information supplied with each vial, Parke-Davis thrombin is prepared as follows. “Thrombin. Topical (bovine origin) is prepared from prothrombin isolated from bovine blood by special chemical techniques. The prothrombin is activated with bovine thrombokinase in the presence of required amounts of calcium chloride and sodium chloride.” Since tissue factor (thrombokinase) is used in the activation of the prothrombin, then, according to published data, Factors V, VII, and X must be present in the reaction mixture for the formation of prothrombin activator. As noted previously, some of these clotting factors were actually detected in the commercial thrombin preparation. Since, in the presence of activated Factor X or thrombin, one cannot specifically test for other clotting factors participating earlier in the coagulation sequence, what evidence can be provided to indicate that neither the thrombin nor the activated Factor X peaks contain these other clotting factors? This problem was approached indirectly. Bovine

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3 E. T. Yin and S. Wessler, unpublished data.
plasma barium sulfate eluate rich in Factors II, VII, IX, and nonactivated X was freshly prepared and chromatographed on a DEAE-cellulose column under the identical conditions employed for the Parke-Davis thrombin preparation (Fig. 2). The first protein peak did not contain any clotting factors, and Factors II, VII, IX, and nonactivated X were confined to the second peak (Fig. 4). Furthermore, no coagulation activity was detected in the zone where activated Factor X was eluted from the Parke-Davis thrombin.

Extent of Activated Factor X Contamination in Parke-Davis Thrombin Preparation—To determine quantitatively other clotting factors that participate earlier in the coagulation sequence in a fraction containing thrombin, this enzyme first must be removed from the test material. However, qualitative analyses for these factors can be performed with some reliability, if the concentrations of the clotting factors to be tested are sufficiently great to permit removal of the effect of the thrombin by dilution of the test sample.

The experiments shown in Table I were performed to demonstrate the amount of activated Factor X present in the commercial bovine thrombin preparation. Two activities were differentiated, the direct action of thrombin on fibrinogen (Column 2) and the capability of the activated Factor X, present as a contaminant, to convert prothrombin to thrombin in the presence of Factor V and phospholipids (Column 4). The substrate used in Column 4 was the same as that of Column 3. At a thrombin concentration of 0.5 NIH unit per ml, giving a fibrinogen clotting time of 165 sec, the presence of activated Factor X in the same fraction was capable of giving a clotting time of 17 sec in the activated Factor X assay system, whereas the same fraction clotted the Factor X assay substrate (Seitz plasma) in 463 sec in the absence of added phospholipids and calcium. The latter test acted as a control to indicate the influence of the thrombin on the Seitz plasma. At a thrombin concentration of 0.05 NIH unit per ml, the fibrinogen clotting time was 1140 sec, whereas the activated Factor X contaminant was capable of yielding a Factor X assay clotting time of 47 sec. The fraction did not clot the Seitz plasma in 7200 sec. The buffer time for the activated Factor X assay was 565 sec. Thus, in a diluted fraction, thrombin activity was negligible, and the activated Factor X activity was still present in appreciable amounts.

Proteolytic Activity—The purified thrombin was assayed on fibrin plates (41) and no lysis was observed. It was also assayed by the a-casein method for proteolytic activity in the presence and absence of streptokinase (42); however, no significant hydrolysis occurred. These assays were kindly performed by Dr. Norma Alkjaersig, Department of Medicine, Washington University School of Medicine, St. Louis.

**DISCUSSION**

Besides other unidentified proteins, preparations of bovine thrombin have been reported to contain tissue factor, plasminogen and its activator, and even prothrombin (43, 44). The techniques available to prepare thrombin are lengthy, and therefore the majority of investigators turn to the readily available commercial products. Fig. 1 illustrates the impurities present in the Parke-Davis thrombin product. The elution profile of the fraction on Sephadex G-200 is typical of that of either serum or plasma. Three distinct protein peaks representing 19 S and 7 S globulins and albumin are obtained. Rather infrequently have investigators, using commercial thrombin preparations, taken the precaution of further purification before use. Perhaps the most widely employed method for thrombin purification is that described by Rasmussen (32), but this technique does not demonstrate that the purified thrombin fraction is free of other clotting factors. Recently, Cole, Koppel, and Olwin (27) reported that Parke-Davis bovine thrombin is highly contaminated with activated Factor X. Despite this report, several investigators have recently published data using untreated Parke Davis thrombin to determine the effect of this enzyme in blood coagulation kinetics and its effects on animals in experiments in vivo.

The technique described in the present communication yields both thrombin and activated Factor X free from each other and also from other clotting factors, as well as from plasminogen and plasmin. These thrombin fractions are also free of tissue thromboplastin or phosphatides, since these materials are excluded in the void volume on Sephadex chromatography (11). The chromatographic techniques described are simple, and reproducible results can be obtained when the procedures are performed.
with care. Although the specific activity of thrombin obtained with this technique is low as compared to that obtained by Seegers (45), our thrombin fractions are demonstrably free of contaminating clotting factors, particularly activated Factor X. This latter enzyme possesses esterase activity and can hydrolyze a variety of synthetic basic amino acid esters also hydrolyzed by thrombin.

It is possible to prepare thrombin of higher specific activity by recycling the fraction obtained by the methods described herein on a phosphate column (31) or on a resin column (32). However, the DEAE-cellulose-chromatographed thrombin lost more than 50% of its activity during freeze-drying and dialysis. These were some of the factors contributing to the low specific activity of our final Sephadex-chromatographed thrombin product. Similar findings have been reported by Seegers (46).

The definition of a unit of activated Factor X activity has been arbitrarily defined in an earlier section. The activated Factor X peak as depicted in Fig. 2 contained a total of 3020 units of activity and 2.76 mg of protein, recovered from 2 vials of Parke-Davis topical thrombin. Assuming (on the basis of our method for the purification of venom- or trypsin-activated Factor X on a DEAE-cellulose column with the same buffer) a 75% recovery of this activity and more than 95% of the protein from the column, each vial of Parke-Davis thrombin should contain approximately 1.5 mg of activated Factor X. This would represent 18% of the total protein content in 1 vial of the commercial thrombin. Although small in terms of protein contamination, this amount of activated Factor X is large in terms of biological activity. Parke-Davis thrombin at a concentration of 0.05 unit per ml of thrombin activity yields a fibrinogen clotting time of 1140 sec, but this same fraction also results in a clotting time of 47 sec, as measured in the activated Factor X assay (Table I). This latter assay determines the ability of the activated Factor X present in the fraction together with Factor V, phospholipids, and calcium, to convert the prothrombin present in the Seitz plasma to thrombin. From the data in Table I, the contamination of activated Factor X in Parke-Davis thrombin is large. Accordingly, any investigations using such preparations in the study of coagulation and enzyme kinetics or in experiments in vivo can be misleading.

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REFERENCES
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E. T. Yin and S. Wessler


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