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# Blood Coagulation Induced by the Venom of *Bothrops atrox*. 1. Identification, Purification, and Properties of a Prothrombin Activator†

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ABSTRACT: In this paper, we show that the procoagulant action of *Bothrops atrox* venom is due in part to a protein component that activates prothrombin. The venom prothrombin activator was purified by ion-exchange chromatography and gel filtration. It was separated from a protease by affinity chromatography in a *p*-aminobenzamidine–CH-Sepharose column. It is a protein of about $M_r$ 70 000, consisting of a single polypeptide chain. We have studied the kinetics of activation of prothrombin under different experimental conditions. The prothrombin activator from *B. atrox* venom is insensitive to reagents of serine and thiol proteases but is inactivated by ion chelators and by various divalent ions. These results suggest that it is a metalloenzyme. The prothrombin activator from *B. atrox* venom is inactive on the chromogenic substrates S-2337 and S-2238, and it is selective for prothrombin since it does not act on other blood coagulation factors such as fibrinogen and factor X. We have also studied the pattern of peptide cleavages produced in the human prothrombin molecule during the activation by the activator from *B. atrox* venom and compared it to that obtained with ecarin, a prothrombin activator from *Echis carinatus* venom. In the presence of thrombin inhibitors, e.g., hirudin, we found that the activators from *B. atrox* venom and ecarin act in a similar, or identical, manner by producing a thrombin intermediate, meizothrombin. In the absence of thrombin inhibitors, several peptides are generated, and $\alpha$-thrombin is produced as a consequence of meizothrombin action.

Snake venoms, particularly those belonging to the families Crotalidae and Viperidae, induce blood clotting. In the case of *Bothrops* species, this activity has been attributed to a fibrinogen clotting enzyme, batroxobin (Stocker &, Egberg, 1973; Stocker & Barlow, 1976; Holleman & Weiss, 1976), which cleaves fibrinopeptide A from fibrinogen (Funk et al., 1971). An other enzyme purified from *Bothrops atrox* venom, thrombocytin, activates blood platelets but has a poor fibrinogen clotting activity (Niewiarowski et al., 1977, 1979; Kirby et al., 1979). In addition to their action on fibrinogen, the venoms of many *Bothrops* species can convert prothrombin into thrombin, either directly or indirectly (Eagle, 1937; Janzky, 1950; Breda et al., 1951; Michl, 1954; Nahas et al., 1964). Recently, we have identified and partially purified a factor X activator from *B. atrox* venom (Hofmann et al., 1983). This procoagulant component resembles that of Russell's viper (*Vipera russelli*) since it activates factor X in a calcium-dependent manner and is not sensitive to diisopropyl fluorophosphate (DFP)[1] (Hofmann et al., 1983).

We also demonstrated that the DFP-treated venom of *B. atrox* clots plasma in the absence of calcium (Hofmann et al., 1983). This indicates that in addition to batroxobin and thrombocytin, which are sensitive to DFP (Kirby et al., 1979), and in addition to factor X activator, which is calcium-dependent (Hofmann et al., 1983), the venom contains another component that acts on fibrinogen and/or prothrombin. The observation that *B. atrox* venom treated with DFP does not clot purified fibrinogen (Hofmann et al., 1983) favors the hypothesis that this venom contains a prothrombin activator.

In this study, we submitted the venom of *B. atrox* to ion-exchange chromatography and analyzed the effects of its fractions on purified fibrinogen, prothrombin, and factor X.

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[1] Abbreviations: DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; S-2238, H-D-Phe-Pip-Arg-*p*-nitroanilide hydrochloride; S-2337, benzoyl-Ile-Glu-Pip-Gly-Arg-*p*-nitroanilide hydrochloride; TAME, *N*-tosylarginine methyl ester; TLCK, *N*-*p*-tosyllysine chloromethyl ketone; TPCK, *N*-*p*-tosylphenylalanine chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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We identified a fraction that activates prothrombin and purified to homogeneity the protein responsible for this coagulant activity. We determined its molecular and enzymatic properties and analyzed its mechanism of action on purified human prothrombin. The prothrombin activator from *B. atrox* venom resembles ecarin, a similar activator that was purified from *Echis carinatus* venom (Franza et al., 1975; Kornalik & Blombäck, 1975; Morita et al., 1976; Morita & Iwanaga, 1978, 1981; Briet et al., 1982; Rhee et al., 1982).

## Experimental Procedures

*Materials.* The venoms from *Bothrops atrox* and *Echis carinatus* were from the stock of the Pasteur Institute. Ecarin, the purified prothrombin activator from *E. carinatus* venom, was a generous gift of Dr. Franck Kornalik (University of Karlovy, Prag, CSSR).

Bovine plasma deficient in factor II was purchased from Diagnostica Stago (Asnières, France). Bovine factor X, human prothrombin, and hirudin were obtained from Laboratoires Stago (Asnières, France), bovine thrombin was from Hoffmann-La Roche (Basel, Switzerland), and bovine fibrinogen was from Povite (The Netherlands).

The chromogenic substrates benzoyl-Ile-Glu-Pip-Gly-Arg-*p*-nitroanilide hydrochloride (S-2337) and H-D-Phe-Pip-Arg-*p*-nitroanilide hydrochloride (S-2238) were purchased from Kabi Diagnostica (Amsterdam, The Netherlands). DEAE-cellulose (DE-52, microgranular) was obtained from Whatman Biochemicals Ltd. (Springfield Hill, United Kingdom); Sephadex G-150 and CH-Sepharose were from Pharmacia (Uppsala, Sweden); *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide hydrochloride and egg lecithin were from Merck A.G. (Darmstadt, Federal Republic of Germany); benzamidine hydrochloride, *p*-aminobenzamidine hydrochloride, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), phenylmethanesulfonyl fluoride (PMSF), *N*-*p*-tosyllysine chloromethyl ketone (TLCK), *N*-*p*-tosylphenylalanine chloromethyl ketone (TPCK), *N*-tosylarginine methyl ester (TAME), and diisopropyl fluorophosphate (DFP) were from Sigma (St. Louis, MO), and acrylamide, *N*,*N'*-methylenebis(acrylamide), and dithiothreitol (DTT) were from Fluka (Bachs, Switzerland). All other chemicals, salts, and solvents were of the best available quality, obtained from Merck (Darmstadt, FRG) or from Prolabo (Paris, France).

*Venom Fractionation. B. atrox* venom (3 g) was dissolved at 4 °C in 10 mM Tris-HCl, pH 7.5 (37 mL), containing 5 mM benzamidine and dialyzed during 16 h against a large volume of the same buffer. The small precipitate (less than 1% of the starting material) was eliminated by centrifugation (25 min at 7000 rpm in a Sorvall centrifuge), and the supernatant was applied to a DEAE-cellulose column (3 × 20 cm) previously equilibrated with the same buffer. Chromatography was performed at 4 °C. Elution was achieved with 10 mM Tris-HCl, pH 7.5, containing 5 mM benzamidine (600 mL) and then with a linear NaCl gradient from 0 to 0.2 M in the same buffer (1500 mL). The protein concentration was estimated from the absorbance at 280 nm, NaCl concentration by conductimetry, the activity of fibrinogen clotting enzyme by its ability to clot purified bovine fibrinogen, the activity of the prothrombin activator by its capacity to activate prothrombin, and the activity of the factor X activator by its activity on purified bovine factor X. Proteolytic activities were monitored by using the two chromogenic substrates S-2238 and S-2337. The phospholipase $A_2$ activity was estimated by the colorimetric method described by Bon and Saliou (1983).

The various fractions were pooled according to their coagulant and enzymatic properties as indicated in Figure 1, lyophilized, dissolved in a small volume of distilled water, and extensively dialyzed against large volumes of 10 mM Tris-HCl, pH 7.5, containing 5 mM benzamidine. Occasionally, a small precipitate was observed after dialysis; it never exceeded 10% of the sample protein and was removed by centrifugation at 7000 rpm for 20 min in a Sorvall centrifuge. The various fractions were frozen at −20 °C and stored up to 1 year in well-closed vials.

*Purification of the Prothrombin Activator.* Fraction II (Figure 1), which was found to have the highest content in prothrombin activator, was applied to a Sephadex G-150 column previously equilibrated with 50 mM Tris-HCl, pH 7.5, containing 5 mM benzamidine and 100 mM NaCl. Elution was performed at 4 °C with the same buffer. Fractions were pooled as described in Figure 1, concentrated, dialyzed, and stored as previously described.

Affinity chromatography was performed in a *p*-aminobenzamidine column: the pH of CH-Sepharose 4B (2 g) suspended in distilled water was adjusted to pH 4.75 with HCl; 1 mL of a solution containing 0.5 g of *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide, also at pH 4.75, was slowly added drop by drop, and the pH of the reaction medium was maintained at the same value by addition of 1 N HCl; 0.2 g of *p*-aminobenzamidine in 1 mL of distilled water was then added, and the mixture was incubated for 5 h at room temperature; the beads were successively washed with large volumes of 2 M NaCl, 1 M acetic acid, 1 mM NaOH, and 50 mM Tris HCl, pH 8.3, containing 0.4 M NaCl. Fraction II-c (Figure 2) was equilibrated with this last buffer by filtration in a small Sephadex G-25 column and applied to the *p*-aminobenzamidine column (1 × 3 cm). Elution was performed with 20 mL of 50 mM Tris-HCl, pH 8.3, containing 0.4 M NaCl and then with 30 mL of 50 mM Tris-HCl, pH 8.3, containing 0.1 M NaCl and 0.15 M benzamidine. The fractions containing prothrombin activator were pooled, concentrated by lyophilization, dialyzed, and stored at −20 °C.

*Assay for Fibrinogen Clotting Activity.* The activity on fibrinogen of the venom and of the fractions obtained during fractionation was determined according to Furukawa and Hayashi (1977) with the modifications described by Hofmann et al. (1983).

*Activation of Purified Prothrombin.* The activation of purified prothrombin was followed by measuring either the amidolytic activity or the coagulant activity of the thrombin which was formed. Purified human prothrombin (20–350 μg/mL) was incubated at 25 °C in 50 mM Tris-HCl, pH 7.5, containing 50 mM NaCl with the prothrombin activator to be tested. Aliquots (1–150 μL) were removed at various times, and their amidolytic activity was immediately tested in 950 μL (final volume) of 200 mM Tris-HCl, pH 8.3, containing 150 mM NaCl, 10 mM EGTA, and 0.10 mM of substrate S-2238. The formation of *p*-nitroaniline was monitored by its absorption at 405 nm. The concentration of the activator was very small compared to that of prothrombin, so that the initial rate of activation was proportional to the concentration of activator. The activation of prothrombin was also determined by the appearance of coagulant activity. The coagulant activity of the aliquots (0.1 mL) was estimated by measuring their ability to induce the coagulation of a human plasma deficient in prothrombin (0.1 mL).

*Activation of Purified Factor X.* The activation of purified factor X was followed by measuring the amidolytic activity of the factor Xa that was formed. Purified bovine factor X (10 μg/mL) was incubated at 25 °C in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, and an appropriate con-

Table I: Specific Activity and Yield of Prothrombin Activator at Different Stages of Purification[a]

| fraction | protein | | activation of prothrombin | | sp act. | purification factor |
|---|---|---|---|---|---|---|
| | mg | % | nmol/min | % | [nmol/(min·mg)] | |
| venom | 2830 | 100 | 500 | 100 | 0.18 | 1 |
| II (DEAE-cellulose) | 27 | 1 | 110 | 22 | 4 | 22.7 |
| II-c (Sephadex G-150) | 2.2 | 0.08 | 40 | 7.8 | 17.9 | 101 |
| II-c-1 (affinity chromatography) | 0.5 | 0.02 | 30 | 5.7 | 57.5 | 326 |

[a] The activity of prothrombin activator was measured as described under Experimental Procedures by its ability to activate purified human prothrombin. It is expressed in nanomoles of prothrombin cleaved per minute. The protein concentration was determined from the absorbance at 280 nm, assuming $A_{280}(1\%) = 10$.

centration of activator. Aliquots (1–50 μL) were removed at various times, and their amidolytic activity was immediately determined in 950 μL of 200 mM Tris-HCl, pH 8.3, 150 mM NaCl, 10 mM EGTA, and 0.25 mM S-2337 by recording the formation of *p*-nitroaniline.

*Enzymatic Assays.* The proteolytic activity of the venom and of its fractions was determined with the chromogenic substrates S-2238 and S-2337. The assay was performed at 25 °C in 950 μL of buffer containing 200 mM Tris-HCl, pH 8.3, 150 mM NaCl, and 0.10 mM S-2238 or 0.25 mM S-2337. The reaction was followed spectrophotometrically by recording the formation of *p*-nitroaniline at 405 nm. Phospholipase activity was determined either by a colorimetric method as described by Bon and Saliou (1983) or by the titrimetric method of Desnuelle et al. (1955) as described by Radvanyi and Bon (1982).

*Polyacrylamide Gel Electrophoresis.* Polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate (SDS) in 1.0–1.5 mm thick plates, according to the method of Laemmli et al., (1970) with the modifications described by Li and Bon (1983). The following proteins were used as molecular weight standards: myosin from rabbit muscle (140 000), bovine serum albumin (67 000), catalase from bovine liver (62 000), chicken ovalbumin (43 000), aldolase (39 000), lactic dehydrogenase from rabbit muscle (35 000), pig chymotrypsinogen) A (25 000), soybean trypsin inhibitor (20 000), and ribonuclease from pig pancreas (15 000).

*Enzyme Inhibitors.* Serine proteases were inactivated by incubating the samples at room temperature for 16 h with 25 mM DFP in 50 mM Tris-HCl, pH 7.5. The treated samples were then dialyzed twice for several hours against large volumes of 5 mM Tris-HCl, pH 7.5. Sensitivity to DFP, PMSF, TLCK, TPCK, iodoacetic acid, iodoacetamide, cysteine, DTT, *p*-bromophenacyl bromide, *o*-phenanthroline, and EGTA was tested by incubating prothrombin activator at 30 °C in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and various concentrations of reagent.

## Results

*Purification of Prothrombin Activator.* Figure 1 shows the fractionation of *B. atrox* venom by ion-exchange chromatography in a DEAE-cellulose column. Four procoagulant components were found. The factor X activator that had been previously identified (Hofmann et al., 1983) eluted in two peaks corresponding to fractions V and VI. The fibrinogen clotting activity due to batroxobin (Stocker & Barlow, 1976) eluted mainly in fraction IV and partially in fraction III. Finally, the prothrombin activator eluted at the lowest ionic strength, mostly in fraction II and partially in fraction III. Fractions II and III also contained some protease activity, determined with the chromogenic substrates S-2238 and S-2337 (Figure 1). This enzymatic activity as well as the fibrinogen clotting activity was found to be completely inhibited in the presence of 5 mM benzamidine and inactivated by a DFP treatment, whereas the prothrombin activator was found to be totally insensitive to benzamidine and DFP. The chromatography was therefore performed in the presence of 5 mM benzamidine. None of the coagulant fractions (II–VI) was toxic when injected intravenously into mice, the lethal components of the venom being found mostly in fraction VIII.

Fraction II was further fractionated by gel filtration in Sephadex G-150 (Figure 2A). Four protein fractions were obtained, one of them containing both the prothrombin activator and the protease activity. However, the prothrombin activator eluted as a single symmetrical peak with an apparent $M_r$ of 68 000, i.e., slightly lower than that of the contaminating protease. Fraction II-b and II-c were defined according to their relative proportions in the two components. Polyacrylamide gel electrophoresis indicated that fraction II-c, which is enriched in prothrombin activator, was mainly composed of two components whereas fraction II-b contained an additional band (Figure 2B). The purification of the prothrombin activator from fraction II-c was achieved by affinity chromatography in a *p*-aminobenzamidine column (Figure 3A). The protein peak that did not adsorb to the column (fraction II-c-1) contained all the prothrombin activator and was totally devoid of protease activity, as determined with the chromogenic substrates S-2238 and S-2337. The protease was eluted when 0.15 M benzamidine was applied to the affinity column (fraction II-c-2).

Polyacrylamide gel electrophoresis, performed in the presence of SDS with or without reduction (2% β-mercaptoethanol), indicated that fraction II-c-1, which contains the prothrombin activator, is homogeneous, presenting a single polypeptide of apparent $M_r$ 70 000 (Figure 3B). This value is in good agreement with that obtained by gel filtration, 68 000, indicating that the prothrombin activator of *B. atrox* consists of a single polypeptide of about $M_r$ 70 000. A similar analysis indicated that the protease contained in fraction II-c-2 is a dimer of polypeptides of apparent $M_r$ 34 500.

Table I shows that the specific activity of the prothrombin activator was increased more than 300-fold after purification, indicating that it is a minor component of the *B. atrox* venom. In addition, the yield of the purification was relatively low, about 6%. This is mainly due to the fact that we discarded the prothrombin activator contained in fractions III (Figure 1) and II-b (Figure 2).

*Enzymatic Properties of Prothrombin Activator of B. atrox Venom.* We studied the kinetics of activation of prothrombin by the purified activator from *B. atrox* venom by measuring the amidolytic and the coagulant activities of the activation products that were formed as a function of time. A low concentration (47 nM, 3.3 μg/mL) of activator incubated with a 100-fold excess of purified human prothrombin (4.6 μM, 330 μg/mL) generated both amidolytic and coagulant activities almost simultaneously and without latency (Figure 4). Within the first 30 min the activation proceeded linearly and then

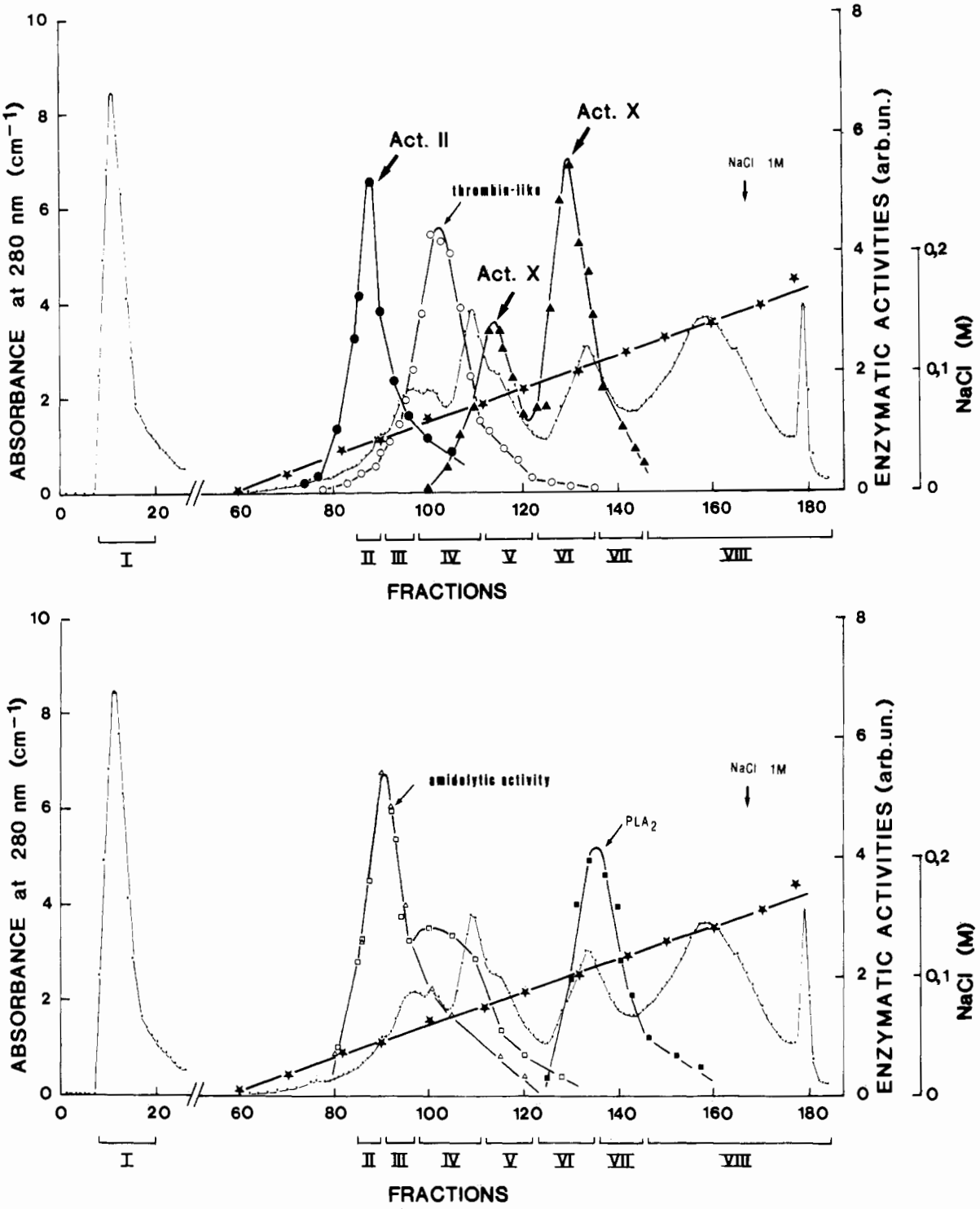



FIGURE 1: Chromatography of *B. atrox* venom in a DEAE-cellulose column. A total of 3 g of lyophilized *B. atrox* venom was dissolved in 37 mL of 10 mM Tris-HCl buffer, pH 7.5, containing 5 mM benzamidine. After dialysis, the solution was centrifuged, and the small pellet was discarded. The supernatant was applied to the DEAE-cellulose column (3 × 20 cm), which has been previously equilibrated with the same buffer. The elution was achieved as described under Experimental Procedures, with a linear NaCl gradient from 0 to 0.2 M (★). The protein concentration was estimated from the absorbance at 280 nm (–·–). Fibrinogen clotting enzyme (○), prothrombin activator (●), factor X activator (▲), phospholipase $A_2$ activity (■), and protease activity on S-2238 (□) and on S-2337 (△) were determined as described under Experimental Procedures. The fractions were pooled as indicated in the figure.

reached a plateau. Further analysis showed that the initial rate of the apparition of amidolytic activity was proportional to the concentration of the prothrombin activator when its concentration was small compared to that of prothrombin (less than a tenth) (results not shown). In addition, we found that the prothrombin activator from *B. atrox* venom was specific for prothrombin since it did not act on other coagulation factors such as fibrinogen and factor X. In addition, it did not possess any hydrolytic activity on the synthetic substrates that we tested: TAME, S-2238, and S-2337.

The activity of the venom activator was maximal between pH 6.5 and pH 8.0. The variation of the activity as a function of pH suggested that the catalytic activity of the activator depends of the protonation of two ionizable groups, with p*K* values of about 5.2 (Figure 5), which may correspond to histidine residues, located either on the activated molecule or on the substrate molecule, prothrombin. The reagents of serine hydrolases, DFP, PMSF, TLCK, and TPCK, were inefficient

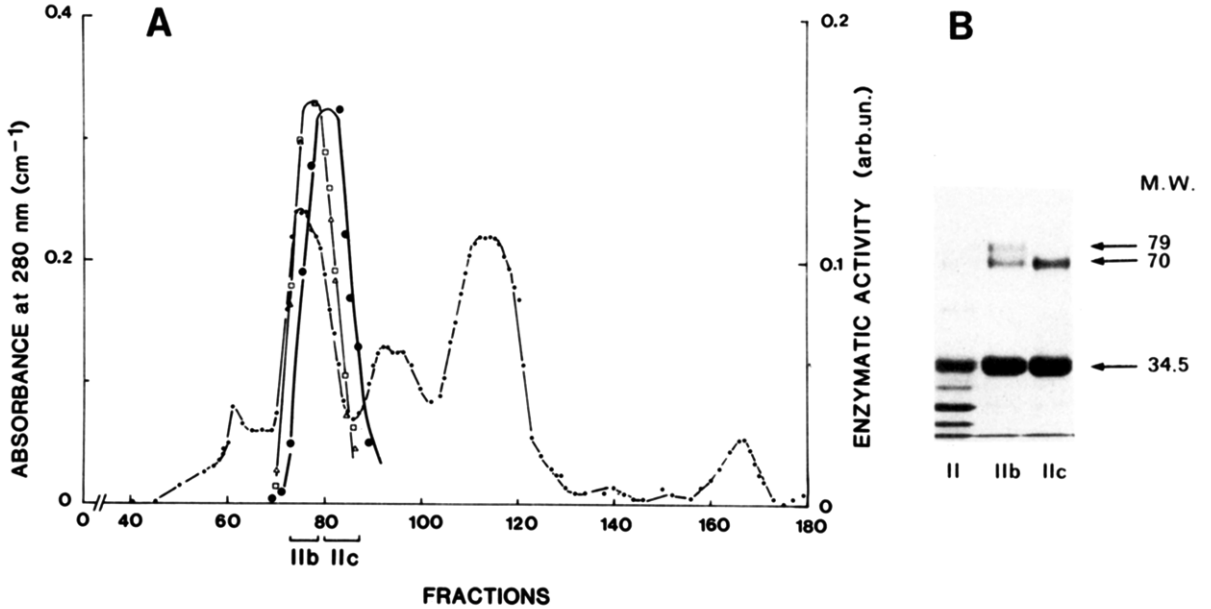



FIGURE 2: Gel filtration of fraction II in a Sephadex G-150 column. (A) Fraction II (27 mg, 2.5 mg/mL) was applied to the top of the Sephadex G-150 column (2.5 × 90 cm) previously equilibrated at 4 °C with 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl and 5 mM benzamidine. Elution was performed with the same buffer at a speed of 8 mL/h. The protein concentration was estimated from the absorbance at 280 nm (–·–). Prothrombin activator (●) and protease activity on S-2238 (□) and on S-2337 (△) were determined as described under Experimental Procedures. The fractions were pooled as described in the Figure. (B) The polypeptide composition of fractions II, II-b, and II-c was analyzed by electrophoresis in polyacrylamide gels (10%) in the presence of SDS (4%) and reducing agent (2% β-mercaptoethanol). Staining was achieved with Coomassie brilliant blue.

Table II: Effect of Various Reagents on Prothrombin Activator from *B. atrox* Venom[a]

| compd | concn (mM) | incubation time (min) | rate of activation (%) |
|---|---|---|---|
| | | 0 | 100 |
| | | 210 | 100 |
| DFP | 25 | 210 | 100 |
| PMSF | 8 | 210 | 100 |
| TLCK | 8 | 210 | 100 |
| TPCK | 1.7 | 210 | 61 |
| iodoacetic acid | 8 | 210 | 93 |
| iodoacetamide | 8 | 210 | 90 |
| *p*-bromophenacyl bromide | 0.8 | 70 | 100 |
| L-cysteine | 4 | 35 | 8 |
| DTT | 4 | 35 | 34 |
| *o*-phenanthroline | 0.8 | 15 | 4.5 |
| EGTA | 5 | 10 | 5 |

[a] Purified prothrombin activator from *B. atrox* venom was incubated at 30 °C for the indicated times in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl with or without (control) the indicated concentration of reagent. The initial rate of activation of a fixed concentration of purified human prothrombin (17 μg/ml, 0.24 μM) by a fixed concentration of treated or nontreated (control) activator was determined by measuring the amidolytic activity of the activated prothrombin with S-2238. In each case, the initial rate of activation was expressed as percent of the control value.

Table III: Inhibition of Prothrombin Activator from *B. atrox* by Divalent Ions[a]

| ion | $I_{50}$ (mM) | ion | $I_{50}$ (mM) |
|---|---|---|---|
| $Mg^{2+}$ | 18 | $Mn^{2+}$ | 4 |
| $Ba^{2+}$ | 12 | $Hg^{2+}$ | 2 |
| $Ca^{2+}$ | 10 | $Cd^{2+}$ | 0.6 |
| $Sr^{2+}$ | 10 | | |

[a] The initial rate of prothrombin activation by the activator from *B. atrox* venom was determined as described in Table II, in the presence of various concentrations of the tested divalent ions. In each case, the concentration of ions reducing the control value by 50% ($I_{50}$) was calculated by interpolation. The concentrations of prothrombin and activator were chosen in such a way that after dilution the divalent ions never significantly inhibited the amidolytic activity of thrombin.

on the prothrombin activator from *B. atrox* venom, even when incubated at high concentrations (more than 1 mM) and for long periods of time (more than 3 h) (Table II). We only observed a slight but significant inhibition with TPCK, at a concentration of 1.7 mM. Similarly, *p*-bromophenacyl bromide and reagents of thiol residues (iodoacetic acic and iodoacetamide) were inactive in the same experimental conditions. On the other hand, the prothrombin activating capacity of the venom activator was rapidly and irreversibly blocked by reagents that reduce disulfide bridges (cysteine and dithiothreitol) (Table II). The enzymatic activity of the prothrombin activator was inhibited by low concentrations (less than 1 mM) of ion chelators such as *o*-phenanthroline and EGTA (Table II). The inhibition by *o*-phenanthroline appeared after a few minutes whereas that by EGTA occurred immediately. In addition, the inhibition by EGTA was reversible either by dilution or by addition of a slight excess of calcium ions within the first hour of incubation but became irreversible after longer incubation times (results not shown). The venom prothrombin activator was inhibited by numerous divalents ions: $Mg^{2+}$, $Ba^{2+}$, $Ca^{2+}$, $Sr^{2+}$, $Mn^{2+}$, $Hg^{2+}$, and $Cd^{2+}$. Table III gives the concentration of these ions that inhibited 50% of the activity.

*Cleavage of Prothrombin by B. atrox Activator.* Catalytic cleavages of the human prothrombin polypeptide by the purified activator from *B. atrox* venom was investigated by polyacrylamide gel electrophoresis in the presence of SDS. We studied the kinetics of the cleavage in parallel with that of the appearance of the amidolytic activity. This study was performed by comparison with the cleavages of prothrombin by ecarin, a prothrombin activator from the venom of *E. carinatus* (Morita et al., 1976; Rhee et al., 1982; Briet et al., 1982).

In order to prevent possible additional cleavages by thrombin and its intermediate precursors, the reactions were performed in the presence of hirudin, a low molecular weight polypeptide isolated from the european leech (*Hirudo medicinalis*), which inhibits thrombin and thrombin intermediates with a very high affinity. Figure 6A shows that no amidolytic activity was measurable in the presence of hirudin, whereas full amidolytic

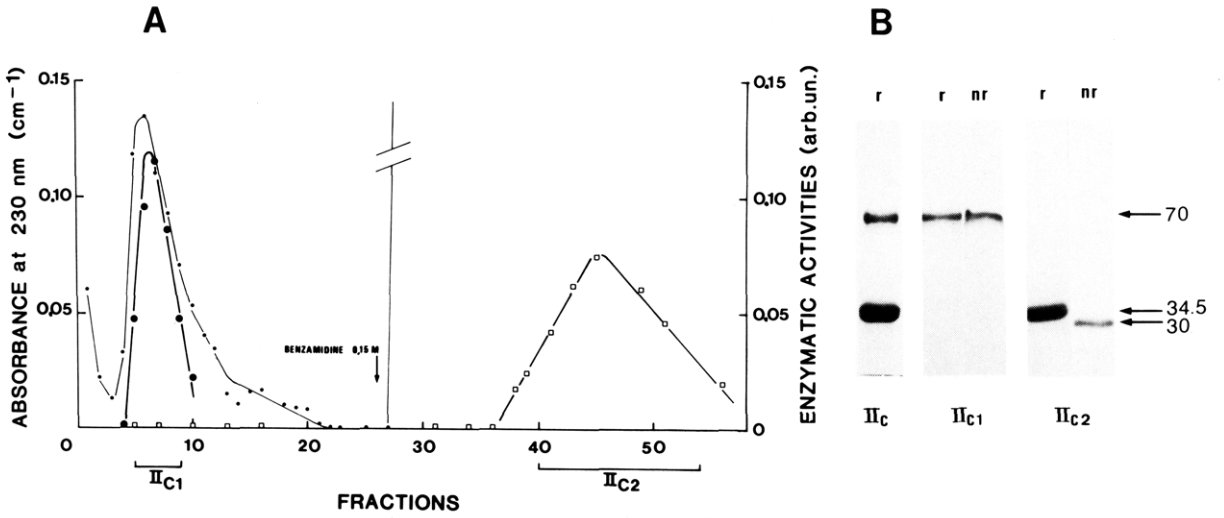



FIGURE 3: Purification of prothrombin activator by affinity chromatography. (A) Fraction II-c (1.4 mg, 0.7 mg/mL) was dissolved in 50 mM Tris-HCl, pH 8.3, containing 0.4 M NaCl and applied to the top of an affinity column prepared with *p*-aminobenzamidine coupled to CH-Sepharose 4B. The elution was performed at a flow rate of 9 mL/h with the same buffer (20 mL) and then with 50 mM Tris-HCl, (pH 8.3, containing 0.1 M NaCl and 0.15 M benzamidine. The protein concentration was estimated from the absorbance at 230 nm (–·–); prothrombin activator (●) and protease activity on S-2238 (□) were determined as previously described. (B) The polypeptide composition of fractions II-c, II-c-1, and II-c-2 was analyzed by electrophoresis in polyacrylamide gels (10%) in the presence of SDS (4%) without reducing agent (nr) or in the presence of 2% β-mercaptoethanol (r).

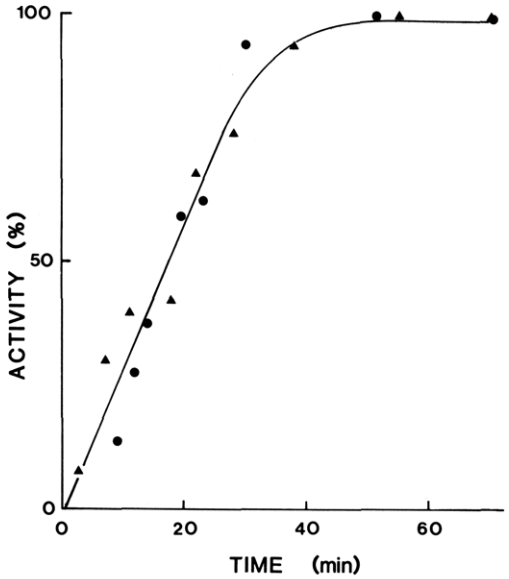



FIGURE 4: Kinetics of activation of human prothrombin by *B. atrox* activator. Purified human prothrombin (330 μg/mL, 4.6 μM) was incubated at 37 °C with prothrombin activator purified from *B. atrox* venom (3.3 μg/mL, 47 nM) in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl. Aliquots were removed at the indicated times, and their amidolytic (▲) and coagulant (●) activities were immediately determined as described under Experimental Procedures, using respectively S-2238 and bovine plasma deficient in factor II. Both activities were expressed as percent of their maximal value.

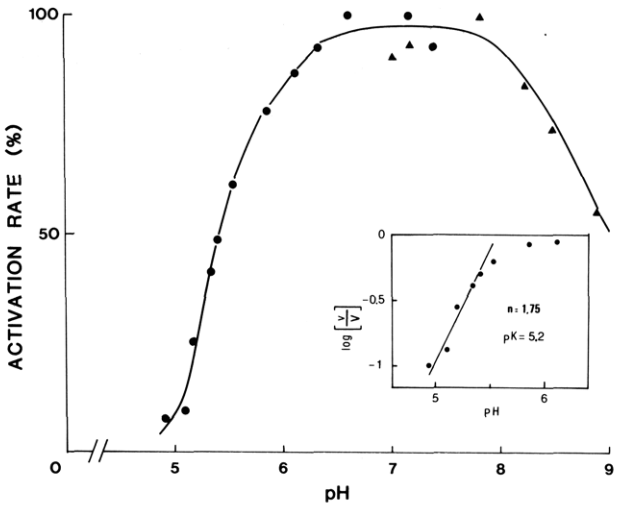
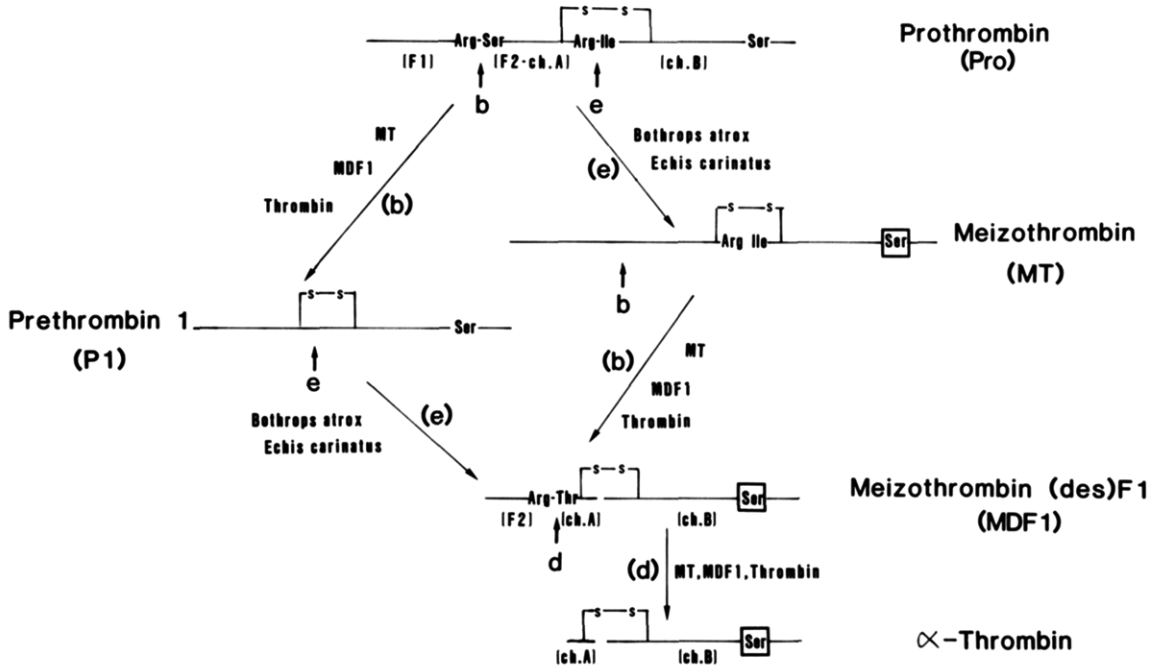



FIGURE 5: pH dependence of activation of prothrombin by *B. atrox* venom activator. The initial rate of activation of a fixed concentration of purified human prothrombin (18 μg/mL, 0.25 μM) by a fixed concentration of activator partially purified from *B. atrox* venom (0.3 μg/mL of fraction II-c) was determined by measuring the amidolytic activity of thrombin with S-2238. Assays were performed at the indicated pH in 20 mM cacodylate (●) or Tris-HCl (▲) buffer containing 150 mM NaCl. The initial rates of activation are expressed as percent of the maximal value obtained. The insert represents a plot of the logarithm of these values as a function of pH.

activity is obtained in less than 20 min in absence of the inhibitor (control samples). Electrophoretic analyses of samples removed during incubation of prothrombin with venom activators in the presence of hirudin showed that in the absence of reduction there was no change in the molecular weight of the prothrombin polypeptide (Figure 6B). On the other hand, Figure 6C shows that after reduction of the samples the polypeptide band of apparent $M_r$ 74 000 corresponding to prothrombin disappeared during activation while two bands of apparent $M_r$ 58 000 and 33 000 appeared simultaneously. This indicates that both venom activators cleave the polypeptide chain of prothrombin at a single site (possibly the same site), located in a loop formed by a disulfide bridge. In the case of the *E. carinatus* activator, ecarin, it has been shown that this cleavage occurs between residues $Arg_{320}$ and $Ile_{321}$ (site e in Scheme I) (Briet et al., 1982; Rhee et al., 1982; Friezner-Degen et al., 1983), forming meizothrombin, which consists of two polypeptides linked by a disulfide bridge: one of these polypeptides is the B chain of thrombin ($M_r$ 32 000) that carries the serine residue of the active site; the other one corresponds to the A chain of thrombin, plus peptides F1 and F2 (Scheme I).

When hirudin, the thrombin and thrombin intermediate inhibitor, was omited, additional cleavages of the prothrombin polypeptide chain were observed (Figure 7). Electrophoretic analyses of aliquots obtained during the activation of prothrombin with *B. atrox* and *E. carinatus* venom activators (Figure 7A) performed in the absence of reduction showed

Scheme I: Cleavage of Human Prothrombin[a]




[a] Cleavage of human prothrombin occurring during activation by activators from the venoms of *B. atrox* and *E. carinatus*. The serine residue of the active site is enclosed in a square when the activation peptide was demonstrated to possess an amidolytic activity.

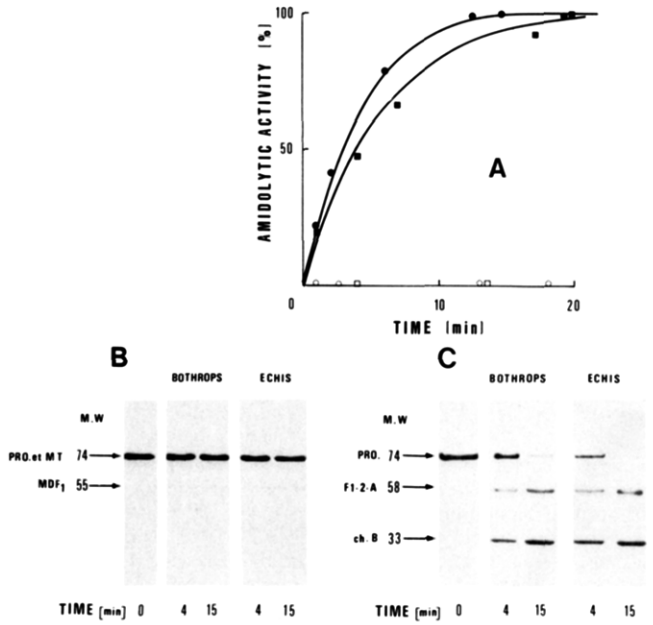



FIGURE 6: Cleavages, in the presence of hirudin, of human prothrombin by activator purified from *B. atrox* venom: Analysis by electrophoresis in polyacrylamide gels. Purified human prothrombin (90 μg/mL, 1.25 μM) was incubated at 25 °C in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl with the activator purified from *B. atrox* venom (6.8 μg/mL, 97 nM) (■, □) or with ecarin from *E. carinatus* venom (4.6 μg/mL) (●, ○) in the presence (empty symbols) or in the absence (full symbols) of hirudin (12.5 μg/mL, 1.25 μM) purified from *Hirudo medicinalis*. Aliquots were removed at the indicated times, their amidolytic activity was immediately determined with S-2238 (A), and their peptide compositions were analyzed by electrophoresis in polyacrylamide gels (12%) in the presence of SDS (4%) in nonreducing (B) and in reducing (C) conditions (2% β-mercaptoethanol). In order to stop the activation reaction, EGTA (13 mM) was added, and the samples were frozen at −18 °C. The apparent molecular weights indicated in the figure were determined in parallel experiments. The abbreviations are defined in Scheme I.

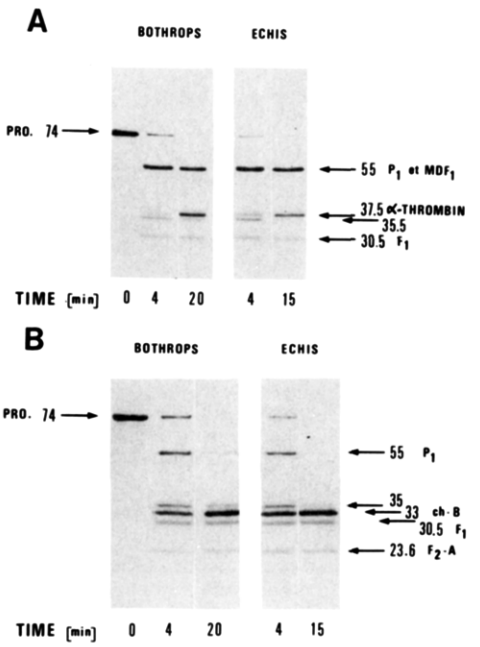



FIGURE 7: Cleavage of human prothrombin by activator from *B. atrox* venom without thrombin inhibitors: Analysis by electrophoresis in polyacrylamide gels. Purified human prothrombin (90 μg/mL, 1.25 μM) was incubated at 25 °C in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl with activator from *B. atrox* venom (6.8 μg/mL, 97 nM) or ecarin from *E. carinatus* venom (4.6 μg/mL). Aliquots were removed at the indicated times and analyzed by electrophoresis in polyacrylamide gels (12%) in nonreducing (A) or in reducing (B) conditions (2% β-mercaptoethanol). The abbreviations are as defined in Scheme I.

similar patterns. These experiments indicate that prothrombin (apparent $M_r$ 74 000) was converted into a polypeptide of apparent $M_r$ 55 000, which corresponds to prethrombin 1 and/or to meizothrombin lacking peptide F1, called meizothrombin(desF1) (Scheme I). The complementary peptide F1 corresponds to a band of $M_r$ of 30 500, which is poorly stained. Analysis of the same activation samples in reducing conditions (Figure 7B) again showed that both venom activators acted in the same manner: the $M_r$ 74 000 band disappeared with the concomitant formation of three bands of $M_r$ 33 000, 30 500, and 23 600, corresponding to meizo-

thrombin(desF1) ($M_r$ 33 000 and 23 600) and peptide F1 ($M_r$ 30 500). The fact that the band corresponding to prethrombin 1 ($M_r$ 55 000) was transient indicated either that the cleavage of prothrombin into prethrombin 1 did not occur significantly or that prethrombin 1 was rapidly converted into meizothrombin(desF1). We also observed the formation and the accumulation of $\alpha$-thrombin, which corresponds to a band of apparent $M_r$ 37 500 in nonreducing conditions (Figure 7A and Scheme I) and an additional and transient component of apparent $M_r$ 35 000, possibly corresponding to prethrombin 2 (Rhee et al., 1982), in both reducing and nonreducing conditions. Therefore in the absence of thrombin inhibitor, two additional cleavages due to a thrombin derivative also occur, and the final product is $\alpha$-thrombin. In the case of derivatives generated by activation by *E. carinatus* venom, these cleavages are known to occur between residues $Arg_{155}$ and $Ser_{156}$ (site b in Scheme I) (Morita et al., 1976; Mann et al., 1981) and between $Arg_{284}$ and $Thr_{285}$ (site d in Scheme I) (Franza et al., 1975).

## Discussion

Our results show that different components of the *B. atrox* venom activate blood clotting at the three final steps of the coagulation cascade. Thus, in addition to the fibrinogen clotting enzyme batroxobin (Holleman & Weiss, 1976; Stocker & Barlow, 1976), which converts fibrinogen into fibrin, we found two activators of factor X, which had been previously described (Hofmann et al., 1983), and one prothrombin activator. In this investigation we focused our attention on the activation of prothrombin by the venom of *B. atrox*. We purified to homogeneity the component responsible for this activity. The *B. atrox* prothrombin activator consists of a single polypeptide chain of about $M_r$ 70 000. This structure differs from that of the physiological prothrombin activator, activated factor X, which consists of two polypeptides of $M_r$ 17 000 and 29 000 linked by a disulfide bridge (Fujikawa et al., 1972; Di Scipio et al., 1977a,b). On the other hand, it may resemble the prothrombin activator from *E. carinatus* venom, ecarin, which is a single polypeptide of about $M_r$ 56 000 (Morita & Iwanaga, 1978; Rhee et al., 1982).

The prothrombin activator from *B. atrox* venom is not sensitive to inhibitors of serine and thiol enzymes but is inhibited by ion chelators and also by numerous divalents ions, indicating that it is not a thiol or serine protease but probably a metalloenzyme. However, we failed to identify the putative metallic ion involved in the catalytic process. The *B. atrox* prothrombin activator is therefore very similar to ecarin, which may also be a metalloenzyme (Morita & Iwanaga, 1978; Rhee et al., 1982), although a determination of the metal content of ecarin by atomic absorption revealed less than 0.01 mol of Co, Cr, Cu, Fe, Mn, Wi, Mo, or Zn per mole (Rhee et al., 1982). The prothrombin activator from *B. atrox* acts in a very similar and probably identical manner as ecarin, which cleaves a specific peptide bond of prothrombin between $Arg_{320}$ and $Ile_{321}$, producing meizothrombin. When the prothrombin activation is carried out with the physiological activator, the number and the order of the cleavages are different and the reaction proceeds in a calcium-dependent manner. In a purified system and in the presence of phospholipids, activated factor V, and calcium ions, the activated factor X nicks the prothrombin molecule first between $Arg_{271}$ and $Thr_{272}$ and second between $Arg_{320}$ and $Ile_{321}$ (Mann et al., 1981).

The venom of *Dispholidus typus* contains a prothrombin activator that has also been reported to act by a mechanism similar to that of the activators from the venoms of *B. atrox* and *E. carinatus* (Guillin et al., 1978). On the other hand, the prothrombin activator found in the venoms of *Notechis scutatus scutatus* and *Oxyuranus scutellatus scutellatus* have been found to act like the physiological activator, activated factor X (Jobin & Esnouf, 1966; Owen & Jackson, 1973; Walker et al., 1980; Tans et al., 1985). The prothrombin activator from *O. scutellatus scutellatus* venom hydrolyzes the chromogenic substrate S-2222 like activated factor X, and it has been postulated that the enzyme is bifunctional (Walker et al., 1980). In the case of the *B. atrox* activator, these two enzymatic activities are carried by two different proteins that have very similar properties in gel filtration and ion-exchange chromatography but were separated by an affinity column of *p*-aminobenzamidine. It therefore appears that at least two classes of prothrombin activators are present in snake venoms: the activators that act on prothrombin in the same manner as the physiological activator and those that proceed through another mechanism involving the formation of meizothrombin. This second type of prothrombin activators may be useful for the determination of the prothrombin level in quantitative deficiency syndromes and may facilitate the analysis of genetic variants of prothrombin.

## Acknowledgments

We are grateful to Dr. B. Boris Vargaftig and Professor Paul Giraudet for their encouragements and for their continuous interest in this work. We thank Dr. François Ravanyi for helpful discussions, Professor Franck Kornalik for his generous gift of purified ecarin, and Bernard Saliou for his help with venom fractionation.

**Registry No.** Batroxobin, 9039-61-6; proteinase, 9001-92-7; phospholipase $A_2$, 9001-84-7; prothrombin, 9001-26-7; ecarin, 55466-26-7; meizothrombin, 69346-19-6; prethrombin 1, 69866-47-3; meizothrombin (des-$F_1$), 105881-83-2; $\alpha$-thrombin, 105881-84-3.

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# Blood Coagulation Induced by the Venom of *Bothrops atrox*. 2. Identification, Purification, and Properties of Two Factor X Activators†

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ABSTRACT: We have characterized and purified the two components of the venom of *Bothrops atrox* that activate the coagulation factor X. Activator 1 and activator 2 were separated by ion-exchange chromatography but otherwise presented similar characteristics. They consist of a heavy polypeptide of $M_r$ 59 000 and either one or two light chains forming a doublet of $M_r$ 14 000–15 000. They are inactive on synthetic substrates and on prothrombin or fibrinogen and thus appear to act specifically on factor X. They are not sensitive to inhibitors of serine proteases or thiol esterases. The activation of factor X is activated by $Ca^{2+}$ ions with a Hill coefficient of 2.4 and is inhibited by $Hg^{2+}$, $Ba^{2+}$, and $Cd^{2+}$. Its pH dependency suggests that the activity depends on the ionization of a group with an apparent p$K$ of 6.9. We studied the cleavage of purified bovine factor X by *B. atrox* activators and compared it to that obtained with the factor X activator from *Vipera russelli* venom. Like the physiological activators, the venom's activators cleave the heavy chain of factor X, producing the activated factor Xa$\alpha$. They produce however two other cleavages: one near the N-terminal end of the heavy chain of factor X, generating factor X$\mu$, and a second one located at one extremity of the heavy chain of factor Xa$\alpha$, generating factor Xa$\nu$.

The venom of *Bothrops atrox* contains several procoagulant components that act at various levels of the blood coagulation cascade. The first procoagulant component that was purified from *B. atrox* venom is a fibrinogen clotting enzyme called batroxobin, a serine protease that converts fibrinogen into fibrin (Stocker & Egberg, 1973; Stocker & Barlow, 1976; Holleman & Weiss, 1976). However, at variance with thrombin, batroxobin cleaves only the A$\alpha$ chains of the fibrinogen molecule, liberating the fibrinopeptide A but not the fibrinopeptide B (Funk et al., 1971). In addition, batroxobin does not share with thrombin the ability to aggregate blood platelets. Subsequently, another serine protease, called thrombocytin, was isolated from the venom of *B. atrox*. It aggregates blood platelets but has limited effects on purified fibrinogen and on factors V and VIII (Niewiarowski et al., 1977, 1979; Kirby et al., 1979). In the preceding paper (Hofmann & Bon, 1987), we reported the purification of a protease from *B. atrox* venom that activates prothrombin by specifically cleaving it into meizothrombin. This prothrombin activator strongly resembles activators previously described in the venoms of *Echis carinatus* and *Dispholidus typus* (Franza et al., 1975; Kornalik & Blombäck, 1975; Morita et al., 1976; Morita & Iwanaga, 1978, 1981; Guillin et al., 1978; Briet et al., 1982; Rhee et al., 1982). The existence in the venom of *B. atrox* of a component that activates factor X has also been reported (Nahas et al., 1964, 1979; Hofmann et al., 1983).

In the preceding paper (Hofmann & Bon, 1987), we described the fractionation of *B. atrox* venom by DEAE-cellulose chromatography and reported that two fractions possess the capacity to activate factor X. In this investigation, we describe a further purification of the two venom components and an-

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