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 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° 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°. 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 s10w of 2.65 S, a molecular weight of 29,000, and an E1% 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 II enzyme, purified on affinity chromatography only, did not activate Factor XIII and was free of thromboplastic-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 s10w of 2.60, and a molecular weight of 31,400.

EXPERIMENTAL PROCEDURE

Materials—p-Aminobenzamidine, N-tosyl-L-arginine methyl ester.HCl, p-nitrophenyl-p'-guanidinobenzoate-HCl, and all com-

1 C. Nolan, Abbott Laboratories, unpublished experiments.

3 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, aniline, thiorbarbituric acid, N-acetylneuraminic acid, and glycine methyl ester-HCl from Sigma; 3,5-diaminodipropylamine from Aldrich, and sodium dodecyl sulfate from Matheson, Coleman and Bell. Miami Serpentarium provided Bothrops aspis mojgas venom, and Bothrops aspis marajoensis was the kind gift of Pentapharm AG, Basel. The venoms were received as dessicated dry crystalline substances which were stable for several months at -20°. Deferase, a partially purified preparation of the thrombin-like activity from Bothrops aspis, 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 matrices for affinity chromatography were prepared by coupling p-aminobenzamidine to Sepharose 4B with either ε-aminoacrylic acid or dimethylaminopropylamine succinate as a spacer. Sepharose 4B was activated with cyanogen bromide (100 mg/ml of agarose) according to the method of Cuatrecasas (11). Either ε-aminoacrylic acid (0.1 mg/ml 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 pH 9.5 for 16 hours. The derivatized agarose was washed sequentially with 2 ml of NaCl and 1 m acetic acid and lastly with water. The amount of ε-aminoacrylic 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-aminoacrylic acid by the method of Cuatrecasas (16) which involved reaction of ε-aminoacrylic acid with the carbodiimide in the presence of glycine methyl ester as a spacer. The color was quantitated with ninhydrin. The aminoalkyl-agarose 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 modifying the method of Cuatrecasas (16) which involved reaction of the agarose derivative with 2,4,6-trinitrobenzenesulfonic acid. The reacted agarose was washed with water and pelleted by centrifuging at 2000 x g for 10 min and the clotting activity.

The reaction was carried out at room temperature and initiated by adding 1 ml of either thrombin, Deferase, or the affinity-purified enzyme. All of the enzyme solutions were adjusted to give equal clotting times of 85 s. After varying periods of time an equal volume of 2% monochloroacetic acid was added to the reaction mixture and the clot agitated on a vortex mixer for 30 s. 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 LKB 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 and the wash fluid was collected at a rate of 0.5 ml/min. Fractions of approximately 2 ml were collected and measured for enzyme activity, absorbance at 280 nm, and pH. The activity peaks were pooled and the ampholine removed by extensive dialysis against 0.02 M citrate and 0.2 M NaCl, pH 6.0.

Titation of Pure Enzyme with p-Nitrophenyl-p'-glutamidoben-
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-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 0.15 M NaCl, pH 7.0. 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 dialysis 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.

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition</th>
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<tbody>
<tr>
<td>p-Aminophenylacetic acid</td>
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</tr>
<tr>
<td>m-Aminophenylpropionic acid</td>
<td>20</td>
</tr>
<tr>
<td>p-Aminophenylbutyric acid</td>
<td>0</td>
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<tr>
<td>p-Aminobenzamidine</td>
<td>69</td>
</tr>
<tr>
<td>γ-Guanidinobutyric acid</td>
<td>3</td>
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<tr>
<td>γ-Guanidinobutyric acid, methyl ester</td>
<td>38</td>
</tr>
<tr>
<td>ε-Guanidinocaproic acid</td>
<td>7</td>
</tr>
<tr>
<td>L-Lysine</td>
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</tr>
<tr>
<td>D-Arginine</td>
<td>21</td>
</tr>
<tr>
<td>ε-Aminocaproic acid</td>
<td>9</td>
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</tbody>
</table>

*All inhibitor concentrations are 0.005 M.*

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**RESULTS**

**Purification of Thrombin-like Activity from Bothrops atrox Snake Venom majojeni**—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-aminohexapeptide 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-aminocaproic acid. When a sample of the crude venom from Bothrops atrox majojeni 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 Cautrecasas (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 diaminodipropylaminomuccinate.

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-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 0.15 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 dialysis 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-
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-diaminopropylamino-agarose which had been equilibrated at 4°C with 0.05 M Tris-HCl, 0.1 M NaCl, pH 9.0, and washed with the same buffer until the A400 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.

Fig. 2. Isoelectric focusing of the thrombin-like activity purified by affinity chromatography. The affinity purified enzyme (5.0 mg) was placed on an LKB 110-ml column and focused in a pH 5 to 9 gradient for 41 hours at 4°C. Enzyme activity (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.

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. 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-acetylglucosamine, sialic acid, and free hexose. Only a trace of N-acetylglactosamine was found. N-Acetylglucosamine and N-acetylglactosaminidase were
These experiments were performed with enzyme which had activity and the properties of the purified enzymes of this Carbohydrate

<table>
<thead>
<tr>
<th>Components</th>
<th>Bothrops atrox</th>
<th>Crotaulus adamanteus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine (residues/29,700)</td>
<td>10.8</td>
<td>7</td>
</tr>
<tr>
<td>Histidine</td>
<td>6.0</td>
<td>6</td>
</tr>
<tr>
<td>Arginine</td>
<td>8.1</td>
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<tr>
<td>Aspartic acid</td>
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<tr>
<td>Threonine</td>
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<tr>
<td>Serine</td>
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<tr>
<td>Glutamic acid</td>
<td>11.9</td>
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<tr>
<td>Proline</td>
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<tr>
<td>Glycine</td>
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<tr>
<td>Alanine</td>
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<tr>
<td>Halt-cystine</td>
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</tr>
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<td>Valine</td>
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<tr>
<td>Methionine</td>
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<tr>
<td>Isoleucine</td>
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<tr>
<td>Leucine</td>
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<tr>
<td>Tyrosine</td>
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<td>Phenylalanine</td>
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</tr>
<tr>
<td>Tryptophan</td>
<td>6.0</td>
<td>4</td>
</tr>
</tbody>
</table>

Carbohydrate (%)

| Hexose (glucose)    | 37.8           |
| N-Acetylamino glucose | 4.0         |
| N-Acetylneuraminic acid | 3.6       |
| Protein (%)         | 73.3           |
| Carbohydrate (%)    | 26.7           |

*Based on 21 aspartic acids using the data of Morkland and Danus (8).

Amino acid and carbohydrate composition of thrombin-like enzymes from Bothrops atrox moojeni and Crotaulus adamanteus

The yields of the purified thrombin-like enzyme from Bothrops atrox, subspecies moojeni (O-O) 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% monochloracetic acid was added and the clot agitated for 30 s. The A280 of the supernatant was determined after centrifugation.

**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-propylaminomaleate. The use of p-aminoabenzamidine 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 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 150 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 0.70 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.
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/mole), 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. Nolan 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 and elegantly demonstrated by Harvey et al. (32), 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 ionic 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 exists as a resonating structure with all nitrogen protons being equivalent. Some likely structures are shown below.

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/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 ability, 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.

REFERENCES
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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