Purification and Properties of a Thrombin-like Enzyme from the Venom of Crotalus adamanteus (Eastern Diamondback Rattlesnake)*

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SUMMARY

A thrombin-like enzyme has been purified to homogeneity from the venom of Crotalus adamanteus (Eastern diamondback rattlesnake). The enzyme acts directly on fibrinogen in vivo (and in vitro) apparently without affecting any of the other proteins involved in blood coagulation. The enzyme has esterase activity on basic amino acid esters and p-nitrophenyl esters of various N-carboxyamino acids. It exhibits no activity with a variety of N-benzoyl or N-methyl amino acid amides. Physicochemical studies indicated that the enzyme is a glycoprotein with a molecular weight of 32,700. Additionally, sedimentation equilibrium in guanidine hydrochloride showed that the enzyme contains a single polypeptide chain. The protein contains approximately 267 residues with a relatively high content of cystine. The enzyme is optimally active near pH 8 and is stable to neutral and alkaline pH; however, it loses activity upon exposure to acid pH. Both clotting and esterase activities are inhibited by diisopropyl phosphofluoridate, showing that the enzyme, like thrombin, is a serine esterase. Furthermore, the chloromethyl ketone of tosyl-L-lysine, a specific inhibitor of trypsin and thrombin, inhibits the venom enzyme indicating that a histidine residue is necessary for the activity of this enzyme. The chloromethyl ketone of tosyl-L-phenylalanine is not an inhibitor of the venom enzyme. Nitration of the enzyme with tetranitromethane causes loss of clotting activity with little effect on esterase activity suggesting that 1 or more tyrosine residues may be involved in the binding site for large substrates. Finally, the importance of disulfide bridges to the structural integrity of the venom enzyme was indicated by the rapid loss of activity in the presence of β-mercaptoethanol.

A major class of proteins found in snake venoms is the proteolytic enzymes (1, 2) including both coagulant and anticoagulant activities (3). Fontana (4) in 1787 was probably the first to classify venoms by their in vitro effect on human or animal blood as coagulant or anticoagulant, although it is now known that the same venom may be either depending on the concentration used. Weir-Mitchell and Reichert (5) at the turn of the century conducted extensive studies on the venom of Crotalus adamanteus (Eastern diamondback rattlesnake) and reported that it destroyed the coagulability of animal blood. Furthermore, he identified the globulin fraction of the venom as being responsible for this effect. Eagle (6), in 1937, reported that the venom of C. adamanteus in coagulating plasma acted directly on fibrinogen and was not dependent on Ca** for this action. Several instances of envenomation of humans by C. adamanteus have been reported (7–9) and in all cases the blood was rendered incoagulable. Weiss et al. (9), recently reported that in vitro the crude venom of C. adamanteus acts like thrombin in converting fibrinogen to fibrin and has no effect on Factors II, VII, or X. However, unlike thrombin, the venom did not cause platelet aggregation.

The incoagulable nature of blood following snake bite is not unique to C. adamanteus (6), and is in fact widely distributed in the Crotalidae family (3). The phenomenon was noted as the outstanding feature of systemic poisoning following the bite of Anchoctron rhodostoma (Malayan pit viper) (10) and dethrombination was identified as the cause of the coagulation defect (11–13). A paradox exists in that the venom acts in vivo as an anticoagulant whereas in vitro it coagulates blood. Recently, the responsible enzyme ("Arvin") was purified from the venom of A. rhodostoma and shown to be a glycoprotein with a molecular weight of about 30,000 (14). Extensive clinical studies with Arvin have been reported (15, 16). A coagulant enzyme has also been purified from the venom of Bothrops jararaca (17). This enzyme (called "reptilase") has been shown to be similar to Arvin in its action (18) although some differences have been noted (19–21).

In view of the possible clinical significance of the anticoagulant action of these enzymes and the relative ease of obtaining venom from C. adamanteus we undertook the isolation of the thrombin-like enzyme from this venom. This report describes the purification procedure, and the physicochemical and enzymatic properties of the purified enzyme. A separate report is concerned with the in vitro and in vivo effect on animals of the purified
Experimental Procedure

Materials—Lyophilized *C. adamanteus* venom, reagent grade Tris, TPCK, TLCK, and purified bovine plasma thrombin (Grade II, approximately 70 National Institutes of Health clotting units per mg) were obtained from Sigma. Human fibrinogen (Cohn Fraction 1) was purchased from Nutritional Biochemicals. Substrates used for enzyme assay and specificity studies were obtained from Cyclo Chemical Company. β-Mercaptoethanol was purchased from Matheson, Coleman and Bell. DTNB was from Aldrich. Ultrapure guanidine-HCl and DFP were purchased from Mann. TNM was obtained from K and L Laboratories, Plainview, New York. Ultrafiltration cells and type UM-2 membranes used for concentrating dilute protein solutions were obtained from Amicon Corporation, Cambridge, Massachusetts. Thiobarbituric acid was obtained from Eastman and N-acetylneuraminic acid was from Pierce (Cambridge, Massachusetts). Thiobarbituric acid was obtained from Matheson, Coleman and Bell. DTNB was from Aldrich. Ultrapure guanidine-HCl and DFP were purchased from Mann. TNM was obtained from K and L Laboratories, Plainview, New York. All other chemicals were of analytical reagent grade.

DEAE-cellulose (DE-52 microgranular) was obtained from Recvac Angel, Company, Clifton, New Jersey. Hydroxylapatite (Bio-Gel HT) was purchased from Bio-Rad Laboratories Richmond, California.

Enzyme Assays—In vitro clotting activity of the enzyme was assayed with human fibrinogen. The venom solution (from 1 to 25 μl of an appropriate dilution) was added to 0.5 ml of a 1% solution of fibrinogen in 0.9% sodium chloride (in some cases containing 0.01 m Tris-chloride, pH 7.4). The time for the first appearance of fibrin strands was measured with gradual shaking at room temperature. A standard clotting curve was obtained by adding varying amounts of thrombin (of known activity) to the fibrinogen solution and measuring the clotting times. Venom-clotting times were used with this standard curve to obtain the venom activity in terms of standard units of Health clotting units.

Esterase activity of the venom enzyme was measured by a titrimetric procedure essentially as described by Glazer (23) with BAE as substrate at 40°C. The volume of the reaction mixture was 10.0 ml and contained 0.1 M CaCl₂ (or KCl) and 0.025 M BAE. The pH was maintained constant at 8.0 by continuous addition of 0.02 N NaOH and the rates were determined from slopes of the plots obtained during the first 5% hydrolysis.

Essentially the same method was used when testing the specificity of the enzyme on a variety of amides or esters. Variations from the standard assay are mentioned in the text. With some esters or amides the titrimetric procedure could not be used because of high spontaneous hydrolysis rates or where no enzymatic hydrolysis was evident. Attempts were made in these cases to identify the products directly by electrophoretic separations from the reaction mixture after incubation with the enzyme. The amides or esters to be tested (0.014 to 0.025 M) were incubated with the enzyme at pH 7.0 in dilute sodium acetate buffer containing 0.1 to 0.5 M potassium chloride and 5 to 10% by volume dioxane. Aliquots were removed at various times and the reaction products separated by electrophoresis on Whatman No. 3MM paper at pH 0.5 (24) and identified by either the Sakaguchi, Pauly, or ninhydrin reagents (24) as appropriate.

Hydrolysis of TAM was followed in a Zeiss PMQ II spectrophotometer at 37°C by the method of Hummel (25).

Hydrolysis of p-nitrophenyl esters of various N-carbobenzoxy amino acids was measured by following the increase in absorbance at 400 nm in a Zeiss PMQ II spectrophotometer at room temperature essentially by the method of Lornand and Condit (26). The esters (final concentration 10⁻⁴ M) were added to 0.033 M Tris-chloride, pH 8.0, containing 20% 2-propanol in a final volume of 2.99 ml; after following the spontaneous rate of hydrolysis for 5 min the venom enzyme (0.1 ml) was added to start the reaction.

Determination of Protein Concentration—Protein concentrations of crude venom preparations were obtained from the 280 nm absorbance by assuming the optical density equal to the protein concentration in milligrams per ml for a 1-cm light path. The extinction coefficient was determined for the purified enzyme and was used for all subsequent protein concentration determinations with the pure preparation.

Inhibition Studies—The effects of diisopropyl phosphofluoridate, tetraneutromethane, and the chloromethyl ketone derivatives of tosyl-L-phenylalanine and tosyl-L-lysine were investigated by incubating the venom enzyme with a 30- to 100-fold molar excess of the reagent at room temperature in 0.05 to 0.15 M Tris buffer between pH 7 and 8.5. Although organic solvents were used for several of the reagents tested, there was never more than 10% by volume of the solvent in the reaction mixture. Aliquots were assayed at various times for both clotting activity and esterase activity. Appropriate controls showed that the inhibitory reagents at the dilutions used did not affect clotting times.

β-Mercaptoethanol (0.06 μl) was incubated with the venom enzyme in 0.08 M tris-chloride, pH 8.5, at room temperature in stoppered vessels under nitrogen. Aliquots were assayed at various times for esterase activity; clotting activity was not measured since the high concentration of β-mercaptoethanol interfered with the fibrinogen-fibrin conversion.

Chromatographic Procedures—Resins were prepared for chromatography by the manufacturers recommended procedures which in all cases involved suspending the resins in the initial chromatography buffer and decanting several times to remove fines. The columns were poured and the initial eluting buffer was washed through for 24 hours before applying the protein sample.

All column operations were performed in the cold (3-5°C) and fractions were collected with the aid of a Fractomat automatic fraction collector (Buchler Instruments Incorporated, Fort Lee, New Jersey). Absorbancies of the fractions at 280 nm were obtained with a Zeiss PMQ II spectrophotometer. Thymol was added as a preservative to all buffers.

Carbohydrate Analysis—Analysis for neutral sugars and hexosamines was performed in duplicate on 0.60-mg samples of the purified enzyme after hydrolysis for 24 hours in 1 ml of 0.25 N sulfuric acid containing 100 mg of Dowex 50-X2 (H⁺ form), 0.1 μmole of L-lysine, 0.2 μmole of D,L-norleucine, and 20 μg of...
neutral sugars were identified by gas chromatography and hexosamines by amino acid analysis as described (27).

Sialic acid was determined by the thiobarbituric acid assay of Warren (28, 29) with 0.43 and 1.05 mg of the venom enzyme. A standard curve was prepared between 5 and 30 μg of N-acetylgalactosaminic acid. All samples and standards were hydrolyzed in 2 ml of 0.1 n sulfuric acid at 80°C for 1 hour prior to the colorimetric determination.

Column effluents were occasionally analyzed for carbohydrate by the phenol-sulfuric acid method described by Hirn (30).

Phosphate Analysis—Phosphate analysis was performed by the method of Ames and Dubin (31) on 0.3 and 0.6 mg of enzyme. A standard curve prepared from stock 10⁻³ M potassium monohydrogen phosphate indicated that 0.01 μmole of phosphate gave an absorbance of 0.24 at 820 nm.

Amino Acid Analysis—Amino acid analyses were performed by the method originally described by Spackman, Stein, and Moore (32) and Moore and Stein (33) with the use of an accelerated system (34), the Beckman model 121 automatic amino acid analyzer equipped with programmer, automatic sample injector, and model 105 integrator. Salt-free venom enzyme was prepared for hydrolysis by dialyzing exhaustively against water. Aliquots containing 0.8 mg of protein were hydrolyzed for 24 and 69 hours in duplicate.

Tryptophan Determination—Tryptophan was determined spectrophotometrically with a Cary model 14 recording spectrophotometer by the method of Bencze and Schmid (35). Spectra were recorded 30 min and 20 hours after adjusting a solution of venom enzyme (0.53 mg per ml) to pH 13.2 with 1 N NaOH. Tryptophan was also determined by duplicate analyses of 0.44-mg samples of protein by the method of Matsubara and Sasaki (36). In our adaptation of this method a column, 54 X 0.9 cm, of Beckman AA-15 resin in 0.35 M sodium citrate buffer, pH 5.28, was used to separate tryptophan from amino sugars present in the hydrolysate of the venom enzyme. Since in our hands the recovery of tryptophan from subtilisin BPN’ (which is known to have 3 tryptophan residues (37)) hydrolyzed in this manner was assumed to be 70%, values for the tryptophan content of the snake venom enzyme were corrected assuming 70% recovery.

Cysteine and Cystine Determination—Free sulfhydryl groups were determined by the method of Ellman (38) either with 0.35 mg of the venom enzyme in 1.0 ml of 0.10 M potassium phosphate buffer (0.5 mM EDTA), pH 8.0, with or without 0.8 M urea or with 0.62 mg of the enzyme in 5.7 M guanidine-HCl, pH 7.6, with 0.005 M EDTA. When denaturating agents were used the protein was initially incubated in these solutions for 60 min prior to the addition of 5, 5’-dithiobis(2-nitrobenzoic acid). The color development at 412 nm was followed over a period of 18 hours with a Zeiss PMQ II spectrophotometer.

Half-cystine was determined as cysteic acid by amino acid analysis after performic acid oxidation and acid hydrolysis of 0.8 mg of the venom enzyme in duplicate (39).

Molecular Weight Determination—Weight average molecular weights (Mav) were determined at 20°C in the Spinco model E analytical ultracentrifuge equipped with a temperature control unit (RTIC) and Raleigh interference optics with the high speed equilibrium method described by Yphantis (40). A centerpiece with three double channels was used, with sapphire windows and column heights of about 2.7 mm. The samples were run at 28,000 or 35,000 rpm with protein concentrations ranging from 0.4 to 0.8 mg per ml.

The enzyme was prepared by dialysis against either 0.10 M Tris-nitrate, pH 7.0 to 7.6, containing 0.5 mM EDTA, or 6 M guanidine-HCl containing 0.5% N-mercaptoethanol, pH 7.5, and centrifugation was performed on both samples.

Interference patterns were recorded on Kodak metallographic plates with a 35-s exposure and read on a Gaertner microcomparator. Equilibrium was considered attained when fringe distances within each channel remained constant for a period of 24 hours or more.

Partial specific volume was calculated from the amino acid composition and carbohydrate content presented in this report. It was assumed that there was no change in partial specific volume in 6 M guanidine-HCl (41).

The molecular weight was also determined on 8-μg samples of the venom enzyme by SDS gel electrophoresis run in triplicate with the method of Weber and Osborn (42). The gels were calibrated with standard proteins with the molecular weights given by Weber and Osborn (43). SDS acrylamide gel electrophoresis was kindly performed by Dr. Derek Chignell of the Biological Chemistry Department, University of California, Los Angeles.

Results

Enzyme Purification

The crude lyophilized venom (6.5 g) was dissolved in 35 ml of 0.9% NaCl and a yellow slightly turbid solution containing about 20,000 clotting units was obtained. This was immediately applied to Sephadex G-100 previously equilibrated with 0.10 M NaCl containing 0.04 M sodium acetate, pH 6.0 (Fig. 1). The active fractions were pooled, yielding 5,000 absorbance units and 26,200 clotting units. A slight increase in activity was observed after gel filtration. This could possibly be attributed to the removal of another protein that interfered with the clotting assay. The pooled sample was concentrated by precipitation with 50% ammonium sulfate and the precipitate was dissolved in a minimal volume of 0.005 M sodium acetate, pH 6.0, containing 0.5 mM EDTA. This solution was dialyzed for 48 hours against 0.005 M sodium acetate, pH 7.0, and a white precipitate formed which contained some activity. After centrifugation the supernatant (containing 26,000 clotting units) was applied to a DEAE-cellulose column previously equilibrated with 0.005 M sodium acetate, pH 7.0, and elution was performed with this buffer. The enzyme was eluted with a linear gradient (Fig. 2) and the active fractions were pooled, concentrated by ultrafiltration, and dialyzed overnight against 0.5 mM potassium phosphate, pH 7.0, containing 0.5 mM EDTA. About 29,900 units of clotting activity were recovered, again representing a slight activation of unknown origin. The dialyzed protein

We are indebted to Doctors Robert Carlsen and John G. Pierce of the Biological Chemistry Department, University of California, Los Angeles, for assistance with the carbohydrate analyses.
Fig. 1. Elution profile of crude *Crotalus adamanteus* venom from Sephadex G-100 column, 5.0 X 90.0 cm. Elution was performed with 0.04 M sodium acetate-0.10 M NaCl, pH 6.0 (upward flow) and fractions of 9.5 ml were collected at a flow rate of 55 ml per hour. The absorbance at 280 nm is shown by the solid line and clotting activity by the dashed line. The first peak contained material that was yellow in color, the other peaks contained no colored material. Fractions pooled are indicated by the solid bar.

Fig. 2. Chromatography of partially purified *Crotalus adamanteus* venom on DEAE-cellulose (DE-52, column, 1.8 X 55 cm). The venom, after dialysis against 0.005 M sodium acetate, pH 7.0, was applied to the column and initially eluted with buffer of the same composition as that used for dialysis. At 400 ml a linear gradient was initiated with 425 ml of the starting buffer in the mixing chamber and 425 ml of 0.10 M sodium acetate, pH 7.0, in the reservoir. At 1250 ml the gradient was increased to 0.70 M sodium acetate, pH 7.0, with 400 ml of this buffer in the reservoir and 400 ml of 0.10 M sodium acetate, pH 7.0, in the mixing chamber. Arrows indicate the position of gradient application. Fractions of 4.0 ml were collected at a flow rate of 24 ml per hour. The absorbance at 280 nm is shown by the solid line, the clotting activity by the dashed line. Fractions pooled are indicated by the solid bar.

Fig. 3. Chromatography of pooled fractions from Step IV (Table I) on hydroxylapatite column, 1.8 X 50 cm. Elution was initially performed with 0.50 mM potassium phosphate buffer (containing 0.5 mM EDTA), pH 7.0, and fractions of 3.4 ml were collected at a flow rate of 20 ml per hour. A linear gradient was initiated at 175 ml with 500 ml of the starting buffer in the mixing chamber and 500 ml of 0.05 M potassium phosphate buffer, pH 7.0 (containing 0.5 mM EDTA), in the reservoir. At 1175 ml the gradient was increased by adding 500 ml of the 0.05 M potassium phosphate buffer to the mixing chamber and 500 ml of 0.50 M potassium phosphate buffer, pH 7.0, to the reservoir. Arrows indicate the point where the second gradient was started; the initial part of the profile is omitted as no 280-nm absorbing material was eluted in this region. Clotting activity is indicated by the dashed line, absorbance at 280 nm by the solid line. Solid bars indicate the fractions pooled for Peaks 1 and 2.

A small amount of a high molecular weight component was removed by passing the concentrated material through a column, 3.8 X 52 cm, of Sephadex G-100 in 0.10 M NaCl containing 0.02 M sodium acetate, pH 7.0. The pooled active fractions, contain-
ing about 13,200 units, were concentrated by ultrafiltration and
dialyzed overnight against 0.005 M sodium acetate, pH 7.0.
Final purification was obtained by rechromatography on DEAE-
cellulose under conditions identical with the first run except that
a slower linear gradient was utilized. The elution profile (Fig. 4)
shows a single peak with a small shoulder; the leading edge
(Peak 1, Fig. 4) when pooled yielded 11,600 units of the thrombin-
like enzyme in a homogeneous state. Additionally about 3300
units of activity were recovered from Peak 2 (Fig. 4). However,
both SDS (42) and standard acrylamide (43) gel electrophoresis
indicated a minor contaminant was present in the Peak 2 frac-
tion and ion exchange chromatography on SE-Sephadex was
utilized to further purify this fraction. As shown in Fig. 5 the
Peak 2 fraction gave a single symmetrical peak after chromato-
tography on SE-Sephadex and SDS acrylamide gel electrophoresis
of this peak indicated a homogeneous protein. Analysis of the
elluent fractions of the SE-Sephadex column for carbohydrate
(30) (Fig. 5) indicated that the venom enzyme was a glycoprotein
since peaks of clotting activity, _A₈₅₀_, and carbohydrate were
superimposable. It was also found that after dialysis against
the pH 5.0 buffer and subsequent chromatography at this pH the
venom enzyme lost over 50% of its clotting activity, however,
the activity was regained after dialysis at pH 7.6 in 0.10 M
Tris-nitrate buffer and the specific clotting activity (clotting
units per _A₈₅₀_ unit) was identical with that of the homogeneous
protein from Peak 1 of the DEAE-cellulose chromatogram (Fig.
4).

Gel electrophoresis patterns of the venom preparation at
various stages of purification are given in Fig. 6A; Fig. 6B shows
the SDS acrylamide gel pattern obtained with the DEAE-
cellulose Peak 1 enzyme (Fig. 4). Table I summarizes the
purification steps and shows that the specific clotting activity
increased by about 75-fold with a recovery of approximately
57%. The highest specific activity that was obtained was
about 220 clotting units per _A₈₅₀_ unit and this was achieved with
both Peaks 1 and 2 (after SE-Sephadex chromatography) of the
DEAE-cellulose column (Fig. 4). Unless otherwise noted,
however, all subsequent studies were performed with the Peak
1 enzyme.
TABLE I

Purification of thrombin-like enzyme from C. adamanteus venom

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein</th>
<th>Total enzyme clotting units</th>
<th>Specific activity</th>
<th>Enzyme recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Crude venom</td>
<td>6,500b</td>
<td>19,750</td>
<td>3.0</td>
<td>100%</td>
</tr>
<tr>
<td>II Chromatography Sephadex</td>
<td>5,000</td>
<td>26,200</td>
<td>5.2</td>
<td>100%</td>
</tr>
<tr>
<td>G-100 (I)</td>
<td>782</td>
<td>26,900</td>
<td>54.4</td>
<td>103</td>
</tr>
<tr>
<td>III to 50% Ammonium sulfate precipitate</td>
<td>266</td>
<td>29,900</td>
<td>112</td>
<td>114</td>
</tr>
<tr>
<td>IV Chromatography DEAE-cellulose (I)</td>
<td>115</td>
<td>16,300</td>
<td>142</td>
<td>62</td>
</tr>
<tr>
<td>V Chromatography hydroxylapatite</td>
<td>90</td>
<td>13,200</td>
<td>133</td>
<td>50</td>
</tr>
<tr>
<td>VI Chromatography Sephadex</td>
<td>52</td>
<td>11,600</td>
<td>222</td>
<td>44</td>
</tr>
<tr>
<td>VII Chromatography DEAE-cellulose (II)</td>
<td>15</td>
<td>3,300</td>
<td>216</td>
<td>13</td>
</tr>
</tbody>
</table>

* Clotting units per A 280 unit.
* Dry weight 0.5 g.
* Increase in activity occurred at this step. 26,200 units were established as 100% recovery level.

Criteria of Homogeneity

SDS acrylamide gel electrophoresis (42) of the highly purified enzyme (Peak 1, Fig. 4) showed a single band after staining with Coomassie brilliant blue (blue, Fig. 6B). Acrylamide gel electrophoresis at pH 8.9 by the method of Davis (43) also gave a single band.

Rechromatography of Peak 2 from the DEAE-cellulose run (Fig. 4) on SE-Sephadex gave one major symmetrical peak with constant specific activity throughout the peak tubes. The specific activity after dialysis of the pooled active fractions was identical with that of Peak 1 from the DEAE-cellulose run indicating that a maximum in specific activity had been reached, further supporting the homogeneity of the enzyme preparation. Additionally, SDS gel electrophoresis of this material showed a single protein band with electrophoretic mobility identical with that of the enzyme in Peak 1.

During sedimentation equilibrium of the thrombin-like enzyme, plots of log C versus X² (where C is protein concentration in fringes and X is distance from the axis of rotation to the center of the fringe pattern in centimeters) gave straight lines. This indicates a homogeneous protein solution insofar as molecular weight is concerned.

Physicochemical Properties

Molecular Weight—Sedimentation equilibrium analysis at 28,000 rpm and 20° of the snake venom enzyme in 0.1 M Tris-nitrate containing 5 × 10⁻⁴ M EDTA, pH 7.6, indicated a molecular weight of 33,700 (±1,500). No differences were observed in molecular weights determined after 22 or 46 hours of sedimentation at protein concentrations of 0.4, 0.6, and 0.8 mg per ml; the value given is the over-all average. A partial specific volume of 0.724 was used for the calculations (see below).

After dialysis of the venom enzyme against 6 M guanidine-HCl containing 0.5% β-mercaptoethanol, pH 7.5, sedimentation equilibrium at 35,600 rpm indicated a molecular weight of 34,200 (±1,850). Although amino acid analysis shows that the enzyme has a high content of disulfide bonds there is no change in molecular weight after denaturation and disruption of the disulfide bridges indicating that the protein is a monomer containing a single polypeptide chain whose molecular weight is approximately 33,700.

The molecular weight was also estimated by disc gel electrophoresis in the presence of sodium dodecyl sulfate (42). The electrophoretic mobilities of the venom enzyme and of marker proteins with polypeptide chains of known molecular weights (42) were used to calculate a molecular weight of 31,700 (±400) for the single polypeptide chain of the venom enzyme (Fig. 7). These results confirmed the value obtained by sedimentation equilibrium. An average value of 32,700 was used for molecular weight calculations.

Partial Specific Volume—The partial specific volume of the venom enzyme was estimated from the amino acid and carbohydrate composition (see below) by a modification of the procedure described by Schachman (44). The partial specific volume was calculated to be 0.724 with the formula V = Σnᵢ/Σnᵢ where nᵢ and εᵢ are number of residues of type i and specific volume of residue i, respectively. Included in this calculation are the 11 carbohydrate residues whose partial specific volume was taken as 0.67 (45).

Amino Acid Analysis—Results of amino acid analyses after 24 and 69 hours of hydrolysis are given in Table II. Values for duplicate analyses agreed in all cases to within ±2% and for most amino acids to within ±1%. The composition was calculated by determining the molar ratios of each amino acid...
Table II

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>24-hour average</th>
<th>69-hour average</th>
<th>Ratio to alanine</th>
<th>Ratio to alanine</th>
<th>Average or extrapolated ratio</th>
<th>Minimum residue weight</th>
<th>Residues (average ratio times factor)</th>
<th>Integral number of residues</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>0.0935</td>
<td>0.0935</td>
<td>0.99</td>
<td>1.00</td>
<td>1.00</td>
<td>127.8</td>
<td>11.3</td>
<td>11</td>
<td>1406</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.0715</td>
<td>0.073</td>
<td>0.76</td>
<td>0.79</td>
<td>0.77</td>
<td>105.7</td>
<td>8.7</td>
<td>9</td>
<td>1234</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.0968</td>
<td>0.0945</td>
<td>1.02</td>
<td>1.02</td>
<td>1.02</td>
<td>159.2</td>
<td>11.5</td>
<td>12</td>
<td>1875</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.2353</td>
<td>0.252</td>
<td>2.68</td>
<td>2.71</td>
<td>2.70</td>
<td>310.5</td>
<td>30.5</td>
<td>31</td>
<td>3508</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.115</td>
<td>0.1065</td>
<td>1.21</td>
<td>1.15</td>
<td>1.25e</td>
<td>126.9</td>
<td>14.2</td>
<td>14</td>
<td>1416</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.123</td>
<td>0.099</td>
<td>1.30</td>
<td>1.07</td>
<td>1.43e</td>
<td>124.5</td>
<td>16.2</td>
<td>16</td>
<td>1394</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.1085</td>
<td>0.1805</td>
<td>2.10</td>
<td>2.04</td>
<td>2.07</td>
<td>267.2</td>
<td>23.4</td>
<td>23</td>
<td>2970</td>
</tr>
<tr>
<td>Proline</td>
<td>0.174</td>
<td>0.185</td>
<td>1.84</td>
<td>1.99</td>
<td>1.91</td>
<td>180.0</td>
<td>21.7</td>
<td>22</td>
<td>2137</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.0904</td>
<td>0.1655</td>
<td>1.80</td>
<td>1.78</td>
<td>1.79</td>
<td>102.2</td>
<td>20.3</td>
<td>20</td>
<td>1141</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.170</td>
<td>0.0931</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>71.1</td>
<td>11.3</td>
<td>11</td>
<td>782</td>
</tr>
<tr>
<td>Valine</td>
<td>0.1235</td>
<td>0.135</td>
<td>1.31</td>
<td>1.46</td>
<td>1.46d</td>
<td>212.9</td>
<td>16.6</td>
<td>17</td>
<td>2475</td>
</tr>
<tr>
<td>Half-cystine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.24c</td>
<td>14</td>
<td>2117</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.017</td>
<td>0.015</td>
<td>0.18</td>
<td>0.16</td>
<td>0.16a</td>
<td>24.1</td>
<td>2.1</td>
<td>2</td>
<td>262</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.1275</td>
<td>0.145</td>
<td>1.35</td>
<td>1.56</td>
<td>1.56d</td>
<td>176.4</td>
<td>17.7</td>
<td>18</td>
<td>2057</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.176</td>
<td>0.176</td>
<td>1.86</td>
<td>1.89</td>
<td>1.88</td>
<td>212.4</td>
<td>21.3</td>
<td>21</td>
<td>2377</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.0675</td>
<td>0.0615</td>
<td>0.66</td>
<td>0.66</td>
<td>0.66</td>
<td>107.9</td>
<td>7.5</td>
<td>7</td>
<td>1142</td>
</tr>
<tr>
<td>Phenyldalanine</td>
<td></td>
<td>0.107</td>
<td>0.1085</td>
<td>1.07</td>
<td>1.17</td>
<td>164.5</td>
<td>12.7</td>
<td>13</td>
<td>1913</td>
</tr>
</tbody>
</table>

Sum: 2737.5 267 31,382

* Tryptophan determined by the method of Matsubara and Sasaki (36) after acid hydrolysis in 4% thioglycolic acid.
* The value given is the average value for tryptophan from the two methods used (see text).
* Tryptophan and serine determined by extrapolation to zero time.
* Valine and isoleucine determined from values obtained after 69-hour hydrolysis.
* Half-cystine determined as cysteic acid and methionine as the sulfone after performic acid oxidation by the method of Moore (39).

FIG. 8. Ultraviolet absorption spectrum of the highly purified thrombin-like enzyme from Crotalus adamanteus venom. The enzyme (Peak I, Step VII of Table I) was exhaustively dialyzed against water and identical aliquots were diluted in Tris-chloride buffer, pH 7.4 (Curve 1) or 1.0 N NaOH, final pH 13.2 (Curve 2). The spectra were recorded on a Cary model 14 recording spectrophotometer in quartz cuvettes with path lengths of 1.0 cm. Scanning speed was 5 A per s. The spectrum at the basic pH was recorded after sitting 20 hours at room temperature to insure complete ionization of all tyrosine residues. Protein concentration, 0.5 mg per ml. No visible absorption was seen between 360 and 500 nm in a separate scan.

(Columns 1 and 2, Table II) to alanine which was taken as 1.00 (Columns 3 and 4). The average or extrapolated ratio (Column 5) was multiplied times the residue weight to obtain the minimum residue weight (Column 6). These were summed and divided into the physically determined molecular weight of 31,000 (average value from sedimentation equilibrium and disc gel electrophoresis less the carbohydrate contribution). This gave a factor (11.3) which when multiplied times the average or extrapolated ratios (Column 5) gave the number of residues of each amino acid (Column 7). This was rounded off to the nearest integral residue to give the amino acid composition of the protein (Column 8). The molecular weight determined from the amino acid composition is 31,382 (Column 9) which when added to the molecular weight of the carbohydrate component gives an over-all molecular weight of 33,160.

Spectrophotometric determinations of tryptophan and tyrosine by the method of Benze and Schmid (35) gave 7.10 tryptophan and 6.53 tyrosine residues. These results when combined with those for tryptophan determined by the method of Matsubara and Sasaki (36) and tyrosine determined by amino acid analysis gave an average value of 6 tryptophan and 7 tyrosine residues in the venom enzyme (Table II).

Cysteine was determined by the method of Ellman (38) in the presence or absence of 0.5 M urea or 5.7 M guanidine-HCl. In neither case was there any reaction indicating the complete absence of any titratable sulfhydryl groups. However, amino acid analysis following performic acid oxidation and hydrolysis of the venom enzyme gave 14 half-cystine residues indicating the presence of seven disulfide bridges.

Carbohydrate Analysis—Hydrolysis of the venom enzyme in 0.25 N sulfuric acid and analysis for neutral sugars by gas chromatography and hexosamines by amino acid analysis by the method of Kim et al. (27) gave the following results (average values of two determinations are expressed as moles of carbohydrate per mole of protein assuming a molecular weight of 32,700): Fucose...
TABLE III

**pH effect on stability of venom enzyme**

The venom enzyme (0.2 μg per ml) was incubated in ground glass-stoppered vessels at room temperature in either 0.1 M sodium acetate (from pH 3 to 7) or 0.1 M Tris-chloride (pH 8 to 10). All quot were removed after varying times of incubation and esterase activity assayed at pH 8.0 following 1 to 200 dilution.

<table>
<thead>
<tr>
<th>Time</th>
<th>pH</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.2</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>4.0</td>
<td>98</td>
</tr>
<tr>
<td>80</td>
<td>5.0</td>
<td>99</td>
</tr>
<tr>
<td>320</td>
<td>6.0</td>
<td>94</td>
</tr>
<tr>
<td>320</td>
<td>7.0</td>
<td>99</td>
</tr>
<tr>
<td>320</td>
<td>8.0</td>
<td>99</td>
</tr>
<tr>
<td>320</td>
<td>9.9</td>
<td>99</td>
</tr>
</tbody>
</table>

* Esterase activity expressed as percentage relative to the pH 7.0 zero time control which was taken as 100%.

Phosphate analyses by the method of Ames and Dubin (31) showed that there was no phosphate present in the enzyme.

**Ultraviolet Absorbance and Extinction Coefficient**—The venom enzyme showed a typical protein ultraviolet absorption spectrum with a maximum at 280 nm and a minimum at 250 nm and no visible absorption (Fig. 8). For comparison the spectrum at pH 13.2 is also shown. The ratio of absorbance at 280 to 260 nm was 1.54 indicating essentially no nucleic acid contamination (46).

The extinction coefficient of the venom enzyme was determined from the dry weight of a sample that had been dialyzed exhaustively against water, dried at 110° under reduced pressure, and cooled in a desiccator over NaOH and P2O5 to constant weight. With this procedure and spectrophotometric measurements of aliquots of the same solution a value for ε\textsubscript{280} (280 nm) of 14.8 was obtained.

**Enzymatic Properties**

**Stability**—The venom enzyme is stable on storage having been kept at 5° for over 6 months at pH 7.0 (0.1 M sodium acetate buffer) and a concentration of 7.5 mg per ml with the loss of at most 10% of the clotting activity. The effect of pH on the stability of the venom enzyme was investigated and the results are presented in Table III. The enzyme is stable at neutral and alkaline pH but at acid pH there is a time-dependent loss of activity which increases at more acidic pH. These data reflect the effect of pH on the esterase activity. There was also a loss in clotting activity at slightly acidic pH as shown during chromatography of the venom enzyme on SE-Sephadex at pH 5. However, essentially 100% clotting activity was regenerated after dialysis against pH 7.6 buffer. At more acidic pH, it appears that the loss of esterase (and presumably clotting) activity is irreversible.

**Substrate specificity of thrombin-like enzyme from C. adamantaeus venom**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (μ mole/mg)</th>
<th>pH</th>
<th>Solvent</th>
<th>Enzyme Rate (μ mole/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Benzoyl-L-arginine ethyl ester</td>
<td>0.025</td>
<td>7.0</td>
<td>MeOH</td>
<td>100</td>
</tr>
<tr>
<td>N-Benzoyl-L-lysine methyl ester</td>
<td>0.025</td>
<td>7.0</td>
<td>MeOH</td>
<td>100</td>
</tr>
<tr>
<td>N-Acetyl-L-tyrosine ethyl ester</td>
<td>0.025</td>
<td>7.0</td>
<td>MeOH</td>
<td>100</td>
</tr>
<tr>
<td>N-Toxy-L-arginine methyl ester</td>
<td>0.001</td>
<td>8.1</td>
<td>H2O</td>
<td>100</td>
</tr>
<tr>
<td>N-Benzoyl-L-lysaminde</td>
<td>0.025</td>
<td>7.0</td>
<td>H2O</td>
<td>100</td>
</tr>
<tr>
<td>N-Benzoyl-L-argininimide</td>
<td>0.025</td>
<td>7.0</td>
<td>H2O</td>
<td>100</td>
</tr>
<tr>
<td>N-Acetyl-L-tyrosinamide</td>
<td>0.025</td>
<td>7.0</td>
<td>H2O</td>
<td>100</td>
</tr>
<tr>
<td>N-Benzoyl glycine ethyl ester</td>
<td>0.025</td>
<td>7.0</td>
<td>H2O</td>
<td>100</td>
</tr>
<tr>
<td>N-Benzoyl-L-tryptophan ethyl ester</td>
<td>0.025</td>
<td>7.0</td>
<td>H2O</td>
<td>100</td>
</tr>
<tr>
<td>N-Acetyl-L-tryptophanamide</td>
<td>0.025</td>
<td>7.0</td>
<td>H2O</td>
<td>100</td>
</tr>
<tr>
<td>N-Benzoyl glycimide</td>
<td>0.025</td>
<td>7.0</td>
<td>H2O</td>
<td>100</td>
</tr>
</tbody>
</table>

* Methanol.
* Dioxane.
* Assayed spectrophotometrically by the method of Hummel (25). TPCK trypsin had an activity of 180 μmoles per min per mg of protein when assayed by this method.
The thrombin-like enzyme from C. adamanteus venom was studied for its clotting and esterase activity. The enzyme was purified and its kinetic parameters were determined using the substrate BAE (benzoylarginine ethyl ester) and various N-carbobenzoxy amino acids. The enzyme exhibited high activity towards BAE, with a Michaelis constant (Km) of 1.6 x 10^-4 M and a maximum velocity (Vmax) of 0.41 μmol/min/mg protein.

**Table V: Hydrolysis of p-nitrophenyl esters of various N-carbobenzoxy amino acids**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>C. adamanteus enzyme</th>
<th>Arvinb</th>
<th>Thrombinb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>4.94</td>
<td>5.55</td>
<td>0.41</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.79</td>
<td>5.0</td>
<td>0.43</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.14</td>
<td>0.015c</td>
<td>0.04d</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.040d</td>
<td>0.040</td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.052</td>
<td>1.78</td>
<td>0.4</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.73</td>
<td>0.58</td>
<td>0.013</td>
</tr>
<tr>
<td>Valine</td>
<td>0.020d</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Nitrophenol liberated was calculated from the absorbance at 400 nm with an extinction coefficient of 1.6 x 10^4 liters per mole per cm (26).
* Data from Reference 14 determined at pH 8.0, 37⁰C, and 0.01 M p-nitrophenyl ester.
* The tertiary butyloxycarbonyl amino acid derivative was used.
* Rates were barely above the level judged to be statistically significant.

**Effect of pH**—As shown in Fig. 9, the venom enzyme shows a sigmoid pH activity profile for the hydrolysis of BAE at 40⁰C over the pH range 4 to 9. Maximum activity was exhibited above pH 8. The enzyme catalyzed the hydrolysis of p-nitrophenyl esters of various N-carbobenzoxy amino acids with high specificity. The enzyme was extensively hydrolyzed within 10 min as judged by the appearance of N-benzoyl-L-arginine after electrophoretic separation of the reaction products. N-Benzoyl-L-histidine methyl ester also appeared to be a good substrate for the venom enzyme, hydrolysis being evident after 10 min of reaction. Control experiments showed some spontaneous hydrolysis but the rate was insignificant compared to the enzymatic rate of hydrolysis of this ester.

**Substrate Specificity**—A variety of esters and amides was used to test the substrate specificity of the venom enzyme (Table IV). The titrimetric assay was generally used with the ester or amide to be tested being substituted for BAE in the standard procedure. Methanol or dioxane was used to dissolve some of the substrates but was never present at greater than 10% final volume in the assay system. The pH of the assays was generally 7.0.

The venom enzyme exhibited no activity with amino acid amides and only the basic amino acid esters served as substrates. To ensure further that the basic amino acid amides were not hydrolyzed by the venom enzyme and to see if N-benzoyl-L-histidine methyl ester (which appeared to be hydrolyzed in the titrimetric assay) was a substrate of the enzyme, a direct assay was utilized in which the reaction products were identified after electrophoretic separation (see “Experimental Procedure”). The results confirmed the findings of the titrimetric assay in that neither N-benzoyl-L-argininamide nor N-benzoyl-L-lysinamide were hydrolyzed by the venom enzyme even after 24 or 48 hours of incubation at 37⁰C, respectively. In contrast BAE was extensively hydrolyzed within 10 min as judged by the appearance of N-benzoyl-L-arginine after electrophoretic separation of the reaction products. N-Benzoyl-L-histidine methyl ester also appeared to be a good substrate for the venom enzyme, hydrolysis being evident after 10 min of reaction. Control experiments showed some spontaneous hydrolysis but the rate was insignificant compared to the enzymatic rate of hydrolysis of this ester.

The rate of hydrolysis of p-nitrophenyl esters of various N-carbobenzoxy amino acids was investigated with the Peak 8 enzyme (Fig. 4) which had been further purified on Sephadex. Since hydrolysis of the p-nitrophenyl esters was measured in 20% 2-propanol the esterase activity of the venom enzyme was determined in this solvent and found to be essentially the same as the activity in water. Table V presents the hydrolysis rates of the p-nitrophenyl esters together with a comparison of the rates found by Esnouf and Tunnah (14) for the hydrolysis of these esters by thrombin and Arvin. There are some striking similarities between the specificities of the two venom enzymes; the rates for the hydrolysis of the p-nitrophenyl esters of tyrosine, leucine, and glycine are almost identical. There also appear to be differences in that Arvin seems to have high activity with the three aromatic amino acid esters while the C. adamanteus enzyme has very low activity with the tryptophan ester and only moderate activity with the phenylalanine ester. Thrombin in general has lower activity with the p-nitrophenyl esters than either of the two venom enzymes.

**Km and Vmax Determination**—The Km and Vmax values were determined for the hydrolysis of BAE by the venom enzyme. These values, calculated from Lineweaver-Burk plots (49) (Fig. 10), were 2.05 x 10^-4 M for Km and 130 μmoles per min per mg of protein for Vmax.

**Inhibition Studies**

Disopropyl Phosphofluoridate—A 120-fold molar excess of disopropyl phosphofluoridate was incubated with the venom enzyme in Tris-acetate buffer, pH 7.6, and the effect on both clotting and esterase activity was followed with time. Fig. 11 shows that disopropyl phosphofluoridate simultaneously in-
FIG. 11. Effect of 120-fold molar excess of diisopropyl phosphofluoridate on clotting (O----O) and BAE esterase (O-O) activities of the venom enzyme. The enzyme (0.26 mg) was incubated in a final volume of 0.50 ml containing 0.05 M Tris-acetate, pH 7.6, and 4% by volume isopropanol. Aliquots were removed after various times of incubation at room temperature and assayed as described (see "Experimental Procedure").

Habits both activities indicating that the venom enzyme is a serine esterase with the active serine also being involved in the clotting activity like thrombin (50).

Chloromethyl Ketone Derivatives of Tosyl-L-lysine and Tosyl-L-phenylalanine—The chloromethyl ketone derivatives of tosyl-L-lysine and tosyl-L-phenylalanine were incubated separately with the venom enzyme and aliquots were removed at various times for activity assays. As shown in Fig. 12 the chloromethyl ketone of tosyl-L-phenylalanine had no effect on either the clotting or esterase activities, however, the chloromethyl ketone of tosyl-L-lysine caused a 65 to 70% loss of activity within 25 hours. The inhibition by the chloromethyl ketone of tosyl-L-lysine is significant in that thrombin (51-53) and trypsin (52) whose specificities are also directed toward the esters of basic amino acids are both inhibited by this reagent as well. The rate of reaction of the chloromethyl ketone of tosyl-L-lysine with the venom enzyme is much slower than with either thrombin or trypsin but the general features of the reaction as well as the pH activity profile of the venom enzyme suggest the involvement of a histidine residue in the reaction mechanism of this enzyme.

Nitration by Tetranitromethane—The venom enzyme was incubated at pH 8.5 with either 30 or 160-fold molar excess of tetranitromethane. Assays of aliquots removed at various times showed a significant drop in the clotting activity after nitration but little effect on esterase activity (Fig. 13). A similar effect of nitration has been observed with thrombin (54). Although tetranitromethane is known to oxidize sulphhydril groups of proteins and to react at higher molar ratios with histidine, tryptophan, and methionine residues (55), the major reaction is with tyrosyl residues (56). Since there are no free sulphhydril groups in the venom enzyme it would appear, although there is only circumstantial evidence, that tyrosine residues are being modified. If this is so, then apparently 1 or more tyrosine residues are involved in the binding site for large molecules such as fibrinogen, but not for the smaller ester substrates.

Disulfide Bond Reduction—Amino acid analysis indicated seven disulfide bonds in the venom enzyme. To determine whether any of the disulfide bridges were critical to the structural integrity of the enzyme the effect of the disulfide-reducing agent β-mercaptoethanol was investigated. Incubations were performed with the Peak 2 enzyme (Fig. 4) (which had been further purified on SE-Sephadex) as already described (see "Experimental Procedure"). Esterase activity was followed with time and within 2 hours 95% of the activity had been lost.
in the presence of β-mercaptoethanol. Incubations were also performed in the presence of 4.8 M guanidine-HCl and guanidine-HCl plus β-mercaptoethanol; however, over 70% of the activity was lost by exposure to the denaturing solvent alone. Clearly there is at least one disulfide bridge critical to the maintenance of biological activity of the venom enzyme, that is readily available to reducing agents.

**DISCUSSION**

In a separate report we have described the effect on animals of a thrombin-like enzyme purified from the venom of the Eastern diamondback rattlesnake (C. adamanteus) (22). In vivo this enzyme acts at the fibrinogen level as an anticoagulant; presumably a dysfibrinogenemic state is induced resulting in non-clotting blood (22). Studies on the mechanism by which Arvin (a purified thrombin-like enzyme from A. rhodostoma, the Malayan pit viper) produces non-clotting blood suggest that this enzyme produces an aberrant fibrinogen which either (a) cannot be clotted by thrombin (57) or (b) forms microclots that are dispersed in the circulation (58). It is assumed that the enzyme from rattlesnake venom acts in the same manner. Paradoxically, the purified rattlesnake venom enzyme (as well as Arvin) when incubated with plasma (or purified fibrinogen) immediately forms a fibrin clot even in the presence of the anticoagulant heparin (22).

The present paper details the biochemical characterization of the thrombin-like enzyme from rattlesnake venom. The enzyme has been purified to a high degree of homogeneity as indicated by ion exchange chromatography on SE-Sephadex, equilibrium ultracentrifugation, and electrophoresis in acrylamide and SDS acrylamide gels. The molecular weight was determined both by sedimentation equilibrium in the presence and absence of denaturing solvents, and by SDS acrylamide gel electrophoresis, and values of 33,700 and 31,700, respectively, were obtained for the single polypeptide chain. An average value of 32,700 has been taken as the molecular weight. Amino acid analysis gave the composition shown in Table II. During ion exchange chromatography of the venom enzyme, carboxyhydride analysis of the effluent fractions showed a peak that coincided with the protein and clotting activity peaks indicating that the venom enzyme was a glycoprotein (Fig. 5). Quantitative carbohydrate analyses of the purified enzyme gave the following composition: fucose 1, mannose 2, galactose 3, and glucosamine 5, for an overall carbohydrate content of 5.4%. The bound carbohydrate content in a variety of different snake venoms has been recently studied by Oshima and Iwanaga (29). These authors identified the carbohydrate components in the venom of C. adamanteus as galactose, mannose, fucose, glucosamine, and sialic acid. Although the purified venom enzyme did not contain sialic acid the relative ratios of the other components were in fair agreement with those of the whole venom (admittedly there are several glycoproteins in the whole venom and the composition of the thrombin-like enzyme would not be expected to match that of the whole venom).

The thrombin-like enzyme from rattlesnake venom is very stable on storage and in general is quite stable except at acid pH. It is probable that the high content of disulfide bonds in this enzyme accounts at least in part for its stability. The carbohydrate moiety as well may have some control over enzyme stability by shielding amino acid residues critical to the structure or function of the enzyme. It should be noted that unlike the pancreatic proteinases, the proteolytic enzymes of snake venom have no zymogen precursors so it is not too surprising to find that the active enzyme exhibits a high degree of stability. In addition to its clotting activity the rattlesnake venom enzyme was also shown to have esterase activity which, like thrombin (60) was directed toward basic amino acid esters: TAM, BAE, benzoyl-L-lysine methyl ester, and for the venom enzyme even benzoyl-L-histidine methyl ester (Table IV). Furthermore, the venom enzyme exhibited no esterase activity with esters of aromatic or neutral amino acids nor was there amidase activity on a variety of N-benzoyl or N-methyl amino acid amides. However, with the p-nitrophenyl esters of N-carbobenzoxy amino acids, hydrolysis was evident with the tyrosine, phenylalanine, leucine, and alanine derivatives (Table V).

The venom enzyme was identified as a serine esterase by its rapid and complete inhibition with diisopropyl phosphofluoridate (Fig. 11). Furthermore, since both esterase and clotting activity are inhibited simultaneously it appears that the same active site is utilized for both of these functions. Additionally, the pH profile (Fig. 9) suggested that histidine may be involved in the active site of the rattlesnake venom enzyme as was previously found to be the case for thrombin (51-53) and other serine esterases (52, 61, 62). This was substantiated by the finding that the chloromethyl ketone of tosyl-L-lysine inhibited the venom enzyme (Fig. 12) although at a substantially slower rate than either thrombin (51, 53) or trypsin (52). This rate difference probably reflects structural differences around the active histidine residue of the venom enzyme, but nevertheless the inhibitory effect of the chloromethyl ketone of tosyl-L-lysine suggests the involvement of histidine in both the clotting and esterase activities of the venom enzyme. By contrast the chloromethyl ketone of tosyl-L-phenylalanine has no effect on the venom enzyme nor thrombin (53).

Another striking similarity between the rattlesnake venom enzyme and thrombin was the finding that nitration causes a substantial loss in clotting activity of the venom enzyme but only a mild loss in esterase activity (Fig. 13). A similar pattern of inhibition was obtained with thrombin (54). These results can be explained by assuming that the tyrosine residue (or residues) modified is in a secondary binding site for fibrinogen (separate from the active site) which is not involved in the binding of small ester substrates.

Finally, the importance of disulfide bonds to the structural integrity of the venom enzyme, already noted earlier, was convincingly shown by the rapid loss of activity promoted by the disulfide-reducing agent β-mercaptoethanol. Although the number of bonds cleaved was not determined it is apparent that at least one disulfide bridge is readily available for reduction and is crucial to activity. Seegers has reported (55) that at least one disulfide bond is essential to thrombin activity also.

These inhibitor studies point out that in addition to physicochemical and kinetic similarities the venom enzyme is also quite similar to thrombin in terms of its structural requirements for activity.

The defibrinating enzyme from C. adamanteus is also remarkably like Arvin, an enzyme with similar biological activity (10-13) purified from the venom of the Malayan pit viper A. rhodostoma (14). Another enzyme with similar in vivo activity, reptilase (18), has been purified from the venom of B. jararaca.
have similar substrate specificities. Their amino acid compositions also show a degree of similarity although there are some subtle differences in specificities between these enzymes, and thrombin as well.

Recent work has shown that Arvin is inactivated by serum proteins and gradually loses its coagulant activity (72). Arvin was also shown to be a weak antigen (73). It is probable that the rattlesnake venom enzyme behaves in a similar manner to thrombin and Arvin but suggest that there are subtle differences in specificities between these enzymes, and thrombin as well.

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gratifying to find that the venom of *C. adamanteus* contained a thrombin-like enzyme in substantial quantity, although the early work of Weir-Mitchell and Reichert (5) and Eagle (6) had indicated this to be the case. In contrast the venom of *Crotalus atrox* (Texas diamondback rattlesnake), a cousin of *C. adamanteus*, contains little if any of this activity but has instead an active fibrinogenolytic enzyme (6).

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Purification and Properties of a Thrombin-like Enzyme from the Venom of *Crotalus adamanteus* (Eastern Diamondback Rattlesnake)

Francis S. Markland and Paul S. Damus


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