The Thrombin-like Enzyme from Bothrops atrox Snake Venom

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

(Received for publication, August 5, 1979)

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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 $E_{1%}$ 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.

EXPERIMENTAL PROCEDURE

Materials—p-Aminobenzamidine, N-$\text{N}^\text{toyl}-l$-arginine methyl ester·HCl, p-nitrophenyl-$p$-guanidino-benzoate·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 Ost Chemical Co.; ninhydrin, anthrone, thoracicuric acid, N-acetyl- 
alanine, and glycine methyl ester-HCl from Sigma; 3,3'-diaminodipropylamine, succinic anhydride from Aldrich, and sodium dodecyl sulfate from Matheson, Coleman and Bell. Miami Serpentarium provided Bothrops atrox moccas venom, and Bothrops atrox moccas venom. The former was the kind gift of Pentapharm AG, Basel. The venoms were received as desiccated dry crystalline substances which were stable for several months at −30°. Defibrin, 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-amino benzamidine to Sepharose 4B with either ε-amino acrylic acid or diamino- 
propylaminomaleimide-diacetate to give a spacer. Sepharose 4B was activated with cyanogen bromide (100 mg/ml of agarose) according to the method of Cuatrecasas (14). Either ε-amino acrylic acid (0.1 mol/mol of 
agarose) or 3,3'-diaminodipropylamine (100 μmol/ml of agarose) was added to the washed, activated agarose and allowed to react at 4°C and 
15. the reaction mixture was washed successively with 2 M NaCl, 1 M acetic acid, and lastly with water. The amount of succinylation was measured by comparing the color of the pelleted 
agarose with water-soluble carbodiimide. The amount of glycine methyl ester incorporated into the agarose derivative was calculated, after hydrolysis, by comparing its activity to NIH standard thrombin. The extent of substitution of the carbboxyl groups by p-amino benzamidine was quantitated by measuring the free carbboxyl groups remaining after reaction with p-amino benzamidine. The method used was that of Hoare and Koshland (17) which involved derivatization of the 
agarose. The derivatization mixture was made by dissolving 1 g of agarose in 5 ml of 1 N HCl and 0.2 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 thrombin-like-like-like activity. The amount of the thrombin-like activity 
among thrombin or Defibrase was expressed directly in NIH standard units. Standard curves were prepared daily using freshly dissolved fibrinogen solutions.

Esterolytic activities were measured titrimetrically with a Radiome- 
titrator, model TTTI, at 37°C using tos-Arg-OMe as the substrate. The reaction mixture, 2.0 ml, contained 0.005 M tos-Arg-OMe, 0.1 M 
CaCl₂, to give a total volume of 1.0 ml. The reactions were maintained at pH 7.7 by the continuous addition of 0.1 M NaOH and enzyme activities were measured from the initial linear portion of the titration curve. These activities were corrected for the spontaneous hydrolysis of the tos-Arg-OMe which occurred in the absence of enzyme. The inhibitor studies were performed using Defibrase as the enzyme source. The enzyme and inhibitor were incubated in the reaction vessel for 5 min prior to the addition of tos-Arg-OMe.

Factor XIII-activating activity of the pure enzyme was quantitated 
by measuring the amount of fibrin soluble in 0.1 M NaCl, 0.05 M 
NaOH and enzyme activities were measured from the initial linear

Titration of Pure Enzyme with p-Nitrophenyl-p'-g~nidinoben-

The abbreviation used is: tos-Arg-OMe, N-tosyl-L-arginine methyl ester-HCl.
Inhibitor, an affinity support was synthesized with p-aminobenzoic acid linked to agarose via the spacer 8-aminoacetate and benzamidine linked to agarose via the spacer f-aminocaproic acid.

When a sample of the crude venom from Bothrops atrox was chromatographed, a 6 fold molar excess of an inhibitor was used. It was found that spontaneous hydrolysis of p-nitrophenyl-p'-guanidinobenzoate-HCl was corrected for by adding equal amounts of it to the reference and sample cuvette. The absorbance due to reaction of this benzamide 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 gels were soaked overnight in 10% 5-sulfosalicylic acid, stained for 10 min with 1% Amido black, and destained electrophotoically in 10% acetic acid. The relative amounts of the bands were determined by scanning the stained gels with the Deniscord 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°F 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). Triptophan 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 to give the desired concentration, assuming an extinction coefficient of ε: 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 three times, and drying the lyophilized powder to constant weight in an evacuated desicator over P2O5. This dried protein was weighed and dissolved in 0.1 M ammonium bicarbonate. The absorbance of this solution was measured at 280 nm correcting for light scattering by subtracting the 340 nm reading. This correction was very small being less than 2% of the 280 nm reading.

Partial specific volumes were calculated from the amino acid composition and carbohydrate content 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 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-aminobenzamidine was the best inhibitor, an affinity support was synthesized with p-aminobenzamidine linked to agarose via the spacer ε-aminoacaproic 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 Cuatrecasas (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 agarose backbone via the longer spacer of dianinodipropylamino 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. All of the above column chromatography experiments were performed at room temperature. Absorption of the crude venom to the p-aminobenzamidine-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-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 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 benzamidine. The activity was pooled (Fractions 114 to 138) and the benzamidine 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. Polyacrylamide 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.

Isoelectric Focusing of Affinity Purified Enzyme—Separa-
Amino Acid Composition and Carbohydrate Content—The amino acid 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. However, 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-acetylgulosamine, sialic acid, and free hexose. Only a trace of N-acetylgalactosamine was found. N-Acetylgulosamine and N-acetylgalactosamine were

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These experiments were performed with enzyme which had activity and the properties of the purified enzymes of this carbohydrate

<table>
<thead>
<tr>
<th>Component</th>
<th>Bothrops atrax</th>
<th>Crotaulus adamantus</th>
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</thead>
<tbody>
<tr>
<td>Amino acid (%)</td>
<td>26.7</td>
<td>73.3</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>73.3</td>
<td>26.7</td>
</tr>
</tbody>
</table>

**TABLE II**

Amino acid and carbohydrate composition of thrombin-like enzymes from Bothrops atrax moojeni and Crotaulus adamantus

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**Discussion**

The present report outlines an affinity method for the purification of the thrombin-like activity from Bothrops atrax venom. The affinity support consisted of the inhibitor p-amino-phenylbenzamidine linked to agarose with the spacer, 3,3'-diaminodipropylaminomuconate. The use of p-amino-phenylbenzamidine 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 atrax 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 subunits 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.

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**Fig. 4.** Determination of Factor XIII activity. An equal amount of Defibrase (A—A), thrombin (O—O), or the purified fraction from Bothrops atrax, 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 CaCl$_2$, 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 A$_{800}$ of the supernatant was determined after centrifugation.

**TABLE III**

Comparison of Bothrops atrax subspecies, moojeni and marajoensis

<table>
<thead>
<tr>
<th></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>2,200</td>
<td>17,000</td>
</tr>
<tr>
<td>Sedimentation coefficient</td>
<td>2.65</td>
<td>2.60</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>31,400</td>
<td>31,400</td>
</tr>
</tbody>
</table>

*Purified by affinity chromatography only.

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**TABLE II**

Amino acid and carbohydrate composition of thrombin-like enzymes from Bothrops atrax moojeni and Crotaulus adamantus

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Determined as the free amines after hydrolysis in 6 N HCl at 110°C. 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 a $E_{280}$ nm/$A_{260}$ nm ratio of 1.57. An extinction coefficient ($E_{1%}$ (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 and thrombin had similar cross-linking activities, while the affinity purified enzyme was nearly devoid of this activity (Fig. 4). These experiments were performed with enzyme which had not been purified by affinity chromatography, but not further purified by isoelectric focusing.

**Comparison of Two Bothrops atrax Subspecies, moojeni and marajoensis—** All of the data reported to this point have been obtained from Bothrops atrax, subspecies moojeni, which is native to South Central Brazil. Table III compares the venom activity and the properties of the purified enzymes of this 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 atrax venom. The affinity support consisted of the inhibitor p-amino-benzamidine linked to agarose with the spacer, 3,3'-diaminodipropylaminomuconate. The use of p-amino-phenylbenzamidine 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 atrax 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*-propylaminosuccinate and *p*-aminobenzamidine required the carboxyl group. To overcome the unreactive nature of the *p*-amino group in *p*-aminobenzamidine is very unreactive.

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 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.

\[
\begin{array}{c}
\text{NH}_3 + \text{NH}_2 + \text{NH}_2 + \text{NH}_3 \\
\text{NH}_3 + \text{NH}_2 + \text{NH}_3 \\
\text{NH}_3 + \text{NH}_2 + \text{NH}_3
\end{array}
\]

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.

References

Thrombin-like Enzyme from Bothrops atrox Snake Venom

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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