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# AFFINITY CHROMATOGRAPHY OF THROMBIN

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## SUMMARY

Bovine thrombin (EC 3.4.4.13) has been purified by adsorption onto *p*-chlorobenzylamido-ε-aminocaproyl agarose, the ligand having been covalently bound to agarose by the cyanogen bromide technique. Thrombin was eluted from the adsorbant with 1.0 M benzamidine and was recovered by gel filtration through Sephadex G-25 at high ionic strength to prevent matrix interaction. Both yield and purity of the thrombin purified by the affinity technique were facilitated by prior removal of contaminant inhibitors and esterases from commercial preparations by the carboxylic acid resin procedure. Affinity chromatography of the resin-purified thrombin with the substituted agarose resulted in a final product containing 2500 N.I.H. clotting units per mg protein.

## INTRODUCTION

Recent studies in our laboratory have centered on a highly purified Factor VIII (ref. 1) and its modification by thrombin (EC 3.4.4.13). The thrombin effect was first investigated by RAPAPORT *et al.*[2] but has not been examined with a thrombin preparation of high purity and one which is free of clotting contaminants. Accordingly, affinity chromatography was tested as a possible simple and rapid method for thrombin purification since thrombin, even when highly purified, contained multiple components[3–5], some of which may be inactive protein[5].

Thrombin, like other serine proteases, is inhibited by derivatives of benzylamine and benzamidine[6]. Of these, only *p*-chlorobenzylamine has a lower $K_i$ for thrombin than for trypsin or plasmin. Accordingly, this competitive inhibitor was selected for study as an affinity moiety for thrombin purification. This ligand can be linked to agarose by the cyanogen bromide technique[7,8] either directly by its free amino group or after coupling to ε-aminocaproic acid which then forms a bridge between the ligand and the agarose[9].

Elution of proteins from affinity gels can usually be achieved by extremes of pH, increased salt concentrations, or addition of free inhibitor. The pH approach is

Abbreviation: TAME, *p*-tosyl-L-arginine methyl ester.

less useful in displacing bound thrombin since thrombin's stability is limited below pH 5.5 and above pH 9 (ref. 10). Displacement of thrombin by an inhibitor such as benzamidine, however, is effective since it has a lower $K_i$ for thrombin as compared to *p*-chlorobenzylamine[6].

Commercial thrombin is often contaminated by inhibitors[10] as well as other similar esterases including Factor $X_a$ (refs. 11–13). The former might compete with affinity binding and decrease its efficiency, whereas the latter could also be bound and purified along with the thrombin. The carboxylic acid resin purification of RASMUSSEN[14] removes most inhibitors and contaminant esterases because activity yields are frequently in excess of 100% and the breakthrough peak contains *p*-tosyl-L-arginine methyl ester (TAME) esterase activity[15] but no clotting activity[14]. Resin-purified thrombin demonstrates a striking parallel between clotting and esterase inhibition by isopropyl methylphosphonofluoridate (sarin) suggesting that esterase and clotting activities are due to the same enzyme[16]. In contrast, the sarin inhibition of esterase and clotting activity of commercial thrombin does not occur in parallel. This data strongly supports the contention that resin-purified thrombin is free of Factor $X_a$ since TAME esterase and clotting activities of the latter are less sensitive to DFP inhibition than thrombin[17,18].

In the present communication, we wish to describe a procedure for further purification of thrombin which removes most of the contaminants. This procedure is simple, rapid, and gives a high yield of product.

## MATERIALS AND METHODS

Commercial thrombin was obtained from Parke, Davis and Co., Detroit, Mich., in 10 000 unit vials. N.I.H. standard (Lot B3) was kindly provided by Dr. D. L. Aronson, Division of Biologic Standards, National Institutes of Health, Bethesda, Md. Fibrinogen was either bovine Fraction I (Pentex, Kankakee, Ill.) cryoprecipitated as previously described[16] or a 98% clottable bovine preparation with 1 mg NaCl per 1 mg protein as purified by the method of BLOMBÄCK AND BLOMBÄCK[19] and generously supplied by Dr. S. Iwanaga, Institute for Protein Research, Osaka, Japan.

*p*-Chlorobenzylamine and benzamidine·HCl were purchased from Aldrich Chem. Co., Milwaukee, Wisc., and ε-aminocaproic acid from Sigma Chem. Co., St. Louis, Mo., as the *N*-carbobenzoxy derivative. ε-Aminocaproyl-*p*-chlorobenzamide·HCl was prepared by Dr. S. Sakakibara, Institute for Protein Research, Osaka, Japan, utilizing a water soluble carbodiimide procedure*. $E^{1\%}_{275\ nm}$ was 3.78 for this compound. TAME was purchased from Mann Research Laboratories, New York, N.Y.

Agarose gel (Type 1.5M) was supplied by Bio-Rad Labs., Richmond, Calif. CNBr was purchased from J. T. Baker Chem. Co., Phillipsburg, N.J., and sodium 2,4,6-trinitrobenzene sulfonic acid from Aldrich Chem. Co. CG-50 type II (Fischer Scientific)** was the generous gift of Dr. Jules Gladner, Division of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. Sephadex G-25

* Dr. Sakakibara found this compound homogeneous on paper electrophoresis and thin layer chromatography; m.p. 156–157°; (Found: C, 50.0; H, 7.3; N, 9.2. $C_{13}H_{17}N_2Cl \cdot HCl \cdot 1.2\ H_2O$ requires: C, 50.0; H, 7.2; N, 9.0%). Prior to analysis, the compound was dried 10 h at 15° over $P_2O_5$ *in vacuo*.

** This particular type of resin is no longer commercially available and other CG-50 types are unsatisfactory. Success has been reported with Bio-Rex 70 (Bio-Rad)[4] or SE-Sephadex[26].

was purchased from Pharmacia Fine Chemicals Inc., Piscataway, N.J. Clotting activity was determined as previously described[16].

*Specific activities*

A 98% clottable fibrinogen at 1 mg protein with 1 mg NaCl per ml distilled water was used. Assays were performed by pipetting 0.1 ml of the thrombin at an appropriate dilution into 0.2 ml fibrinogen at 28°, and the first visualization of granules was determined as the end point. Triplicate assays over the range of 20 to 60 sec agreed to within 10%. Thrombin samples were diluted in both 0.05 or 0.15 M $NaH_2PO_4$ (pH 7.0) and dilution curves were compared to those of N.I.H. standard thrombin (Lot B3) dissolved and diluted in the same buffers. All dilutions and clotting measurements were performed with siliconized glassware to avoid glass adsorption of thrombin. Protein was determined by absorbance at 280 nm, assuming the extinction coefficient of 19.5 (ref. 21). Corrections were made by subtracting 1.7 times the absorbance at 320 nm, as suggested by BAUGHMAN AND WAUGH[10], and ranged from 15 to 20% prior to affinity chromatography.

Hydrolysis of TAME was measured by a titrimetric method as previously described[16] except the temperature was 22.0 ± 0.5°, and polyethylene reaction vessels were employed. Spontaneous hydrolysis was less than 0.005 $\mu$mole·min$^{-1}$ and hydrolysis with thrombin was in the range of 0.1 $\mu$mole·min$^{-1}$.

*Agarose gel substitution*

The procedure of CUATRECASAS[8] was followed for activation of 55 ml agarose gel by 10 g of CNBr. The ligand, 500 mg $\varepsilon$-aminocaproyl-*p*-chlorobenzamide·HCl (equivalent to 30 $\mu$moles/ml agarose), was added to the activated washed gel in 50 ml 0.1 M $NaHCO_3$ (pH 10.0) and incubated for 12 h at 2°. Absorbance measurements of the reaction filtrate and washings at 275 nm indicated 70% of the ligand was coupled to the agarose. The substituted gel produced a bright orange color by the trinitrobenzene sulfonic acid test[8]. The gel could be reused several times when stored in 0.02% sodium azide at 2°.

*Resin purification of thrombin*

The initial purification of thrombin from commercial preparations involved a slight modification of the procedure of RASMUSSEN[14] as previously described[16]. Important features of this method include type of resin used (CG-50 Type II), extensive equilibration with initial buffer before adding the sample, and a moderate flow rate. In the present experiments, the flow rate was 4–6 ml/h and 20-min fractions were collected at room temperature. Glass wool, columns, and fraction tubes were siliconized to avoid adsorption of the highly purified thrombin.

*Affinity chromatography of resin-purified thrombin*

A 1.2 cm × 3.0 cm $\varepsilon$-aminocaproyl-*p*-chlorobenzamide·HCl column of agarose equilibrated with 0.3 M $NaH_2PO_4$ (pH 8.0) adsorbed all clotting activity from one preparation of resin-purified thrombin containing 3.4 mg in 23 ml effluent buffer. The column was washed with 20 ml 1.0 M NaCl added to the initial phosphate buffer. This removed a faint yellow pigment from the sample on the gel. Benzamidine·HCl (10 ml of 1.0 M in 0.3 M $NaH_2PO_4$, pH 7.0) eluted the thrombin which was recovered

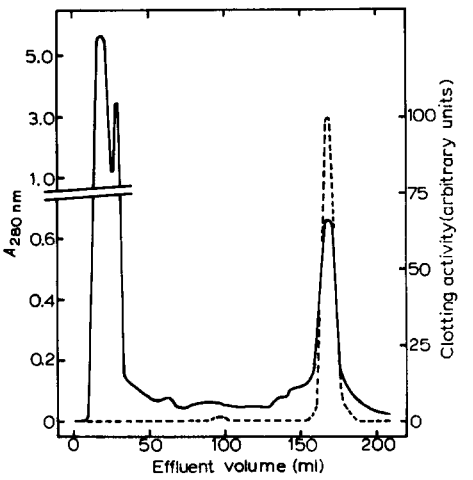


Fig. 1. Resin purification of thrombin. Commercial thrombin (10 000 Parke–Davis units) was applied to a 1.5 cm × 25 cm CG-50 Type II column in 0.05 M $NaH_2PO_4$ (pH 7.0) with stepwise elution by 0.3 M $NaH_2PO_4$ (pH 8.0) as previously reported[14,16]. ———, corrected $A_{280\ nm}$ (ref. 10); — — —, fibrinogen clotting[16], assayed every 5 ml under the main activity peak, every 20 ml elsewhere.

by gel filtration on Sephadex G-25. The Sephadex G-25 was previously equilibrated with 0.75 M NaCl in 0.05 M $NaH_2PO_4$ (pH 7.0).

## RESULTS

### *Resin purification of thrombin*

A typical elution pattern for the chromatography of commercial thrombin on the carboxylic acid resin is presented in Fig. 1. Thrombin activity appeared after 155–160 ml of effluent, while fractions from the breakthrough peak contained no clotting activity. With some preparations, a clotting peak was found between 50 and 100 ml which represented 5% of the total activity recovered. The nature of this early

TABLE I

PURIFICATION OF THROMBIN

| *Thrombin* | *Protein** (*mg*) | *Activity* | |
|---|---|---|---|
| | | *clotting*** (*N.I.H. units·mg⁻¹*) | *Esterase**** (*μmoles·min⁻¹·mg⁻¹*) |
| Commercial | 72.0 | 40 | — |
| Resin-purified | 3.4 | 1600 | 44 |
| Affinity-purified | 2.0 | 2500 | 59 |

* $A_{280\ nm}$ corrected by subtracting 1.7 × $A_{320\ nm}$, assuming the extinction coefficient of 19.5 (ref. 21.)

** Specific activity against 1 mg/ml purified fibrinogen at 28° compared to N.I.H. standard thrombin, Lot B3.

*** TAME esterase activity at 22° using 20 mM MAME in 0.15 M KCl at pH 8.0.

peak was not investigated further since in subsequent lots of commercial thrombin it represented less than 1% of the total activity. Ten thrombin preparations from three lots of commercial thrombin showed nearly identical elution patterns. The recovery of thrombin activity ranged from 100 to 180%. The apparent high yield in some preparations presumably is related to the removal of inhibitory material. The overall purification from the starting preparation was about 20–40-fold (Table I).

*Affinity chromatography*

In preliminary experiments, it was observed that *p*-chlorobenzylamine coupled to ε-aminocaproic acid would inhibit fibrinogen clotting by resin-purified thrombin with an approximate $K_i$ of 10 mM, compared to 3 mM reported for *p*-chlorobenzylamine alone[6]. In contrast, ε-aminocaproic acid alone had no effect at these concentrations. The *p*-chlorobenzylamine attached directly to agarose was less effective in binding thrombin than the ε-aminocaproic acid derivative.

The recovery of thrombin purified by affinity chromatography was greater than 90% with a 1.5-fold increase in protein purification (Table I). The affinity-purified thrombin had no residual absorbance at 320 nm. Attempts to remove thrombin with NaCl at concentrations as high as 3 M in phosphate buffer or by elution with 0.2 M ε-aminocaproyl-*p*-chlorobenzamide·HCl were unsuccessful. Acetic acid removed the protein from the column, but no activity was recovered.

TAME esterase activities were determined to further compare resin-purified and affinity-purified thrombins. Initially, esterase activities of affinity-purified thrombin were variable, but a second gel filtration apparently removed traces of benzamidine and the specific esterase activities then increased. The increase in specific esteras activity is similar to the degree of protein purification achieved by affinity purification. Representative results are presented in Table I.

## DISCUSSION

Competitive inhibition of trypsin-like enzymes by benzylamines and benzamidines was thought to require a free amino function[6]. Since ε-aminocaproyl-*p*-chlorobenzamide·HCl inhibited thrombin nearly as well as *p*-chlorobenzylamine alone and whereas ε-aminocaproic acid had no effect, the coupled compound probably inhibits by a similar mechanism. Because the competitive inhibitor benzamidine could elute thrombin from the affinity column, it seems likely that both inhibitors can occupy the same active site. Other analogues of these inhibitors should prove useful for affinity chromatography of other serine proteases. The *p*-amino derivatives might be particularly well suited because of the lower p$K$ of the aromatic amine.

The specific activity of thrombin is difficult to assess due to the lack of a pure standard and of an accurate assay method. N.I.H. standard thrombin contains but 20 units/mg which is even less than the commercial variety. Contaminant esterases preclude ester assays, whereas inhibitors would influence clotting at different fibrinogen concentrations. The clotting assay also depends upon the non-enzymatic polymerization of fibrin with its lower p$K$ and sensitivity to changes in ionic strength. With a carefully controlled clotting assay, BAUGHMAN AND WAUGH[10] estimated their specific activity as ±300, whereas BATT *et al.*[4] questioned the significance of a 700-

unit difference. Nevertheless, the N.I.H. standard thrombin assayed according to the 1946 manual[22] remains the best means of comparing various thrombin preparations.

In this study, the specific activity of affinity-purified thrombin was increased over the resin-purified form and was somewhat higher than any previously reported. Theoretically, inactive thrombin should be removed by the affinity step and this may relate to the loss of absorbance at 320 nm. The increase in esterase activity paralleling protein purification also suggests loss of inactive protein. Thus, the present thrombin purification procedure, in addition to being rapid and efficient, may well be one of the most effective procedures thus far described. Success of the affinity step, however, is dependent upon an efficient first step, such as the RASMUSSEN[14] procedure. Attempts to purify commercial thrombin directly by affinity chromatography have been much less satisfactory.

Affinity-purified thrombin does modify highly purified Factor VIII, activity being determined by the kaolin recalcification time of Factor VIII-deficient plasma. When 0.03 $\mu$g of thrombin was incubated with 12.5 $\mu$g Factor VIII at 2° with phospholipid, Factor VIII activity rapidly increased in 3 min to 50-fold greater than control activities without thrombin (A. R. THOMPSON, E. P. KIRBY AND E. W. DAVIE (1971), unpublished experiments). This activity was stable for 1 h at 2° before gradually declining.

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