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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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 diaminodi-propylaminosuccinate. 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_{20,w} of 2.65 S, a molecular weight of 29,000, and an E_{1%26;280} 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_{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^{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 (6, 7) 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 (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 peptidohydrolases.

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-tosyl-L-arginine methyl ester·HCl, p-nitrophenyl-p'-guanidinobenzoate·HCl, and all com-

1 Available from Pentapharm AG, Basel, Switzerland.
pounds used for the inhibition study were obtained from Cyclo Chemical Co.; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl from Ott Chemical Co.; ninhydrin, anthrone, thiorbarbituric acid, 2,4,6-trinitrobenzenesulfonic acid were purchased from Eastman Chemical Co. The 90% clottable human fibrinogen was obtained from Cutter Laboratories and Sepharose 4B from Pharmacia Ltd. Mann; benzamidine-HCl, cyanogen bromide, ε-aminocaproic acid, and succinic anhydride from Aldrich, and sodium dodecyl sulfate from Matheson, Coleman and Bell. Miami Serpentarium provided Bothrops atrox major venom, and Bothrops atrox 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°. Defibrinogenated, a partially purified preparation of the thrombin-like activity from Bothrops atrox, was obtained from Pentapharm AG, Basel. The NIH standard thrombin used was Lot R 3. All other chemicals were reagent grade or the best grade available. All aqueous 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-aminobenzamidine to Sepharose 4B with either ε-aminocaproic acid or diaminodipropylamine to a spacer. Sepharose 4B was activated with cyanogen bromide (100 mg/ml of agarose) according to the method of Cuatrecasas (14). Either ε-aminocaproic acid or 3,3'-diaminodipropylamine (100 μmol/ml of agarose) was added to the washed, activated agarose and allowed to react at 4° 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 ε-aminocaproic 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 110° 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 ε-aminocaproic acid on the basic column of a Beckman model 220 amino acid analyzer. ε-Aminocaproic acid eluted just prior to lysine. The incorporation of diaminodipropylamine was quantitated with ninhydrin. The aminoalkyl-agarose (0.1 ml) was diluted to 2 ml with water, and 5 ml of a ninhydrin solution (0.15 ml) was added. 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 × 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 succinylation was estimated by comparing the color of the pelleted succinylated agarose with similar pellets obtained from reaction mixtures consisting of varying amounts of unmodified agarose and aminoalkyl-agarose.

The inhibitor, p-aminobenzamidine, was covalently linked to the carboxyl groups of ε-aminocaproic acid-agarose or succinylaminodipropylamine-agarose by reaction with the water soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The agarose derivative was suspended in 1 volume of distilled water and 5 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/100 ml bed volume of agarose was added. The suspension was adjusted to pH 4.75 with 1 M HCl and incubated at room temperature for 15 min. At this point p-aminobenzamidine·HCl (1 μg/100-ml bed volume of agarose), dissolved in a mixture of water and 5% sodium hydroxide, was added dropwise to the stirred suspension over a 30-min period. After stirring at room temperature for 5 hours and maintaining the pH at 4.75 by adding 1 N HCl, the agarose was washed thoroughly with water and resuspended in 1 volume of water. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (2.5 μg/100 ml bed volume of agarose, 5 g/100 ml bed volume) was added as described above and allowed to react at room temperature for 5 hours and overnight at 4°. After washing the agarose with water this second reaction was repeated at the same reactant concentrations. This derivatized agarose, after washing successively with 2 M NaCl, H2O, 1 M acetic acid, H2O, 0.001 M NaOH, H2O, and lastly with the column buffer system, was used for enzyme affinity purification.

The extent of substitution of the carbboxyl groups by p-aminobenzamidine was quantitated by measuring the free carbonyl groups remaining after reaction with p-aminobenzamidine. The method used was that of Hoare and Koshland (17) which involved derivatization of the agarose groups with glycine methyl ester in the presence of a water-soluble carbodiimide. The amount of glycine methyl ester incorporated into the agarose derivative was calculated, after hydrolysis, by measuring glycine on the amino acid analyzer. The hydrolysis procedure was identical to that used for ε-aminocaproic acid-agarose. The analysis of the p-aminobenzamidine-succinyl-diaminodipropylamine-agarose gave 11.2 μmol of free carbonyl groups/ml of agarose after succinylation and 0.6 mol after derivatization with p-aminobenzamidine, indicating that there were 10.6 μmol of p-aminobenzamidine/ml of settled agarose.

Enzyme Assays—The thrombin-like activities were measured at 25° using 0.5 ml of 90% clottable fibrinogen dissolved in 0.05 M Tris·HCl and 0.1 M NaCl, pH 7.4, to give a protein concentration of 2 mg/ml. The concentration of fibrinogen was based on an extinction coefficient of 14.8 for a 1% solution at 282 nm. Heparin (1 mg/ml) was also included in the assay mixture for the purpose of inhibiting the thromboplastin-like activity. The amount of the thrombin-like activity incorporated into the agarose was calculated assuming a plot of reciprocal clotting time versus units of activity yielded a straight line. The activity of a lot of Defibrase (Pentapharm AG) was determined by comparing its activity to NIH standard thrombin. The two plots obtained for thrombin and Defibrase were parallel from 25 to 200 s and therefore the thrombin-like activity of Bothrops atrox venom was expressed directly in NIH standard units. Standard curves were prepared daily using freshly dissolved fibrinogen solutions.

Esterolytic activities were measured titrimetrically with a Radiometer titrator, model TTT1, at 37° using 4-nitrophenyl-p'-g~nidinoben-
Inhibitor, an affinity support was synthesized with p-aminobenzamide linked to agarose via the c-aminocaproic acid. When a sample of the crude venom from Bothrops atrox maojeni 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 Cuganos (16) has shown that increasing the length of the spacer can increase the degree to which an enzyme binds to an affinity absorbent, p-aminobenzamide was attached to the agarose backbone via the longer spacer of diaminodipropylaminosuccinate.

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-aminobenzamide-agarose 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-aminobenzamide 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 0.4 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 benzamide at a concentration of 0.15 M. The active enzyme trailed behind the benzamide front and eluted in approximately 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 benzamide. The activity was pooled (Fractions 114 to 138) and the benzamide removed by exhaustive dialyses against three changes of 0.02 M citrate and 0.2 M NaCl, pH 6.0. The pool represented 91% of the starting activity and had an activity of 210 to 230 NIH units/mg.

<table>
<thead>
<tr>
<th>Substrate, tos-Arg-OMe, is 0.005 M, pH 7.7.</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound*</td>
<td>Inhibition</td>
</tr>
<tr>
<td>p-Aminophenylacetic acid</td>
<td>0</td>
</tr>
<tr>
<td>m-Aminophenylpropionic acid</td>
<td>20</td>
</tr>
<tr>
<td>p-Aminophenylbutyric acid</td>
<td>0</td>
</tr>
<tr>
<td>p-Aminobenzamide</td>
<td>69</td>
</tr>
<tr>
<td>y-Guanidinobutyric acid</td>
<td>3</td>
</tr>
<tr>
<td>y-Guanidinobutyric acid, methyl ester</td>
<td>38</td>
</tr>
<tr>
<td>y-Guanidinopropionic acid</td>
<td>7</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>9</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>21</td>
</tr>
<tr>
<td>D-Arginine</td>
<td>9</td>
</tr>
</tbody>
</table>

All inhibitor concentrations are 0.005 M.

**RESULTS**

**Partition of Thrombin-like Activity from Bothrops atrox maojeni**—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 I. Also tested but without any inhibitory activity were the peptides Arg-Val and Gly-Lys as well as p-methoxybenzylamine, spermidine, and 7-aminohexanoic acid. Assays using fibrinogen as substrate gave similar results. As p-aminobenzamide was the best inhibitor, an affinity support was synthesized with p-aminobenzamide linked to agarose via the spacer c-aminocaproic acid. When a sample of the crude venom from Bothrops atrox maojeni 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 Cuganos (16) has shown that increasing the length of the spacer can increase the degree to which an enzyme binds to an affinity absorbent, p-aminobenzamide was attached to the agarose backbone via the longer spacer of diaminodipropylaminosuccinate.

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**Isoelectric Focusing of Affinity Purified Enzyme—Separation**

<table>
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<tr>
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<th>Inhibition</th>
</tr>
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<tbody>
<tr>
<td>p-Aminophenylacetic acid</td>
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<tr>
<td>p-Aminophenylbutyric acid</td>
<td>0</td>
</tr>
<tr>
<td>p-Aminobenzamide</td>
<td>69</td>
</tr>
<tr>
<td>y-Guanidinobutyric acid</td>
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</tr>
<tr>
<td>y-Guanidinobutyric acid, methyl ester</td>
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<td>D-Arginine</td>
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</tbody>
</table>

All inhibitor concentrations are 0.005 M.
Thrombin-like Enzyme from Bothrops atrox Snake Venom

FIG. 1. Affinity chromatography of Bothrops atrox venom at 4°C. Bothrops atrox venom, 20 ml at 100 mg/ml, was placed on a column (2.5 cm x 56 cm) of p-aminobenzamidine-succinyl-diaminodipropylamino-agarose which had been equilibrated at 4°C with 0.05 M Tris-HCl, 0.4 M NaCl, pH 9.0, and washed with the same buffer until the A280 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 units/ml.

Partition of the three components present in the affinity purified enzyme was accomplished by isoelectric focusing, the results of which are shown in Fig. 2. It was noted that the thrombin-like activities and the protein bands corresponded exactly. The three active fractions were pooled and used for further studies. The results of polyacrylamide disc gel electrophoresis on these samples are shown in Fig. 3. The position of the individual bands isolated by isoelectric focusing corresponded with those found by disc gel electrophoresis of the protein prior to isoelectric focusing. The bands located at the bottom of gels B and D were coincident with the dye marker and were due to incomplete removal of the ampholines from the protein solutions.

Partial Specific Volume, Sedimentation Coefficient, and Molecular Weights—From the amino acid composition and carbohydrate content given in Table II (see below) a partial specific volume of 0.700 ml/g was calculated. The protein portion had a partial specific volume of 0.731 ml/g. Sedimentation equilibrium molecular weight experiments on Pool II yielded a molecular weight of 29,100. The slope of log, the recorder deflection versus the square of the radius yielded a straight line from meniscus to the bottom of the cell, indicating that the protein was molecularly homogeneous. Sedimentation velocity experiments on this material yielded a single symmetrical boundary and a sedimentation coefficient of 2.66 S. The molecular weight was also estimated by disc gel electrophoresis in the presence of sodium dodecyl sulfate (21). The results of this experiment, where mobilities of marker proteins of known molecular weight were compared with the thrombin-like activity yielded a value of 32,400. This value is higher than that obtained by sedimentation equilibrium (29,100). This discrepancy is not unexpected for a glycoprotein (see “Discussion”).

Amino Acid Composition and Carbohydrate Content—The amino acid and carbohydrate composition of the main protein fraction purified by isoelectric focusing (Pool II) is shown in Table II. The amino acid analysis was based on an aspartic acid content of 22 residues/mol of protein. Tryptophan determination gave 6.0 residues/mol. Cysteine, determined as cysteic acid, yielded 7.9 residues/22 aspartic acid residues. Because of the small amount of protein isolated by isoelectric focusing it was not possible to do an extensive and complete amino acid analysis of Pools I and III. 24-hour hydrolysates of Pools I and III gave results very similar to those obtained for the 24-hour hydrolysate of the main pool. Within experimental error the amino acid compositions of the three proteins were identical. Carbohydrate analyses show that the thrombin-like activity of Bothrops atrox is a glycoprotein containing 27% carbohydrate. The carbohydrate includes N-acetylglucosamine, sialic acid, and free hexose. Only a trace of N-acetylgalactosamine was found. N-Acetylglucosamine and N-acetylgalactosamine were
These experiments were performed with enzyme which had activity and the properties of the purified enzymes of this

<table>
<thead>
<tr>
<th>Carbohydrate (%)</th>
<th>26.7</th>
<th>29.7</th>
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</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>37.8</td>
<td>37.8</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>4.9</td>
<td>4.0</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>73.3</td>
<td>73.3</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>26.7</td>
<td>26.7</td>
</tr>
</tbody>
</table>

*Based on 21 aspartic acids using the data of Markland and Damus (8).

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.

**Titration 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 a A280 nm/A260 nm ratio of 1.57. An extinction coefficient (E1% (280 nm)) of 15.6 was measured from the dry weight of an aqueous sample. This value was used for all determinations of protein concentrations.

**Factor XIII-activating Activity**—When equal amounts of thrombin, Defibrase and, affinity purified enzyme were tested for their ability to cross-link fibrin monomer, Defibrase (▲), thorubin (□—□), or the purified fraction from Bothrops atrox, subspecies moojeni (●—●) was added to a test tube containing 4 mg of fibrinogen dissolved in 1.0 ml of 0.17 M Tris-HCl, 0.03 M NaCl, 0.005 M cysteine, and 0.01 M CaCl2, pH 7.5. After the indicated time period 1.0 ml of 2% monochloroacetic acid was added and the clot agitated for 30 s. The A490 of the supernatant was determined after centrifugation.

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

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>moojeni</th>
<th>marajoensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH thrombin units/g of venom</td>
<td>2,200</td>
<td>17,000</td>
</tr>
<tr>
<td>NIH thrombin units/mg of pure enzyme</td>
<td>210-250</td>
<td>900-1,100</td>
</tr>
<tr>
<td>Sedimentation coefficient</td>
<td>2.65</td>
<td>2.60</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>29,100</td>
<td>31,400</td>
</tr>
</tbody>
</table>

*Purified by affinity chromatography only.

subspecies with the 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.

**DISCUSSION**

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-amino- benzamidine linked to agarose with the spacer, 3,3'-diaminodi- propylaminobenzoate. The use of p-amino- benzamidine affinity supports have been reported for the purification of thrombin and trypsin (29, 30) as well as acrosomal 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°-(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 ionic 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-diaminodi-propylaminosuccinate 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.

\[
\text{NH}_2 + \text{NH}_2 \text{H} \quad \text{NH}_2 \quad \text{NH}_2 + \text{NH}_2 \text{H} \quad \text{NH}_2 \text{H}
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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 Crotalid 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/mol 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 ability, like thrombin, to activate Factor XIII (35). However, the results shown in Fig. 4, where Defibrase, thrombin, and affinity purified enzyme were 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.

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