The Purification of Thrombin and Isolation of a Peptide Containing the Active Center Histidine*

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SUMMARY

A chromatographic purification of bovine thrombin from commercial starting material is described which yields preparations judged to be essentially pure, that is, with a clotting activity of 2100 to 2500 NIH units per mg. In addition to the two-chain form of thrombin, the purified enzyme contains a three-chain form arising from cleavage of the B chain at arginine-73 (or 76). This form must be fully active since the specific clotting activity of various preparations does not vary with the content of the three-chain form, amounting at times to 70%.

Inactivation of thrombin with Na-tosyl lysyl chloromethyl ketone and Na-(p-nitrobenzyloxycarbonyl)arginyl chloromethyl ketone results in alkylation of nitrogen 3 of the active center histidine with loss of both clotting and esterase activities. A radioactive peptide has been isolated from thrombin inactivated with tritiated Na-tosyl lysyl chloromethyl ketone and shown to contain histidine-43.

Thrombin is a proteolytic enzyme whose esterase action resembles trypsin with respect to a preference for esters of arginine (1) and lysine (2) whereas its action on polypeptide and protein substrates is considerably more limited. Thrombin rapidly cleaves only four Arg-Gly bonds in its natural substrate fibrinogen although 200 to 300 potential points of tryptic cleavage (3) are present. We were interested in the effect of the specific inhibitors [active-site directed] of trypsin, 1-chloro-3-tosylamido-7-amino-2-heptanone (4), and the arginine analogue of TLCK,1 the chloromethyl ketone derived from Nγ-(p-nitrobenzyloxycarbonyl)arginine (5) on thrombin, hoping to obtain information about active center residues by chemical modification. For this purpose purified thrombin was essential.

The chromatography of thrombin on a carboxylate ion exchange resin was introduced by Rasmussen (6). This method has yielded highly active preparations of thrombin when semipurified prothrombin has been used (7, 8). However, with the convenient, commercially available topical bovine thrombin from Parke-Davis as a starting material, this method of chromatography is less effective. An alternate method of purification of the commercial material has been reported by Baughman and Waugh (9). At the time of a preliminary account of our investigation (10), Mann and Bahl (11) also suggested a modified procedure. However, the method we describe, employing Bio-Rex 70 carboxylic resin (—400 mesh) succeeds in removing species of thrombin which have lost clotting activity and provides a convenient and reliable means of obtaining highly active preparations comparable to the best isolated from plasma.

The thrombin thus obtained was shown to contain the two-chain form of thrombin described by Magnusson (12) along with an additional, fully active form cleaved in the B chain.

Thrombin was inactivated by the affinity-labeling reagents TLCK (4) and p-NOZACK (5) both of which alkylated N-3 of a histidine residue. Histidine-43 was identified as the active center histidine through isolation of a radioactive peptide from 3H-TLCK-inhibited thrombin.

MATERIALS AND METHODS

Materials

Proteins were purchased as follows: topical thrombin (bovine), Parke-Davis; twice crystallized trypsin and a-chymotrypsin, Worthington; crystalline bacterial protease Novo, Novo; pepsin and bovine fibrinogen, Fraction I, 65%, clottable, Pentex, diisopropyl fluorophosphate-treated carboxypeptidase A and B, Worthington.

Benzyloxycarbonyl ethyl hydrochloride was purchased from Mann, imidazole from Eastman, and gum arabic from Fisher. Sephadex derivatives were purchased from Pharmacia; cellulose ion exchange derivatives Cellex-P and Cellex-D as well as Bio-Rex 70, —400 mesh, carboxylic resin, from Bio-Rad; Rexyn 102 H², 200 to 400 mesh, carboxylic resin from Fisher.

The thrombin purification column was made of cast acrylic plastic pipe (Franklin Fibre-Lamitex Corporation, Wilmington, Delaware; 7.5 cm, inner diameter × 30 cm). It was closed at either end with No. 14 rubber stoppers equipped with plastic fittings and stopcocks (Pharmaseal Laboratories, Glendale, California) to fit hospital tubing (Bard-Parker Company, Rutherford, New Jersey). The resin was supported on porous
polyethylene discs (Scientific Glass Company) cut to the exact inside diameter of the column. The thrombin-concentrating column was a 50-cm plastic syringe (Beckton, Dickinson and Company, Rutherford, New Jersey) fitted in the same manner. All other columns were of glass, with sintered glass discs for packing support.

The principal buffers used for thrombin chromatography were a pH 7.0 buffer, 0.05 M in sodium phosphate and a pH 8.0 buffer, 0.3 M in sodium phosphate. For the concentration step, 1 M sodium chloride buffered with 10 ml of 0.5 M potassium phosphate, pH 7.0, per liter was used.

TLCK, tritiated TLCK (H-TLCK), and p-NO2-ZACK were prepared as described (5).

Methods

The clotting activity of thrombin was measured according to the method of Kline (13) by means of a standard curve of clotting time versus thrombin concentration in NIH units per ml, using NIH standard Lot 8-3 for this purpose. The curve was valid as long as the same bottle of fibrinogen was used. Standard weighed samples of fibrinogen were stored at –20° until used.

Thrombin concentrations were measured by titration with the active site titrant p-nitrophenyl p-guanidinobenzoate according to the method of Chase and Shaw (14) with the use of a Cary 15 spectrophotometer.

A molecular weight of 36,000 was used to calculate thrombin concentrations in terms of milligrams per ml for specific activity comparisons.

Esterase activity was determined on benzoylarginine ethyl ester spectrophotometrically (15) by means of a Beckman DB spectrophotometer equipped with a Leeds and Northrup recorder.

Disulfide bonds were cleaved and carboxymethylated according to the method of Crestfield, Moore, and Stein (16); the reaction mixture was acidified with acetic acid and applied directly to a-G 75 columns wrapped in aluminum foil for simultaneous separation of the peptide chains and desalting. Cyanogen bromide splitting was accomplished by a published procedure (17). Amino acid analyses were performed with a Beckman-Spinco analyzer on the products of a 22-hour hydrolysis in 6 N hydrochloric acid in evacuated, sealed tubes.

The subtractive Edman procedure as described by Edzuga, Lai, and Hirs (18) was used for NH2-terminal and amino acid sequence determinations. Carbohydrate was qualitatively determined by the phenolsulfuric acid test for glycoproteins (19). Peptides were detected by alkaline hydrolysis in polypropylene tubes and the ninhydrin reaction according to the method described by Hirs (20). Carboxy-terminal sequences were determined by the use of carboxypeptidase A and B as described by Amblor (21).

Resin and Columns for Thrombin Purification

Bio-Rex 70, 400 mesh, resin was pre-cycled according to the method of Hirs (22), except that fines were removed after 1 to 2 hours of settling in view of the small particle size of this resin. The acid form of the resin was stirred with 2 volumes of pH 7.0 buffer and the pH was adjusted to 7.0 with 50% sodium hydroxide. When the pH remained constant for 1 hour, the resin was poured into the column as a slurry. When about 2 cm of resin had settled, a slow flow of buffer was started and a column of 20 to 25 cm of resin was allowed to settle. The column was washed with 4 liters of pH 7.0 buffer before use.

Cellex-P and Cellex-D resin were pre-cycled and regenerated as recommended by the manufacturer. The thrombin-concentrating column (described under “Materials”) was poured by means of a glass extension tube (1.5 X 100 cm) through which a 1:1 slurry of Cellex-P and buffer adjusted to pH 7.0 was poured with the stopcock fully opened until a bed height of 11 cm under a 100-cm head of buffer was obtained. Before use the column was washed with 400 ml of pH 7.0 buffer.

Thrombin Chromatography—The contents of thirty 10,000-NIH unit vials of topical thrombin were dissolved in 100 ml of pH 7.0 buffer; the buffer was first used to rinse the vials after the solid had been removed. After adjusting the pH to 6.9 with 0.1 M sodium hydroxide, the solution was applied to the Bio-Rex 70 column and rinsed in with buffer. (Occasionally the top of the resin bed cracked and separated as the thrombin was applied in which case the first 5 cm of resin bed was stirred with buffer and allowed to resettle. This was not detrimental to the subsequent chromatography.) Elution was started with pH 7.0 buffer; 20-nl fractions were collected at 10-min intervals. When the absorbance at 280 mg dropped below 0.05 (usually after 16 to 20 hours), the pH 8.0 buffer was introduced and elution continued at the same rate; 16-nl fractions were collected in polyethylene tubes at 6-min intervals. Thrombin was obtained in the last peak eluted (usually after 30 to 36 hours). The tubes with specific activities >700 NIH units per absorbance unit were pooled for concentration. (Baughman and Waugh (9) have discussed the relationship between the various methods of expressing clotting activities; our absorbance values were uncorrected.)

Concentration of Dilute Thrombin—The pooled thrombin from Bio-Rex 70 was diluted 4.3-fold and passed through the Cellex-P concentrating column as rapidly as it would flow (a 2-m head of buffer was used). The absorbed thrombin was eluted with 1 M sodium chloride; 2-ml fractions were collected in polyethylene tubes. Fractions of specific activities >800 to 1000 NIH units per absorbance unit were pooled giving solutions of 10 to 14 absorbance units per ml or 5 to 7 mg per ml.

Ammonium Sulfate Precipitation of Thrombin—The thrombin pool in 1 M sodium chloride was treated at 0° with 0.55 g of powdered ammonium sulfate (reagent grade) per ml in a polyethylene beaker with gentle stirring at 0° until all had dissolved. After transferral to 50-ml polyethylene centrifuge tubes with washings of five 1-ml portions of saturated ammonium sulfate, the suspension was centrifuged at 10,000 rpm for 15 min in the cold. The precipitated thrombin was stored in the frozen state at –20° after solution in 0.05 M sucrose (5 ml); the concentration was about 15 mg per ml.

Inhibition of Thrombin with TLCK and p-NO2-ZACK—Purified thrombin at a concentration of 6.47 × 10−4 M and a specific activity of 1830 NIH units per mg was used.

TLCK Thrombin—A solution of 9 ml of thrombin and 1 ml of 0.5 M potassium phosphate, pH 7.0, was treated with TLCK (9 × 10−4 M). Loss of esterase activity reached 83% in 65 min and was essentially complete in 120 min.

p-NO2-ZACK Thrombin—A mixture of 4 ml of thrombin and 0.5 ml of 0.5 M potassium phosphate, pH 7.0, was treated with 16.9 mg of a p-NO2-ZACK preparation (5) (this quantity corresponded to 0.8 × 10−9 moles of actual p-NO2-ZACK determined by titration with β-trypsin (5)) giving a final inhibitor concentration of 1.5 × 10−4 M. The inhibition was complete within 2 hours.
The above reaction mixtures were extensively dialyzed against deionized water and lyophilized. An aliquot of the TLCK-inhibited thrombin and all of the p-NO₂-ZACK-inhibited thrombin was oxidized with performic acid (23).

**Inhibition of Thrombin with H-TLCK**—The H-TLCK used had a specific activity of $8.75 \times 10^6$ dpm per amole. Thrombin was in 1 M sodium chloride as obtained from the Cellex-P concentrating column. To each 10 ml of protein solution was added 54 mg (3.9 $\times$ 10⁻⁴ moles) of sodium phosphate monohydrate, 87 mg (6.1 $\times$ 10⁻⁴ moles) of disodium phosphate, and 18.5 mg of H-TLCK (5 $\times$ 10⁻⁵ moles) giving 0.1 M phosphate buffer, pH 7.0, and 5 $\times$ 10⁻⁴ M H-TLCK. When loss of esterase activity reached 99% (5 to 6 hours) the solution was dialyzed exhaustively against deionized water and lyophilized.

**Peptic Digestion of Cyanogen Bromide Peptide 52-84 from the B Chain H-TLCK Thrombin**—The peptide (1.98 pmoles), obtained as described in Fig. 6, was digested with 1.5 mg of pepsin at 25° in 3.5 ml of 5% formic acid (24) for 22 hours. The reaction mixture was evaporated in a vacuum and the residue was gel filtered on Sephadex G-50 (Fig. 6).

**RESULTS**

Thrombin has previously been purified to high specific activity by ion exchange chromatography on a carboxylate resin (Amberlite IRC-50) (7, 8). However, the starting material for such preparations was obtained by activation of the partially purified zymogen, prothrombin, obtained from fresh plasma. This is generally not a convenient starting material and we turned our attention to the preparation of thrombin at high specific activity from a more convenient but relatively crude commercial source, Parke-Davis topical thrombin. Such a method would allow many other laboratories lacking facilities for fractionation of blood ready access to this important and interesting enzyme. The procedure of Baughman and Waugh (9) appeared to solve this problem, since they reported that the purification of Parke-Davis topical thrombin as described, thus is comparable to the Cellex-P column providing 70 to 80 mg of thrombin usually with specific activities of 1,000 to 4,000 NIH units per mg of thrombin. A typical chromatogram of thrombin (Parke-Davis topical, bovine, 300,000 NIH units) is shown in Fig. 1.

The elution pattern is similar to that obtained by Magnusson (8) except that the broad area designated esterase thrombin was not found in his preparations. While esterase thrombin had the same amino acid composition as thrombin eluted subsequently with high specific activity (Table I) it was devoid of clotting activity. Titration of the active center of esterase thrombin with p-nitrophenyl p-guanidinobenzoate (14) gave 40 to 50% of the expected esteratic site content when the thrombin concentration was measured using the extinction coefficient of purified thrombin (29).

Although thrombin has been shown to lose clotting activity more rapidly than esterase activity eventually leaving only a low level of esterase activity (7), this is the first isolation of a highly active esterase thrombin chromatographically. This material has not been further investigated but could provide information about structure-activity relationships in thrombin. It amounted to about 50% of the total initial esterase content.

The active thrombin in the last peak (Fig. 1) was pooled and concentrated by absorption and desorption from a cellulose phosphate column shown earlier to absorb thrombin quantitatively at low ionic strength (9). From the 300 to 400,000 NIH units of crude thrombin applied to the Bio-Rex 70 column, about 40 to 50% of the clotting activity was recovered in purified and concentrated form from the Cellex-P column providing 70 to 80 mg of thrombin usually with specific activities of 1,000 to 1,100 NIH units per absorbance unit. This activity is comparable to that of the most active preparations obtained in other laboratories (9). The thrombin concentration of such preparations was also measured by titration with p-nitrophenyl p-guanidinobenzoate (14) and shown to reach a level of 2,100 to 2,500 NIH units per mg based on a molecular weight of 36,000. Fully active thrombin is generally considered to have a specific activity of about 2,000 NIH units per mg (7-9). The amino acid composition of purified thrombin (Table I) was constant from run to run.

Magnusson (8) reported an average yield of 74 mg of thrombin with an average specific activity of 2,900 NIH units per mg of dry weight from semipurified prothrombin obtained from 20 liters of plasma. Chromatography of 300,000 units of Parke-Davis topical thrombin as described, thus is comparable to the processing of 40 liters of whole blood.

About half of the clotting activity applied to the column was recovered as purified thrombin. Purified thrombin thus obtained could be rechromatographed on Bio-Rex 70 with no change in specific activity or amino acid composition. A third chromatography also gave no change. These results are not in accord with the report that rechromatography of thrombin separates a 75-residue peptide from thrombin with a 2-fold increase in specific activity (30).

![Fig. 1. Ion exchange chromatography of Parke-Davis topical thrombin (bovine) on Bio-Rex 70. The details are presented under "Methods."](image-url)
as in the case of trypsin (4, 5). Both inhibitors completely in-
and p-NOs-ZACK would give irreversible inhibition of thrombin
respect to amino acid side chains, it was expected that TLCK
activated clotting and esterace activities of thrombin. In each
from the current sequence studies of Magnusson (25) or rc-
all glucosamine (25).

Parke-Davis thrombin contains some enzymic constituent(s)
catalyzing the formation of esterase thrombin. The relative
low specific activities of our preparations of stock thrombin
from the failure to separate esterase thrombin from clotting
prepared by the method of Baughman and Waugh (9) results
undergo autodigestion at $1.4 \times 10^{-4}$ M,

Since thrombin and trypsin have comparable specificity with
other groups (26-28). Our preparations were also
rechromatography, along with the fact that thrombin loses
only about 20% of its clotting activity in 48 hours if allowed to
undergo autodigestion at $1.4 \times 10^{-4}$ M, pH 7.0, indicates that
Parke-Davis thrombin contains some enzymic constituent(s)
catalyzing the formation of esterase thrombin. The relatively
low specific activities of our preparations of stock thrombin
prepared by the method of Baughman and Waugh (9) results
from the failure to separate esterase thrombin from clotting
thrombin since both forms adhere to and are eluted from phos-
phocellulose under the same conditions.

The amino acid compositions of esterase thrombin, stock
thrombin, and purified thrombin arc in good aggre-
ment with each other (Table I) and with that of bovine thrombin calculated
in agreement with the report that thrombin contains
found to contain from 2 to 4 moles of glucosamine per mole of
3H-TLCK thrombin on Sepha-
radioactivity was not used for stoichiometry.) Gel filtration of
a progressive loss of radioactivity was observed. Therefore
radioactivity was not used for stoichiometry.) Gel filtration of reduced and carboxymethylated $^3$H-TLCK thrombin on Sepha-
active center histidine for compa-
ion of the inhibited enzyme gave 0.48 residue and 0.74 residue
per mole of TLCK- and ZACK-inhibited thrombin, respectively.

In order to facilitate isolation of a peptide containing the
active center histidine for comparison of its sequence with that
of other serine proteases and to establish its location in the
primary sequence, purified thrombin was labeled with tritiated TLCK. One mole of $^3$H-TLCK was incorporated per mole of
inhibited enzyme. (During subsequent degradation procedures
a progressive loss of radioactivity was observed. Therefore
radioactivity was not used for stoichiometry.) Gel filtration of reduced and carboxymethylated $^3$H-TLCK thrombin on Sepha-
dex G-75 (Fig. 2) gave unexpected results. Magnusson has
shown (12, 25) that thrombin arises by cleavage of the single
polypeptide chain of prothrombin in two places, releasing active
thrombin in a form consisting of two chains, A and B (Fig. 3),
connected by a disulfide bridge. In gel filtration of reduced,
carboxymethylated samples of $^3$H-TLCK-inhibited thrombin,
more than the two expected chains were consistently resolved as
in Fig. 2. The fragment corresponding to the 49-residue A
chain in composition was invariably found intact and unlabeled
(Fig. 2, peak at right). However, the radioactivity appeared
consistently distributed between two peaks which varied recip-
rocally in amount from one preparation to the next. The smaller
chain accounted for 10 to 70% of the radioactivity. It became

| Table I
Amino acid composition of thrombin preparations described in this paper compared with those of other workers |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Magnusson       | This paper      | Miller et al.   | Laki et al.     | Schrier et al.  | "Stock" thrombin|
|                 |                 |                 |                 |                 |                 |                 |
| Lysine          | 25              | 24.2            | 24.9            | 22.9            | 23.9            | 22.1            | 24.7            |
| Histidine       | 7               | 6.92            | 6.39            | 6.29            | 6.71            | 6.90            | 6.45            |
| Arginine        | 22              | 21.3            | 22.7            | 20.3            | 22.1            | 19.6            | 20.0            |
| Aspartic acid   | 27              | 28.9            | 28.3            | 29.0            | 27.1            | 29.4            | 28.8            |
| Threonine       | 13              | 14.1            | 14.0            | 14.5            | 13.6            | 14.9            | 13.6            |
| Serine          | 17              | 16.2            | 16.2            | 16.5            | 14.9            | 14.2            | 20.4            |
| Glucosamine     | 34              | 35.6            | 33.9            | 31.5            | 34.7            | 35.0            | 37.8            |
| Proline         | 16              | 16.1            | 16.1            | 14.6            | 13.2            | 14.4            | 15.2            |
| Glycine         | 25              | 26.8            | 25.5            | 25.0            | 27.9            | 28.0            | 31.8            |
| Alanine         | 15              | 15.1            | 16.2            | 16.0            | 14.3            | 13.3            | 16.7            |
| Half cystine    | 8               | 6.89            | 7.95            | 9.30            | 8.72            | 7.13            |
| Valine          | 23              | 20.6            | 16.4            | 14.0            | 20.6            | 20.2            | 20.0            |
| Methionine      | 5               | 4.82            | 4.94            | 5.17            | 5.31            | 4.24            | 4.65            |
| Isoleucine      | 16              | 13.7            | 12.3            | 9.79            | 14.5            | 13.4            | 13.5            |
| Leucine         | 27              | 28.1            | 27.3            | 26.3            | 30.2            | 25.0            | 28.0            |
| Tyrosine        | 11              | 11.8            | 11.9            | 11.6            | 13.7            | 12.2            | 10.7            |
| Phenylalanine   | 12              | 13.0            | 11.5            | 14.3            | 11.6            | 13.1            | 12.9            |
| Tryptophan      | 10              | 8.2             | 6.90            | 5.27            |                 |                 |                 |
| Glucosamine     | 2               | 2.4             | 2.69            |                 |                 |                 |                 |

a Calculated from the sequence in Reference 25.
b Average of 12 different preparations; values are uncorrected for destruction or resistance to hydrolysis.
c Calculated from data in Reference 26.
d Calculated from data in Reference 27.
e Calculated from data in Reference 28.
f Average of nine preparations purified according to the method of Baughman and Waugh (9).
g Average of six preparations obtained from Bio-Rex 70 (Fig. 1).

We are grateful to Dr. Darrell Liu for determinations of glu-
cosamine and tryptophan.
of a COOH-terminal arginine. Since a methionine is at position 17 of the B chain (25), cyanogen bromide cleavage of B-1 was expected to yield smaller derivatives with a composition corresponding to residues 1 through 17 and 18 through 73 and such evidence was obtained (Table II). In addition, the carbohydrate side chain of thrombin is known to be attached to a single asparagine residue near the NH$_2$-terminal of the B chain, possibly residue 50 (25). The B-1 chain was found to contain from 2 to 4 glucosamine residues, the total content found in the thrombin preparation from which it was derived.

The position of the proteolytic split within the B chain was localized through the use of carboxypeptidase A and B (21). Only the latter released free amino acids, providing 1 eq of arginine. On this basis, splits at arginine 71, 73, or 76 approximately accommodate the amino acid composition calculated for the B-1 chain (Table II). However, of these, arginine-73 is the best candidate due to the consistent indication of 3 residues of threonine rather than the two required by arginine-71. Arginine-76 was less probable due to values of arginine and glutamic acid consistently 1 residue less than required by a split at that point. Our data thus favor arginine-73; however, an additional tyrosine must be accounted for. A decision between arginine-73 and arginine-76 must await further work.

As mentioned above, only part of the radioactivity of H-TLCK-inactivated thrombin appeared in the B-1 peak. The intact (two-chain) form of thrombin often predominated. To conserve material for degradation studies use was therefore made of the labeled B chain obtained mixed with R-2 in the initial peak of gel filtration (Fig. 3). It was considered likely that each form of thrombin was labeled at the same histidine residue and that the presence of methionine at positions 17 and 84 of the B chain would permit the isolation of a radioactive cyanogen bromide fragment encompassing the same labeled histidine.

These have, however, been purified by successive gel filtrations in 5% acetic acid on G-75 columns such as that in Fig. 2. The amino acid compositions recorded in Table II are in general agreement with those expected.
Amino acid compositions of A, B, B-1, and B-2 chains of thrombin and of some selected peptides obtained by cyanogen bromide cleavage compared to compositions calculated from sequence in Reference 25

| Peptide | B-chain a | B-1 chain b | B-2 chain c | CNBr d B-chain e | CNBr B-1 chain f | Peptide g 1-17 18-84 40-44 |
|---------|-----------|-------------|-------------|----------------|----------------|-----------------|
| Lysine  | 21        | 21.1        | 3           | 3.41           | 18             | 17.2            |
| Histidine | 5         | 5.07        | 1           | 1.16           | 4              | 4.08            |
| Arginine | 20        | 20.1        | 5           | 4.86           | 15             | 13.0            |
| CMC g   | 7         | 7.09        | 2           | 1.60           | 5              | 4.45            |
| Aspartic acid | 23 | 24.7        | 6           | 6.03           | 17             | 18.0            |
| Threonine | 10        | 10.8        | 3           | 4.90           | 7              | 8.47            |
| Serine  | 15        | 12.8        | 6           | 4.43           | 9              | 8.06            |
| Glutamic acid | 22 | 21.5        | 7           | 7.04           | 15             | 15.0            |
| Proline | 14        | 15.6        | 4           | 3.72           | 10             | 11.1            |
| Glycine | 21        | 21.6        | 4           | 4.69           | 17             | 17.2            |
| Alanine | 13        | 12.6        | 4           | 3.98           | 9              | 9.03            |
| Half cysteine | 22 | 19.2        | 7           | 5.15           | 15             | 13.0            |
| Valine  | 21        | 19.2        | 7           | 5.15           | 15             | 13.0            |
| Methionine | 5         | 4.64        | 1           | 0.90           | 4              | 3.43            |
| Isoleucine | 15        | 13.2        | 3           | 2.61           | 12             | 10.4            |
| Leucine | 24        | 25.0        | 10          | 8.68           | 14             | 16.3            |
| Tyrosine | 10        | 9.91        | 1           | 1.98           | 9              | 7.58            |
| Phenylalanine | 6      | 7.0         | 2           | 2.32           | 4              | 3.30            |
| Glucosamine | 1.51      | 1-2         | 0           | 1              | 1.99           | 1.59            |

a One preparation (see text, Footnote 3).
b Average of 17 preparations (Fig. 2, Peak II).
c One preparation (see text, Footnote 3).
1 Average of five preparations (Fig. 5, Peak II), alanine = 3.00 residues.
1 One preparation (same procedure as in Fig. 5).
1 Cysteic acid, from air oxidation of S-carboxymethylcysteine.
1 CMC, carboxymethylcysteine.

residue obtained in B-1. Fractionation of the cyanogen bromide peptides obtained from the mixture of B and B-2 (Fig. 3) provided a radioactive peak similar in composition to the region 18-84 of sequence (25) (Table II) as expected.

The B-1 peptide and the cyanogen bromide fragment of the B chain contain two histidines, namely residues 43 and 69, out of the seven present in thrombin. To determine which of these two had been alkylated by Trl-CLK, further degradation by proteolytic action was sought. The cyanogen bromide peptide from B (residues 18-84) was digested with trypsin and chymotrypsin. However, even prolonged and repeated digestions released only a small amount of radioactivity which gave no promise of yielding a pure peptide on chromatography. On the other hand, subtilisin gave extensive digestion but the labeled peptides formed were too numerous for profitable resolution by ion exchange chromatography. Use was finally made of pepsin to obtain a suitable digest.

The pepsin digest of the labeled cyanogen bromide peptide (18-84) was fractionated on Sephadex G 50 (Fig. 6) giving 91% recovery of radioactivity digested with 65% conversion to a pool of smaller peptides (Fig. 6, Peak II). These were re-chromatographed on Bio Gel P 4 (Fig. 7) with 86% recovery of radioactivity applied, 77% of which appeared as a single peak. Chromatography of this on DEAE-Sephadex (Fig. 8) provided a 70% recovery of radioactivity of which 67% was found in Peak III. Rechromatography at lower ionic strength on DEAE-Sephadex gave a symmetrical peak representing 95% of the radioactivity recovered in the two peaks (only 57% of the radioactivity applied was recovered). At this point 17% of the radioactivity in the small peptides released by pepsin remained (Fig. 6, Peak II). Amino acid analysis revealed that this material represented a fairly clean peptide corresponding to the result described below. Rechromatography on IRC-50 gave further fractionation (Fig. 9). All of the radioactivity was recovered with 60% in the major peak. Analysis of a peptide with the composition Thr, Ala, Cys (Table II) in which the additional presence of the alkylated histidine was indicated by radioactivity and the formation of 0.34 residue of 3-carboxymethylhistidine on performic acid oxidation. This yield of histidine oxidation product was considered adequate for 1 residue in view of the yield of 0.48 residue obtained from Trl-CLK inhibited thrombin itself and earlier experiences (31). This
peptide corresponds to residues 40 to 44 of the B chain having the sequence Thr, Ala, Ala, His, Cys. In the isolated material, of course, the histidine was alkylated with 3H-TLCK. Four cycles of the subtractive Edman procedure, with performic acid oxidations to give 3 carboxymethylhistidine and cysteic acid from TLCK histidine and 5 carboxymethylcysteine, respectively, confirmed this sequence. The active center histidine of thrombin was thus identified as histidine-43.

**Fig. 5.** Gel filtration on Sephadex G-75 of the cyanogen bromide peptides from the mixture of the B and B-2 chains of 3H-TLCK-thrombin from the first peak in Fig. 2. The column was the same as that described in Fig. 2. The tubes indicated by the brackets were pooled.

**Fig. 6.** Gel filtration on Sephadex G-50 of the peptic digest of cyanogen bromide peptide 18-84 (the third peak in Fig. 5) from 3H-TLCK-thrombin. The column (0.9 X 20 cm) was equilibrated with 50% acetic acid. The flow rate was 10 ml per hour. The bracketed tubes were pooled.

**Fig. 7.** Gel filtration on Bio-Gel P-4 of the peptides in Peak II, Fig. 6. The column (0.9 X 200 cm) was equilibrated with 0.2 M acetic acid. The bracketed tubes were pooled.

**Fig. 8 (left).** Ion exchange chromatography of Peak I, Fig. 7 on DEAE-Sephadex. The column (0.9 X 20 cm) was equilibrated with pH 7.0 buffer prepared by adding N-ethylmorpholine (about 13.6 ml) to a solution which contained 0.1 M pyridine and 0.1 M acetic acid when made up to 1-liter volume. Elution with the same buffer was carried out at a flow rate of 6 ml per hour. The bracketed tubes were pooled.

**Fig. 9 (right).** Ion exchange chromatography of peptic peptide on Bio-Rex 70. The column (0.9 X 20 cm) was equilibrated with buffer made by adjusting the pH of a solution of 10 ml of N-ethylmorpholine per liter of H2O to 6.5 with acetic acid. Elution, with the same buffer, was at a flow rate of 6 ml per hour.

**DISCUSSION**

The method of purification of bovine thrombin described makes this enzyme accessible in the state of purity desirable for studies on the relationship of structure to function. Since a large number of proteases are present in plasma, misleading results could easily be obtained particularly with the customary use of simple substrates in biochemical studies involving enzyme function.

The finding that thrombin may exist in more than one form
in a fully active state, the difference consisting in a limited proteolytic cleavage that converts a two-chain form to a three-chain form, has considerableprecedence in the pancreatic pro-
etolytic enzymes (32, 33). It is not known whether the new form described has physiological significance since the enzymes used experimentally for the activation of prothrombin are not necessarily the ones that function in blood clotting.

Mann and Batt (11) have reported that a number of molecular species are found in Parke-Davis topical thrombin when purified by ion exchange chromatography on IRC-50, followed by tri-
ethylaminoethylcellulose to remove some nonthrombin impurity. On the basis of electrophoretic analysis and chain separations, the authors conclude that their preparations contain three thrombin species that vary in proportion from batch to batch. However, since their preparations had relatively low specific activities (about 1400 NIH units per mg), it seems probable that esterase thrombin had not been removed and that this accounted for the extra heterogeneity. Components A and B as designated by Mann and Batt possibly correspond to the forms described in this paper.

In view of the known similar esterase activities of thrombin and trypsin, it was expected that substrate-derived alkylation agents such as TLCK and N-O-ZACK would complex with thrombin as they do with trypsin (4, 5), leading to a subsequent specific alkylation. The results in this regard were completely analogous to those obtained with trypsin, namely, inactivation by alkylation of N-3 of a particular histidine residue. The pH rate profile of the esterase action of thrombin indicates the essentiality of a group with pK near 6.5 (depending on the sub-
strate studied) (35, 36). In addition, the fact that thrombin is a serine protease (37) with certain kinetic similarities to trypsin and chymotrypsin has strengthened the impression that a common hydrolytic mechanism is at work in all three enzymes. The observation that inactivation of thrombin results from the alkylation of histidine-43 of the B chain indicates that this residue is the active center residue whose function has been implied in the earlier kinetic studies.

It is of considerable interest that the sequence studies in progress on thrombin provide a model in which the structural relationship to the pancreatic enzymes is made clear. The additional molecular weight of thrombin over trypsin or chymo-
trypsin, namely about 10,000, can be accounted for by a poly-
peptide (the A chain) and a single carbohydrate side chain that are probably externally attached to a central globular structure formed by the B chain (25). The B chain sequence suggests considerable homology with chymotrypsin and therefore probably has a similar three-dimensional structure (38). In this sequence comparison histidine-43 of thrombin is homologous to histidine-57 of chymotrypsin (25). The results with TLCK provide functional evidence supporting the sequence data.

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REFERENCES

1. Sherry, S., and Troll, W., J. Biol. Chem., 201, 10 (1954).
2. Elmore, D. T., and Curragh, E. F., Biochem. J., 85, 9P (1963).
3. Blombäck, B., Blombäck, M., Hessel, B., and Iwanaga, S., Nature, 215, 1445 (1967).
4. Shaw, E., Marks-Out, M., and Cohen, W., Biochemistry, 4, 2210 (1965).
5. Shaw, E., and Glover, E., Arch. Biochem. Biophys., 139, 286 (1970).
6. Rasmussen, P. S., Biochem. Biophys. Acta, 16, 157 (1955).
7. Berger, W. H., Levine, W. G., and Sheppard, R. S., Can. J. Bio-
chem. Physiol., 35, 603 (1958).
8. Magnusson, S., Ark. Kem., 24, 349 (1965).
9. Baughman, D. J., and Waugh, D. F., J. Biol. Chem., 242, 5572 (1967).
10. Glover, G., and Shaw, E., Abstracts of the American Chemical Society Meeting, New York, September, 1969, Abstr. Biol. 322.
11. Mann, K. G., and Batt, C. W., J. Biol. Chem., 244, 6555 (1969).
12. Magnusson, S., Biochem. J., 110, 25 (1968).
13. Klune, D. J., in S. P. Colowicr and N. O. Kaplan (Editors), Methods in enzymology, Vol. II, Academic Press, New York, 1955, p. 139.
14. Chance, T. C., and Shaw, E. N., Biochemistry, 8, 2212 (1969).
15. Schweit, G. W., and Takekawa, Y., Biochem. Biophys. Acta, 16, 570 (1955).
16. Crestfield, A. M., Moore, S., and Stein, W. H., J. Biol. Chem., 238, 622 (1963).
17. Tanaschi, H., and Amfinsen, C. B., J. Biol. Chem., 241, 4366 (1966).
18. Elginga, M. G., Lai, C. Y., and Hirs, C. H. W., Arch. Bio-
chem. Biophys., 123, 553 (1968).
19. Hirs, C. H. W., in C. H. W. Hirs (Editor), Methods in enzymol-
ogy, Vol. XI, Academic Press, New York, 1967, p. 412.
20. Hirs, C. H. W., in C. H. W. Hirs (Editor), Methods in en-
zymology, Vol. XI, Academic Press, New York, 1967, p. 325.
21. Ambler, R. P., in C. H. W. Hirs (Editor), Methods in en-
zymology, Vol. XI, Academic Press, New York, 1967, p. 438.
22. Hirs, C. H. W., in C. H. W. Hirs (Editor), Methods in en-
zymology, Vol. XI, Academic Press, New York, 1967, p. 113.
23. Hirs, C. H. W., in C. H. W. Hirs (Editor), Methods in en-
zymology, Vol. XI, Academic Press, New York, 1967, p. 197.
24. Smyth, D. G., in C. H. W. Hirs (Editor), Methods in enzymol-
ogy, Vol. XI, Academic Press, New York, 1967, p. 215.
25. Magnusson, S., in P. Desnuelle, H. Neurath, and M. Ottesen (Editors), Structural aspects of thrombin and pro-
thrombin—structure-function relationships of proteolytic enzymes, Macrae, Copenhagen, 1970, p. 138.
26. Miller, K. D., Brown, R. K., Casillas, G., and Seegers, W. H., Thromb. Diath. Haemorrh., 3, 362 (1959).
27. Laki, K., and Gladner, J. A., Physiol. Rev., 44, 127 (1964).
28. Schrier, E. E., Broomfield, C. A., and Scheraga, H. A., Arch. Biochem. Biophys., 99, Suppl. 1, 209 (1963).
29. Winzor, D. J., and Scheraga, H. A., J. Phys. Chem., 68, 338 (1964).
30. Seegers, W. H., McCoy, L., Kipper, R. K., and Murano, G., Biochem. Biophys. Res. Commun., 21, 612 (1965).
31. Desnuelle, P., in P. D. Boyer, H. Lardy, and K. Myrback (Editors), The enzymes, Vol. IV, Academic Press, New York, 1960, p. 92.
32. Schroeder, D. D., and Shaw, E., J. Biol. Chem., 243, 2943 (1968).
33. Batt, C. W., Mikulka, T. W., Mann, K. G., Guarracino, C. L., Altiero, R. J., Graham, R. G., Quigley, J. P., Wolf, J. W., and Zafonte, C. W., J. Biol. Chem., 245, 4857 (1970).
34. Keddy, F. J., Lorand, L., and Miller, K. D., Biochemistry, 4, 2392 (1965).
35. Rando, J. B., Curragh, E. F., and Elmore, D. T., Biochim. Biophys. J., 96, 733 (1965).
36. Miller, K. D., and van Vunakis, H., J. Biol. Chem., 233, 227 (1960).
37. Sigler, P. B., Blow, D. M., Matthews, B. W., and Henderson, R., J. Mol. Biol., 35, 143 (1968).