PREPARATION AND PARTIAL CHARACTERIZATION OF TWO FORMS OF BOVINE THROMBIN[1]

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SUMMARY: A chromatographic procedure has been developed for the separation and purification of bovine α- and β-thrombin. α-Thrombin has a specific activity of 2400-3000 NIH U/mg while β-thrombin has only approximately 100 NIH U/mg. When assayed with an ester substrate, the two forms have equivalent activity while β-thrombin has only 30% of the activity of α-thrombin toward an anilide substrate. Previous studies have suggested that the degradation of α-thrombin produces a species in which a peptide fragment containing a disulfide bridge is lost. The amino acid composition determined in the present study indicates that the content of cysteine is identical in the two forms of the enzyme thus permitting the proposal of a structure for β-thrombin which differs from that currently in the literature. It is suggested that changes in the environment of the active site histidine residue in the two species is largely responsible for the observed changes in the catalytic activity.

It has become increasingly apparent that a preparation of bovine thrombin, although functionally homogeneous, may contain two or more species with vastly differing specific activities toward protein and ester substrates (1-6). With the exception of electrophoretic and chromatographic characterization, very little information concerning the structural and enzymatic properties of these proteins has been obtained. The present communication describes the isolation and partial characterization of two forms of bovine thrombin which differ in catalytic activity.

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MATERIALS AND METHODS

Crude bovine thrombin was either obtained from Parke-Davis or from the activation of crude prothrombin in 25% (w/v) trisodium citrate pH 7.2 and further purified by procedure previously described (7). Crude prothrombin was obtained from bovine blood utilizing the procedure of Bajaj and Mann (8) as far as the ammonium sulfate fractionation step. The prothrombin-containing fraction from this step was dialyzed extensively against 0.01 M NH_4HCO_3 and lyophilized. Sulfopropyl (SP)-Sephadex was obtained from Pharmacia Fine Chemicals. Tosyl-L-arginine methyl ester (TosArgOMe) was a product of Schwarz-Mann while benzoyl-L-arginine-p-nitroanilide (BzArgNan) was obtained from BaChem Inc. All other chemicals were of reagent grade and used without further purification.

Samples were prepared for amino acid analysis as described by Moore and Stein (9). Cysteine was determined as cysteic acid after performic acid oxidation (10). Amino acid analysis was performed using single-column methodology on a Glenco 100-AS amino acid analyzer. Enzymatic activity against fibrinogen or TosArgOMe was determined as previously described (7). Thrombic activity against BzArgNan was determined at pH 8.4 (0.05 M Tris) by following the release of nitroaniline at 410 mμ (12). Protein concentration was routinely estimated by absorbance at 280 nm or more accurately by the ninhydrin reaction (13) after alkaline hydrolysis (14) using crystalline bovine serum albumin as a standard.

RESULTS

The results of the chromatography of thrombin which has been partially purified using stepwise elution (10) on SP-Sephadex followed by gradient elution is shown in Figure 1. It is apparent that the thrombin can be resolved into two distinct components. The leading peak (β-thrombin) is characterized by low fibrinogen-clotting activity but significant esterase activity while the second peak (α-thrombin) possesses considerably more fibrinogen-clotting activ-

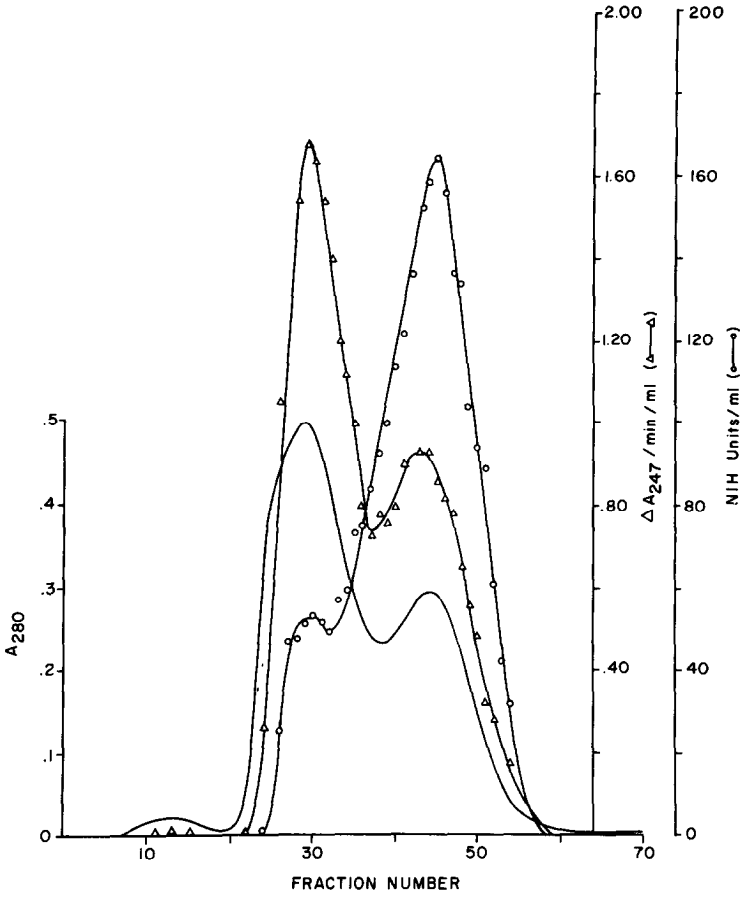

Figure 1. The separation of α- and β-thrombin by gradient chromatography on Sulfopropyl Sephadex C-50. The partially purified thrombin was obtained as described in the text and, after dialysis against 0.05 M sodium phosphate, pH 6.5, applied to a 2.5 x 30 mm column of SP-Sephadex C-50 previously equilibrated with the sample solvent. After elution of one column volume, the solvent was change to 0.15 M sodium phosphate, pH 6.5. After the elution of a second column volume a linar gradient from 0.15 M to 0.45 M sodium phosphate (200 ml/Chamber) was initiated. The flow rate was 60 ml/hr. Assays for catalytic activity was performed as described in the text.

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ity and comparable esterase activity. This type of a separation of thrombin into several components using similar media has been described by other investigators (3, 5). The starting material for the above experiment was obtained commercially. The same type of separation can be obtained on thrombin which has been obtained from crude prothrombin upon activation in 25% trisodium

citrate. Early in the citrate activation (e.g. 24-36 hrs.) the thrombin obtained is primarily α-thrombin but as the reaction proceeds (48-124 hrs.), increasing amounts of β-thrombin are observed. Allowing the citrate activation to proceed for even longer periods of time results in the formation of an additional form of thrombin which elutes prior to β-thrombin on SP-Sephadex. This has been tentatively identified as γ-thrombin (5). It is presumed that the conversion of α-thrombin to the other thrombin species involves proteolysis. It has been suggested that this is an autolytic process (2, 6). We have not been able to demonstrate autolysis either in the present study or in previous studies (15). It was then of interest to see if limited proteolysis of α-thrombin with a well-characterized endopeptidase such as trypsin could result in the formation of a species resembling β-thrombin. Figure 2 shows the results of the chromatographic fractionation of an α-thrombin preparation treated with an insoluble trypsin (11) as compared to a control preparation. The separation of species seen here is similar to that seen with both the commerical thrombin preparations as well as with "citrate-activated" bovine prothrombin.

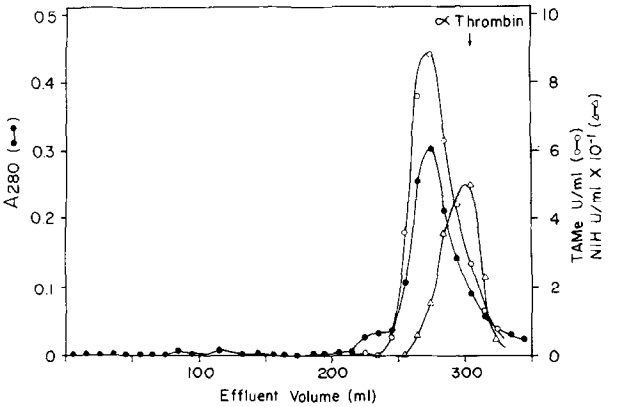

Figure 2. The effect of Tryptic digestion on the Chromatographic Behavior of α-Thrombin. α-Thrombin was prepared as described under Figure 1. It was then passed over a column of trypsin-Sepharose as previously described (11), and subjected to rechromatography on a column of SP-Sephadex C-50 as described under Figure 1.

It is clear from an examination of the data presented thus far that the several species of thrombin possess significantly different specific activities with respect to fibrinogen-clotting and esterase activity. Table I lists the kinetic paramenters of α- and β-thrombin toward three substrates. β-Thrombin has significantly less fibrinogen-clotting activity than α-thrombin but equivalent or greater esterase activity. In spite of a slightly increased value for K_m (obsvd.), the activity of β-thrombin toward the anilide substrate is substantially less than that of α-thrombin. In this latter instance both the value for K_m (obsvd.) and k_{cat} are different, with β-thrombin having a value for K_m (obsvd.) approximately 6-fold greater than that for α-thrombin.

The amino acid composition of α- and β-thrombin are shown in Table II. The primary object of interest in this determination is in the content of cysteine. We find a value of 8 cysteine residues for both α- and β-thrombin. This suggests that at least a portion of the A chain is conserved in the transition of α- and β-thrombin. Furthermore, since the data of Magnusson (16) place two cysteine residues in the amino terminal 76 residues of the B chain, it is unlikely that this entire portion of the α-thrombin molecule could be deleted upon the conversion of α-thrombin to β-thrombin.

DISCUSSION

The present investigation supports prior reports on the existence of multiple forms of bovine thrombin (1-6). The present information suggests that neither the two chain structure derived from α-thrombin upon the deletion of the complete A chain (5), nor the more recent suggestion that the formation of a species of thrombin with esterase but no clotting activity upon removal of the amino terminal 76 residues (B1 chain) from the B chain (6) are correct. The present information suggests an alternative: that β-thrombin is a three chain structure in which two chains are derived from the parent B chain through the excision of a peptide containing the carbohydrate moiety (5) and the third chain resulting from extensive proteolytic degradation of the A chain. It

TABLE I

ACTION OF THROMBINS ON VARIOUS SUBSTRATES

Thrombin Species	NIH U/mg	TosArgOMe μmoles/min/mg	TosArgOMe μmoles/min/μmole	TosArgOMe K_m (obsvd.)	BzArgNan μmoles/min/mg	BzArgNan K_m (obsvd.)
α-Thrombin	2500	37.8	1361	1.7×10^{-4}M	.18	1.43×10^{-4}M
β-Thrombin	134	62.9	1760	3.0×10^{-4}M	.06	8.4×10^{-4}M

TABLE II

THE AMINO ACID COMPOSITION OF BOVINE THROMBINS

	ALPHA-THROMBIN	BETA-THROMBIN
ASP	27	23
THR	14	11
SER	18	13
GLU	32	26
PRO	17	14
GLY	27	23
ALA	16	12
CYS	8	8
VAL	20	17
MET	5	4
ILE	16	14
LEU	29	25
TYR	11	9
PHE	14	9
LYS	23	19
HIS	7	5
ARG	23	19
TRP	8	8

should be noted that this would result in the formation of a peptide which probably could not be visualized upon gel electrophoresis.

The existence of multiple thrombins with different catalytic activities as described herein is similar to the multiple forms of trypsin (17) which differ significantly in their kinetic properties (18). The hydrolysis of TosArgOMe by both trypsin and thrombin is considered to be rate-limited in the deacylation step while the hydrolysis of amide substrates including

nitroanilides is rate-limited in the acylation step. In serine proteases it is thought that the active site histidine primarily functions in the acylation step by increasing the nucleophilicity of the active site serine residue. Thus, a condition which would result in a decrease in the ability of histidine to function in catalysis would be expected to have a greater effect on the hydrolysis of anilide (and protein) substrates than ester substrates. This, in fact, is observed in the comparison of α- and β-thrombin. Since it is clear that the conversion of α- to β-thrombin does involve cleavage of the B chain between the active site histidine and serine residues (5), it is likely that this would result in a change in the relationship of the two residues thus affecting the above described changes.

Further work is currently in progress to elucidate fully the structure and kinetic differences between these various species of thrombin.

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