The Thrombin-like Enzyme from Bothrops atrox Snake Venom

PROPERTIES OF THE ENZYME PURIFIED BY AFFINITY CHROMATOGRAPHY ON p-AMINOBENZAMIDINE-SUBSTITUTED AGAROSE

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(Received for publication, August 5, 1975)

The thrombin-like activities from the snake venoms of two subspecies of Bothrops atrox, moojeni (type I) and marajoensis (type II), were purified to homogeneity by affinity chromatography on a support consisting of the inhibitor, p-aminobenzamidine, linked to Sepharose 4B with a spacer of diaminodipropylaminosuccinate. At room temperature the enzyme was not bound to the affinity support but rather was retarded in relation to the unbound protein. As a result the thrombin-like activity eluted in a large volume following the main protein fraction. However, at 4°C the enzyme was absorbed to the affinity support and could be eluted specifically with the ligand benzamidine (0.15 M). Optimal conditions for column loading and washing were 0.05 M Tris-HCl/0.4 M NaCl, pH 9.0, at 4°C. The type I enzyme isolated in this manner showed a single major band on pH 8.9 disc gel electrophoresis as well as two minor bands. Further purification by isoelectric focusing yielded one major and two minor components. All three protein fractions had identical thrombin-like activities and amino acid compositions. The major band had a specific activity of 210 to 230 NIH thrombin units/mg, a s\textsubscript{20,w} of 2.65 S, a molecular weight of 29,000, and an E\textsubscript{124} of 15.6. This protein has a carbohydrate content, measured as hexose, glucosamine, and sialic acid, of 27%. From the amino acid and carbohydrate composition a partial specific volume of 0.700 ml/g was calculated. The type I enzyme, purified on affinity chromatography only, did not activate Factor XIII and was free of thromboplastin-like activity. The type II enzyme behaved very differently from the type I on pH 8.9 polyacrylamide disc gels yielding two major bands and two minor bands. The relative amounts of these four bands were not a function of purity. The type II enzyme had a specific activity of 650 to 700 NIH thrombin units/mg, a s\textsubscript{20,w} of 2.60, and a molecular weight of 31,400.

Fontana in 1787 (11, as cited by Weiss et al. (21, was probably the first to show that the venoms of many snakes had the ability to clot human and animal blood. Eagle in 1937 (31 did an extensive study and showed that many snakes of the family Crotalidae coagulated titrated horse plasma indicating that coagulation was not due to the conversion of prothrombin to thrombin, as this reaction required Ca\textsuperscript{2+}. These and later studies (4) showed that this ability to clot blood is due to the direct action of the venom on the fibrinogen components of the blood.

Several instances of envenomation of humans by venomous snakes leading to blood which is noncoagulable have been reported. Two examples of this phenomenon are Crotalus adamanteus (Eastern diamondback rattlesnake) (3) and Agkistrodon rhodostoma (Malayan pit viper) (5) where defibrination has been identified as the cause for the noncoagulable nature of the blood. The thrombin-like enzyme from Agkistrodon rhodostoma has been purified\textsuperscript{1} and its physical and enzymatic properties characterized (6, 7) as have the thrombin-like enzymes from Crotalus adamanteus (8) and Bothrops atrox (9). The enzyme isolated and purified from the venom of Bothrops atrox is available commercially as Defibrase and/or Reptilase.\textsuperscript{2}

The enzymes isolated from the above snake venoms are all serine esterases and are inhibited by reagents which react with active-site serine (7–10). Like thrombin they clot fibrinogen, but unlike thrombin, which releases fibrinopeptides A and B from fibrinogen, these preparations release Peptide A only (8, 11, 12). A nomenclature for these enzymes does not currently exist but the World Health Organization's committee on "Nomenclature of Thrombin and Thrombin-like Enzymes, their Peptide Chains and Zymogens Thereof" has recommended the generic name of peptidohydrolase.

The current procedure for the purification of the thrombin-like enzyme from the venom of Bothrops atrox is lengthy and results in very low recoveries of starting activity. This report describes an affinity method for the purification of this enzyme to homogeneity and the physiochemical and enzymatic properties of the purified enzyme as isolated from two subspecies of Bothrops atrox, moojeni and marajoensis. A preliminary account of this work has been published (13).

EXPERIMENTAL PROCEDURE

Materials—p-Aminobenzamidine, N\textsuperscript{4}-tosyl-L-arginine methyl ester·HCl, p-nitrophenyl-p'-guanidinobenzoate·HCl, and all com-

\textsuperscript{1}C. Nolan, Abbott Laboratories, unpublished experiments.

\textsuperscript{2}Available from Pentapharm AG, Basel, Switzerland.
pounds used for the inhibition study were obtained from Cyclo
Chemical Co.; N-ethyl-3-(3-dimethylaminopropyl)carbodiimide .
HCl, disodium hydrogen phosphate, and dithiothreitol from Sigma;
L-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl from Aldrich,
and sodium dodecyl sulfate from Matheson, Coleman and Bell. Mi-
ami Serpentarium provided Bothrops atrorax mossae venom, and
Bothrops atrorax marajoensis was the kind gift of Pentapharm AG, Basel. The venoms were received as desiccated dry crystalline substances which were stable for several months at -20°C. Diffragram, a partially purified preparation of the thrombin-like activity from Bothrops atrorax, was obtained from Pentapharm AG, Basel. The NIH standard thrombin used was Lot B 3. All other chemicals were reagent grade or the best grade available.

Affinity equilibria of solutions of buffers and reagents were dissolved in triple-distilled water.

Preparation of Agarose Derivatives—The insoluble matrixes for affinity chromatography were prepared by coupling p-aminobenzi-
damide.HCl to Sepharose 4B with either e-aminoacaproic acid or diamino-
dipropylaminomuccinate as a spacer. Sepharose 4B was activated with
2 M NaCl and 1 M acetic acid and lastly with water. The amount of
carboxyl groups in the free form and one when coupled to agarose.

The inhibitor, p-aminobenzamidine, was covalently linked to the
agarose derivative with 2 M NaCl and 1 M acetic acid and lastly with water. The amount of e-aminoacaproic acid incorporated was measured by hydrolyzing 1 ml of
agrose, 1 ml of l-ethyl-3-(3-dimethylaminopropyl)carbodiimide/l00-ml bed volume
was added to the washed, activated agarose and allowed to react at 4°C and
pH 9.5 for 16 hours. The derivatized agarose was washed sequentially
with 2 M NaCl and 1 M acetic acid and lastly with water. The amount of e-aminoacaproic acid incorporated was measured by hydrolyzing 1 ml of
washed, settled agarose in an evacuated, sealed tube with 6 ml of 6 N
HCl at 1 10° for 22 hours. The hydrolysate was evaporated twice and
the residue dissolved in 5 ml of 0.2 M citrate buffer, pH 2.2. This sample was clarified and analyzed for e-aminoacaproic acid on the basic column of a Beckman model 220 amino acid analyzer. e-Aminoacaproic acid eluted just prior to lysine. The incorporation of diamino-
dipropylamine was quantitated with ninhydrin. The aminoolyl-agarose (0.1 ml) was diluted to 2 ml with water, and 5 ml of a ninhydrin
solution (15 ml). The mixture was heated in a boiling water bath for
20 min, diluted with 7 volumes of 50% alcohol, and the insoluble agarose removed by centrifuging at 2000 x g for 5 min. This procedure, which was repeated until the wash was colorless, yielded a yellow pellet. The intensity of the color was a measure of the amino groups which were not succinylated. The degree of
coupling was measured by comparing the color of the pellet and
agaro
ing with similar pellets obtained from reaction
mixtures consisting of varying amounts of unmodified agarose and
aminooolyl-agarose.

The inhibitor, p-aminobenzamidine, was covalently linked to the
carboxyl groups of c-aminocaproic acid-agarose or succinyl-
diaminodi-propionyl-agarose by reaction with the water soluble carbodiimide
1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (0.25 g/l00-ml bed volume
of agarose) was added. The suspension was adjusted to pH 4.75 with 1
molar or 100-ml bed volume of agarose, l-ethyl-3-(3-dimethylaminopropyl)-
carbodiimide.HCl, the agarose was washed
and applied in the center of the sucrose gradient. After 41 hours at 600
volts the gradient was removed from the column by a peristaltic pump
and pH. The activity peaks were pooled and the ampholine removed by
dialysis against 0.02 M citrate and 0.2 M NaCl, pH 6.0. The precipitate was spun down at 10,000 x g for 10 min and the A280 of the supernatant measured. Zero
time points were obtained by adding the monochloroacetic acid prior to
the clotting activity.

Isoelectric Focusing—Isoelectric focusing was performed at 4°C in an
LKD 110 ml column using an ampholine concentration of 1% and
pH range of 5 to 8. The ampholine was stabilized in a sucrose (1 to 45%)
gradient prepared with the use of a two-chamber gradient mixer. The sample for isoelectric focusing was dialyzed overnight against 0.02 M
citrate and 0.2 M NaCl, pH 6.0. This sample was made 30% in sucrose and applied in the center of the sucrose gradient. After 41 hours at 600 volts the gradient was removed from the column by a peristaltic pump
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moqui }—The titration of the enzyme purified by affinity chromatography and isoelectric focusing was done according to the method of Chase and Shaw (19). The reaction was performed at pH 8.3 in a 0.1 M Veronal buffer and the absorbance of the sample followed at 410 nm on a Cary 14 spectrophotometer. A 30-fold molar excess of inhibitor to protein was used. Spontaneous hydrolysis of p-nitrophenyl-p'-guanidinobenzoate HC1 was corrected for by adding equal amounts of it to the reference and sample cuvette. The absorbance due to reaction of this benzoate with the enzyme was obtained by extrapolating the recorder tracing to the time at which the enzyme was added to the sample cuvette.

**Analytical Methods**—The procedures of Davis (20) for 7% polyacrylamide disc gels at pH 8.9 and 4.3 were utilized. Disc gel electrophoresis in the presence of sodium dodecyl sulfate was done according to the method of Weber and Osborn (21). After electrophoresis at 8 mA/tube for 6 hours, the sodium dodecyl sulfate gel was soaked overnight in 10% 5-sulfosalicylic acid, stained for 10 min with 1% Amido black, and destained electrophotographically in 10% acetic acid. The relative amounts of the bands were determined by scanning the stained gels with the Densiscord densitometer made by Photovolt Corp. Samples too dilute for disc gel electrophoresis were concentrated with Amicon concentrator cells.

Amino acid analyses were determined with a Beckman model 120B amino acid analyzer. Samples were hydrolyzed in 6 n HCl at 110° for 24, 48, and 72 hours in nitrogen-flushed evacuated tubes. Phenol was added to protect tyrosine during hydrolysis, and half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively (22). Tryptophan was determined spectrophotometrically by the method of Edelhoch (23). Amino-sugars were quantitated with a Beckman model 120B amino acid analyzer by extrapolating the values found at 24 and 48 hours of hydrolysis time to zero hydrolysis time. Sialic acid was determined by the thiobarbituric acid method of Warren (24) using N-acetylneuraminic acid as standard and neutral sugars by the anthrone reaction of Roe (25) using glucose as standard.

Analytical sedimentation data were obtained using a Spinco model E ultracentrifuge equipped with a split-beam automatic photoelectric scanning optical system. The sedimentation experiments were performed in 12- and 30-mm double sector cells. Enzyme samples were prepared by dissolving the stock enzyme in buffer and diluting this stock to give the desired concentration, assuming an extinction coefficient of E1%1cm equals 15.6. Sedimentation coefficients were corrected to standard conditions according to the method of Schachman (26). Sedimentation equilibrium experiments were run at 18,000 rpm for at least 24 hours to assure that equilibrium was attained. Molecular weights were obtained from a graph of log of concentration versus radius squared.

The extinction coefficient of the thrombin-like activity from Bothrops atrox venom was determined on a dried, weighed sample obtained by thoroughly dialyzing the enzyme against 3 changes of 0.1 M ammonium bicarbonate, lyophilizing to dryness, and then redissolving in 0.1 M ammonium bicarbonate. The absorbance of this solution was measured at 340 nm correcting for light scattering by subtracting the 340 nm reading. This correction was very small being less than 2% of the 340 nm reading.

Partial specific volumes were calculated from the amino acid composition and carbohydrate content using the values for the individual amino acids given by Cohn and Edsall (27) and the values given for carbohydrate residue by Gibbons (28).

**RESULTS**

**Purification of Thrombin-like Activity from Bothrops atrox moqui**—The results of a study done on the possible inhibitory effects of several organic compounds on Defibrase, as quantitated using the esterolytic assay described under "Experimental Procedure," are compiled in Table 1. Also tested but without any inhibitory activity were the peptides Arg-Val and Gly-Lys as well as p-methoxybenzylamine, spermidine, and 7-aminoheptanoic acid. Assays using fibrinogen as substrate gave similar results. As p-aminobenzamidine was the best inhibitor, an affinity support was synthesized with p-aminobenzamidine linked to agaroarose via the spacer ε-aminoacapric acid. When a sample of the crude venom from Bothrops atrox moqui was placed on the column and eluted at room temperature with 0.05 M Tris-HCl and 0.15 M NaCl, pH 7.0, the thrombin-like activity did not bind to the resin, but slightly trailed behind the main protein peak which eluted in one column void volume. As Cuetrecasas (16) has shown that increasing the length of the spacer can increase the degree to which an enzyme binds to an affinity absorbent, p-aminobenzamidine was attached to the agaroarose backbone via the longer spacer of diaminodipropylamino succinate.

The thrombin-like activity did not bind to this affinity support but the activity was substantially retarded in relation to the main protein fraction. The activity was spread out over several column void volumes and when pooled had a specific activity of only 30 NIH units/mg. The crude venom had a specific activity of 6 NIH units/mg. All of the above column chromatography experiments were performed at room temperature. Absorption of the crude venom to the p-aminobenzamidine-agaroarose derivative at 4° resulted in total binding of the thrombin-like activity. However, a great deal of nonspecific binding also occurred, which was due to the fact that p-aminobenzamidine was positively charged and thus bound proteins with a net negative charge. This effect was minimized by loading and washing the affinity resin with a buffer of high ionic strength and also by choosing a pH which gave optimal binding. The buffer used was 0.05 M Tris-HCl and 0.1 M NaCl, pH 9.0. Higher ionic strengths interfered with the binding of the thrombin-like activity and lower pH values decreased the capacity of the affinity resin. The column was washed with this buffer until the absorbance of the eluant at 280 nm decreased to 0.2. The thrombin-like activity was specifically eluted with a buffer, 0.05 M Tris-HCl/0.1 M NaCl, pH 9.0, which contained the inhibitor benzamidine at a concentration of 0.15 M. The active enzyme trailed behind the benzamidine front and eluted in two column void volumes, as shown in Fig. 1. The absorbance at 280 nm at this point was not due to protein but to the benzamidine. The activity was pooled (Fractions 114 to 138) and the benzamidine removed by exhaustive dialysis against three changes of 0.02 M citrate and 0.2 M NaCl, pH 6.0. The pooled represented 91% of the starting activity and had an activity of 210 to 230 NIH units/mg. Polycrylamide disc gel electrophoresis at pH 8.9 (Fig. 3) showed a major band and two very minor bands. Densitometer scans of the gel indicated that the major band represented 85 to 90% of the total protein.
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FIG. 1. Affinity chromatography of Bothrops atrox venom at 4°. Bothrops atrox venom, 20 ml at 100 mg/ml, was placed on a column (2.5 cm × 56 cm) of p-aminobenzamidine-succinyl-diaminodipropylamino-agarose which had been equilibrated at 4° with 0.05 M Tris·HCl/0.4 M NaCl, pH 9.0, and washed with the same buffer until the A_{280} was less than 0.2. The column was run at 65 ml/hour and fractions of 6.7 ml were collected. The active enzyme was eluted with a solution of 0.15 M benzamidine·HCl, buffered at pH 9.0 with 0.05 M Tris·HCl and 0.1 M NaCl. Enzyme activity (A—A) is expressed as NIH thrombin units/ml.

FIG. 2. Isoelectric focusing of the thrombin-like activity purified by affinity chromatography. The affinity purified enzyme (5.0 mg) was placed on a LKB 110-ml column and focused in a pH 5 to 9 gradient for 41 hours at 4°. Enzyme activity (A—A) is expressed as NIH units/ml. The protein isolated in Pool II was used for all physical and analytical studies.

FIG. 3. Polyacrylamide disc gel electrophoresis (pH 8.9) of purified enzyme fractions. A to D, Bothrops atrox, subspecies moojeni; A, purified by affinity chromatography only; B, C, D, Pools I, II, and III obtained by isoelectric focusing of affinity purified protein (see Fig. 2); E, Bothrops atrox, subspecies marajoensis, purified by affinity chromatography only.
These experiments were performed with enzyme which had activity and the properties of the purified enzymes of this Carbohydrate (%) 26.7 Protein (%) 73.3 determined as the free amines after hydrolysis in 6 N HCl at 110°. From the data presented in Table II a molecular weight of 29,700 was calculated. This is equivalent to 21,700 g of protein and 8,000 g of carbohydrate/mol of glycoprotein.

**Tritium with p-Nitrophenyl-p'-guanidinobenzoate**—Reaction of the purified thrombin-like enzyme (Pool II, Fig. 2) with a 30-fold molar excess of the serine active site titrant p-nitrophenyl-p'-guanidinobenzoate resulted in the loss of 100% of the clotting activity. This reaction resulted in the incorporation of 0.80 mol of inhibitor/mol of enzyme using 29,100 as the molecular weight for the protein.

**Ultraviolet Absorbance and Extinction Coefficient**—The purified enzyme exhibited a typical protein absorption spectra with a maximum at 278 nm, a minimum at 250 nm, and an absorbance of 1.57. An extinction coefficient (E at 280 nm) of 15.6 was measured from the dry weight of an enzyme unit.

**Factor XIII-activating Activity**—When equal amounts of thrombin, Defibrase ( ), and the purified thrombin-like enzyme from Bothrops atrox, subspecies moojeni ( ), or the purified fibrinolytic activity from Bothrops atrox, subspecies marajoensis, native to Northern Brazil, Marajoensis venom contained about seven times as much activity per g of venom and also the purified enzyme had approximately five times the activity per mg using the same extinction coefficient of 15.6 for both preparations. The sedimentation coefficient of the marajoensis subspecies was 2.60 S. Sedimentation equilibrium experiments yielded a molecular weight of 31,400. A partial specific volume of 0.700 ml/g was used for these calculations. The results of disc gel electrophoresis are shown in Fig. 4. The marajoensis enzyme migrated much faster than the moojeni enzyme, although both had similar molecular weights suggesting that the latter had a lower isoelectric point. The four bands were present in a 1:2:2:1 ratio and were present at this ratio in all preparations. No further attempts were made to separate these bands.

**Comparison of Bothrops atrox subspecies, moojeni and marajoensis**

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<tr>
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<th>moojeni</th>
<th>marajoensis</th>
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<tr>
<td>NIH thrombin units/g of venom</td>
<td>2,200</td>
<td>17,000</td>
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<tr>
<td>NIH thrombin units/mg of pure enzyme</td>
<td>210-230</td>
<td>900-1,100</td>
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<tr>
<td>Sedimentation coefficient</td>
<td>2.65</td>
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<tr>
<td>Molecular weight</td>
<td>29,100</td>
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*Purified by affinity chromatography only.

The present report outlines an affinity method for the purification of the thrombin-like activity from Bothrops atrox venom. The affinity support consisted of the inhibitor p-aminobenzamidine linked to agarose with the spacer, 3,3'-diaminodipropylaminomuccinate. The use of p-aminobenzamidine affinity supports have been reported for the purification of thrombin and trypsin (29, 30) as well as ascorosomal proteinase (31). Yields of the purified thrombin-like enzyme from the Bothrops atrox venom of 90% or greater were obtained. The enzyme obtained from the affinity support was enzymatically homogeneous. Disc gel electrophoresis at pH 8.9 yielded one major band and two minor bands. The major band equaled 85 to 90% of the...
total protein as determined by densitometer recordings of the stained gels. The two minor bands, which were separated from the main protein fraction by isoelectric focusing, had specific activities and amino acid compositions identical with the main protein fraction. The data reported here cannot determine if these two minor bands were present in the original venom or arose during the purification procedure or perhaps during the drying of the freshly collected venom. As the purified enzyme contains sialic acid (4 residues/mol), it is possible that the differences between the three activities were due to their content of sialic acid. Only the sialic acid content of the main protein fraction was examined. Nolan1 obtained similar multiband patterns with the purified thrombin-like activity isolated from Agkistrodon rhodostoma venom. Disc gel electrophoresis yielded five bands, and treatment with neuraminidase reduced the five bands to a single band. Such results suggested that the microheterogeneity was due to differing amounts of sialic acid.

The thrombin-like enzyme activity of Bothrops atrox venom did not bind to the affinity support at room temperature but was significantly retarded. However, at 4°C the activity was completely bound and the esterolytic inhibitor benzamidine was required to elute the activity. The tighter binding of the enzyme at low temperature was not unexpected as the binding of a solute from a mobile phase to an insoluble phase is generally exothermic, i.e. binding is favored at low temperature. This effect of temperature on binding was suggested by Cuatrecasas (32) and elegantly demonstrated by Harvey et al. (33), who used linear temperature gradients to separate dehydrogenases and kinases from a column consisting of N\(^4\)-(6-aminohexyl) 5'-AMP agarose. One major problem encountered using affinity supports, such as benzamidine-agarose, which have a charged species as the ligand, is the occurrence of nonspecific ion binding. This nonspecific binding can be partially controlled by carrying out the absorption and eluting steps in the presence of high ionic strength buffers, if the high ionic strength does not interfere with absorption of the protein which one is attempting to isolate. In the case of the binding of the thrombin-like activity of Bothrops atrox venom, considerable nonspecific binding occurred, as evidenced by the trailing of the main protein fraction in the presence of 0.15 M NaCl. Higher NaCl concentrations (0.4 M) decreased this binding without affecting the binding of the thrombin-like activity. Increasing the NaCl concentration to 0.7 M resulted in lesser binding of the thrombin-like activity. The pH of the absorbing and washing buffer was also important for binding, with maximum binding occurring at pH 9.0 and very little occurring at pH 6.0.

The chemical reaction between agarose-diaminodipropylaminosuccinate and p-aminobenzamidine required three reaction cycles in order to obtain a 95% derivatization of the carboxyl group. To overcome the unreactive nature of p-aminobenzamidine a 25-fold molar excess of the carbodiimide and a 4-fold molar excess of p-aminobenzamidine was utilized. The slow reaction is apparently due to the fact that the p-amino group in p-aminobenzamidine is very unreactive. NMR studies substantiated this fact by showing that p-aminobenzamidine exists as a resonating structure with all nitrogen protons being equivalent. Some likely structures are shown below.

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\begin{align*}
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\text{NH}_3 & \quad \text{NH}_3
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The affinity supports prepared in this manner were stable indefinitely and were reused numerous times. The affinity resins showed no decrease in binding ability after being used and washed 15 or more times over a 6-month time period.

Carbohydrate analysis of the pure enzyme revealed a high content of carbohydrate (27%). The thrombin-like enzymes isolated from other members of the Crotalidae family have similar carbohydrate contents (e.g. the enzyme from Agkistrodon rhodostoma is 29% carbohydrate) (7). The thrombin-like enzyme isolated from Crotalus adamanteus has considerably less, 5.4% (6). The amino acid content of the purified enzyme is normal except for the unusually high content of proline. Comparison of the amino acid contents of the Bothrops atrox enzyme and of the Crotalus adamanteus enzyme (8) show a remarkable likeness. Both have a very high proline content (see Table II). The figures given in Table II for Crotalus adamanteus are those of Markland and Damus (8) based on 21 residues of aspartic acid/mole of protein.

The molecular weights for the pure enzyme measured by sedimentation equilibrium and sodium dodecyl sulfate gel electrophoresis do not agree, 29,100 being measured using the former method and 32,400 with the latter. Stocker and Egberg (9) have reported a slightly higher value of 35,300 also using sodium dodecyl sulfate gel electrophoresis. These differences were not unexpected as glycoproteins have an impaired capacity to bind sodium dodecyl sulfate and consequently a lower electrophoretic mobility (34). As a result the molecular weights reported for glycoproteins, using sodium dodecyl sulfate gels, are larger than the molecular weights obtained by sedimentation equilibrium. Therefore, the molecular weight of 29,100 obtained by sedimentation equilibrium is the correct value and was used for the calculations reported here.

It has been commonly accepted that the thrombin-like activity isolated from Bothrops atrox venom also has the activity, like thrombin, to activate Factor XIII (35). However, the results shown in Fig. 4, where Defibrase, thrombin, and affinity purified enzyme are compared definitively show that it was possible to separate the Factor XIII-activating enzyme from the thrombin-like activity. The lack of Factor XIII-activating enzyme was not unexpected as the enzymes isolated from Agkistrodon rhodostoma (36) and Crotalus adamanteus (8) also lack this activity.

Acknowledgments—We are indebted to Mr. Edward Devine for performing all of the assays and also for aiding in the amino acid analysis. We would also like to thank Mr. Grant Barlow for his support and helpful suggestions during the course of this work.

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