Purification and Biochemical Characterization of Atroxase, a Nonhemorrhagic Fibrinolytic Protease from Western Diamondback Rattlesnake Venom†

Todd W. Willis and Anthony T. Tu*

Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80523

Received November 23, 1987; Revised Manuscript Received February 22, 1988

ABSTRACT: *Crotalus atrox* venom contains a variety of proteases which render fibrinogen incoagulable and solubilize fibrin. One of these proteases was purified by using ion-exchange and gel permeation liquid chromatography. The protease, called atroxase, consists of a single nonglycosylated polypeptide chain with a molecular weight of 23 500 and an isoelectric point of pH 9.6. Amino acid analysis indicates atroxase to contain 206 residues with no sulfhydryl groups. Metal analysis found zinc and potassium at 1 mol/mol of protein, and calcium at 0.3 mol/mol of protein. Proteolytic activity is inhibited by ethylenediaminetetraacetate and α_2-macroglobulin. Maximal proteolytic activity occurs at pH 9.0 and 55 °C. Proteolytic specificity, using oxidized insulin B chain, is similar to that of several hemorrhagic toxins found within the same venom, yet atroxase shows no hemorrhagic activity and exhibits low lethality when tested on Swiss Webster mice. Atroxase, an Aα, Bβ fibrinogenase, cleaves the Aα chain of fibrinogen first followed by the Bβ chain and shows no effect on the γ chain. The nonspecific action of the enzyme results in the extensive hydrolysis of fibrinogen which releases a variety of fibrinopeptides. Fibrin solubilization appears to occur primarily from the hydrolysis of α-polymer and unpolymerized α and β chains. Although crude venom induces platelet aggregation, atroxase demonstrated no ability to induce or inhibit aggregation.

Snake venoms, particularly those belonging to Crotalidae and Viperidae families, are known to strongly affect the blood coagulation system. Thrombin-like enzymes have been isolated and characterized from the venoms of *Agkistrodon acutus* (Ouyang et al., 1971), *Agkistrodon contortrix* (Denson et al., 1972), *Agkistrodon rhodostoma* (Esnouf & Tunnah, 1967), *Bothrops atrox* (Devi et al., 1972), *Crotalus adamanteus* (Markland & Damus, 1971), *Crotalus horridus* (Denson, 1969), *Crotalus horridus horridus* (Shu et al., 1983), *Trimeresurus gramineus* (Ouyang & Yang, 1974), and *Trimeresurus mucrosquamatus* (Yang, & Teng, 1976). In vitro, these enzymes act as procoagulants converting fibrinogen to a fibrin derivative; however, in vivo, fibrin produced by the venom proteases may be quickly hydrolyzed by the natural fibrinolytic system, resulting in a state of defibrinogenation (Markland & Pirkle, 1977). Several venoms also contain proteases capable of directly rendering fibrinogen incoagulable or solubilizing fibrin. Anticoagulant proteases with fibrinogenolytic or fibrinolytic activity have been reported from the venoms of *Crotalus atrox* (Bajwa et al., 1980; Pandya & Budzynski, 1984; Sapru et al., 1983; Komori et al., 1985; Nikai et al., 1984, 1985), *A. contortix mokasen* (Moran & Geren, 1981), *A. contortrix contortrix* (Bajwa et al., 1982), *C. horridus horridus* (Civello et al., 1983b), and *Cerastes cerastes* (Daoud et al., 1986).

A variety of fibrinogenolytic and fibrinolytic enzymes found within *C. atrox* venom exemplify the degree of proteases within that venom. Molecular sieve chromatography of crude venom revealed that fibrinolytic activity was equally distributed between two fractions with molecular weights of 21 500 and 60 000 (Bajwa et al., 1980). Four purified proteases, called proteases I, II, III, and IV with molecular weights of 20 000, 31 000, 24 000, and 46 000, respectively, have been isolated which render purified fibrinogen incoagulable, while proteases II and III also degrade fibrinogen in plasma (Pandya & Budzynski, 1984). Hemorrhagic toxins HT-*b* and HT-*g*, with molecular weights of 24 000 and 60 000, are known to extensively hydrolyze the Aα and Bβ chains of fibrinogen (Komori et al., 1985; Nikai et al., 1985), while a third toxin, HT-*f*, hydrolyzes the γ chain (Nikai et al., 1984). It has not been determined whether the other known hemorrhagic toxins within *C. atrox* venom have similar activities.

In this work, a fibrin(ogen)olytic protease within *C. atrox* venom was isolated and characterized. Fractionation of the venom was performed in such a manner as to separate proteases with hemorrhagic activity from fibrin(ogen)olytic proteases containing little or no hemorrhagic activity. The protease isolated, called atroxase, was chemically characterized and its proteolytic activity studied. Proteolytic specificity, using oxidized B chain of insulin, was likewise investigated and compared to several rattlesnake venom proteases.

Materials and Methods

Materials. *Crotalus atrox* venom was purchased in lyophilized form from Miami Serpentarium Laboratories, lot CX12KZ. Diethylaminoethyl (DEAE)[1]- and CM-cellulose (DE52 and CM52) were purchased from Whatman Biochemicals, and Sephadex G-75 resin was purchased from Pharmacia. Human fibrinogen (grade L) and S-2238 were

† This work was supported by National Institutes of Health Merit Award 5 R37 GM15591.

* Address correspondence to this author.

[1] Abbreviations: DEAE, diethylaminoethyl; CM, carboxymethyl; HT, hemorrhagic toxin; Tris, tris(hydroxymethyl)aminomethane; DMC, dimethylcasein; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetate; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; TNBS, trinitrobenzenesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetate; S-2238, H-D-Phe-Pip-Arg-pNA; PRP, platelet-rich plasma; PPP, platelet-poor plasma; MHD, minimum hemorrhagic dose; LD_{50}, lethal dose to 50% of the population; CPI, capillary permeability increasing; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

0006-2960/88/0427-4769$01.50/0

obtained from Kabi Diagnostica, and bovine thrombin was from Parke-Davis. Amino acid analysis reagents and standards of the highest purity grade and Coomassie blue G-250 protein assay reagent were purchased from Pierce Chemical Co. HPLC-grade solvents and water were purchased from Mallinckrodt. HPLC was performed by using a Beckman trinary pump system, Model 334-T. Acrylamide and *N,N'*-methylenebis(acrylamide) were from Serva. Insulin oxidized B chain was obtained from Calbiochem, and α_2-macroglobulin was from Boehringer Mannheim Biochemicals. All other reagents and substrates were from Sigma Chemical Co.

Venom Fractionation. Isolation procedures were performed at 4 °C. Test tube fractions were collected and their protein concentrations determined by their absorbance at 280 nm. After each isolation step, fractions were tested for fibrinogenolytic, fibrinolytic, or hemorrhagic activities as further described.

Crude *C. atrox* venom (2 g) was dissolved and dialyzed against starting buffer; 10 mM borate buffer, pH 9.0, containing 0.1 M NaCl and 2 mM $CaCl_2$. The venom solution was applied to a DEAE-cellulose column (2.5 × 32 cm), previously equilibrated with starting buffer, and eluted at a flow rate of 25 mL/h. A step gradient using 0.4 M NaCl in the same buffer was used to elute bound proteins.

Determination of the thrombin clotting time of a 1% fibrinogen solution in 5 mM Tris buffer, pH 7.4, was introduced as a screening test for fibrinogenolytic activity. Thrombin clotting times were tested by preincubating 0.1 mL of fibrinogen with 0.1 mL of each test tube fraction for 15 min at 37 °C. Clotting times were then recorded after the addition of 0.1 mL of bovine thrombin, 10 units/mL. Controls were performed by replacing the protein fraction with buffer, and the experiment was run under identical conditions.

The first fraction eluted by anion-exchange chromatography was collected, lyophilized, and dissolved in 5 mL of 5 mM Tris buffer, pH 8.5, containing 0.1 M NaCl and 2 mM $CaCl_2$. A 5 × 84.5 cm column was packed with Sephadex G-75 superfine resin and equilibrated with the Tris buffer. The fraction was applied to the column and eluted at a flow rate of 50 mL/h.

The third fraction eluted by gel permeation chromatography was collected, dialyzed against distilled water, and lyophilized. A 1.5 × 47 cm column was packed with CM-cellulose and equilibrated with starting buffer; 10 mM Tris, pH 8.5, containing 2 mM $CaCl_2$. The lyophilized fraction was then dissolved in 2 mL of starting buffer and applied to the column. Bound proteins were eluted by using a linear salt gradient from 0 to 0.2 M NaCl with a total volume of 500 mL.

Homogeneity. Two independent methods were employed to establish homogeneity. These were SDS–PAGE using 11% polyacrylamide gels (Laemmli, 1970) and HPLC using an analytical reverse-phase column (μBondpack C_{18}). Reverse-phase chromatography was performed by using a linear gradient from 0% solvent A, 0.1% TFA in water, to 60% solvent B, 0.1% TFA in CH_3CN.

Protein Assay. Protein was assayed by using Coomassie blue G-250 based reagent according to a modified Bradford assay technique (Read & Northcote, 1981). Bovine serum albumin was used as a standard.

Molecular Weight and Isoelectric Point Determination. SDS–PAGE was performed according to Laemmli (1970) using 11% polyacrylamide gels. Samples were treated with 0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol at 100 °C for 5 min to denature proteins. Molecular weight standards were bovine serum albumin (66 000), egg albumin (45 000), glyceraldehyde-3-phosphate dehydrogenase (35 000), carbonic anhydrase (29 000), trypsinogen (24 000), and trypsin inhibitor (20 100).

Isoelectric focusing was performed at 4 °C using 7.5% polyacrylamide tube gels containing 2% ampholyte with a narrow range pH gradient of pH 8–10.5 (Righetti & Drysdale, 1971).

Amino Acid Content. Amino acid analysis was carried out on a JEOL Model JLC-6AH amino acid analyzer by the method of Spackman et al. (1958). Complete amino acid analysis was performed from a single hydrolysate according to Simpson et al. (1976). Samples were prepared and hydrolyzed for 24, 48, and 72 h with 4 N methanesulfonic acid at 110 °C. The average number of methionine residues, after being corrected for destruction, was selected as 1.00, and the remaining residues were normalized to this value. Tryptophan content was confirmed by the method of Edelhoch (1967) to check for possible destruction which may have occurred during hydrolysis. Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)] was likewise used to confirm the number of free sulfhydryl groups within the protein (Ellman, 1959).

Carbohydrate Content. Neutral sugar content was measured by the method of Morris (1948). A standard curve of various concentrations of glucose was used to determine the neutral sugar content of a 1 mg/mL solution of atroxase.

Metal Content. Metal analysis was determined by inductively coupled plasma atomic emission spectroscopy using a Model 975 plasma Atomcomp spectrometer made by Jarrell-Ash.

Atroxase, 0.5 mg, was dissolved in distilled–deionized water and dialyzed against 4 L of 50 mM HEPES buffer, pH 7.2, containing 50 mM NaCl, for 48 h at 4 °C. Dialysis membranes were pretreated to remove absorbed compounds (Richmond et al., 1985). Metal analysis was performed on the dialysis buffer before and after dialysis. Metal analysis of the postdialysis buffer was subtracted from the analysis containing atroxase.

Dimethylcasein Hydrolysis. The proteolytic activity of atroxase, crude venom, and samples from peak fractions collected during the isolation was determined by using DMC as substrate, a method modified from Lin et al. (1969).

Two milliliters of a 0.1% DMC solution in 0.01 M borate buffer, pH 9.0, was incubated at 37 °C with 0.3 mL of enzyme solution in the same buffer. At various time intervals (0, 15, 30, and 60 min), 0.2-mL aliquots were withdrawn, and the reaction was stopped by immersing the samples for 3 min in a boiling water bath. A solution containing 0.3 mL of H_2O, 0.5 mL of 0.2 M borate buffer, pH 10.5, and 0.02 mL of 1.1 M TNBS was added to each sample followed by incubation at room temperature for 10 min. Five milliliters of 0.2 M NaH_2PO_4, pH 6.87, containing 1.5 mM Na_2SO_3, was then added to each sample. The absorbancies were read at 420 nm relative to the zero-minute incubation mixture. The absorbance of a control mixture consisting of all the components except active enzyme was subtracted from each test sample.

One unit of proteolytic activity was defined as the amount of enzyme which causes a net increase of 1 unit of optical density at 420 nm in 1 h. Specific activity was calculated as units per milligram of venom proteins.

Optimum Proteolytic Activity. The temperature for optimum proteolytic activity was determined by preincubating atroxase and a 1% casein solution in 0.1 M Tris buffer, pH 7.2, for 5 min at 10, 20, 30, 40, 50, 55, 60, and 70 °C. After preincubation, the substrate and 5 μg of enzyme were mixed, and the protease activity was determined according to the method of Kunitz (1947).

The dependence of activity on pH was determined by using a 1% casein solution in 0.15 M phosphate, 0.2 M borate, and 0.1 M citrate buffer, with pHs ranging from pH 5 to pH 11. Proteolytic activity was determined as before.

The effect of added zinc ion on catalytic activity was determined by briefly preincubating 5 μg of enzyme with 1 mL of 0.1 M Tris buffer, pH 7.2, containing 1 or 2 mM $ZnCl_2$ at 37 °C. The enzyme mixture was then added to 1 mL of a 1% casein solution and the proteolytic activity determined as before.

Inhibitors. Inhibition of proteolytic activity was determined by incubating atroxase, 5 μg in 0.25 mL of 0.1 M Tris buffer, pH 7.2, for 30 min at room temperature with an equal volume of the following inhibitors: 10 mM EDTA, 10 mM PMSF, 10 mM 1,10-phenanthroline, 200 μg/mL aprotinin, 200 μg/mL soybean trypsin inhibitor, and 38, 75, 150, or 300 μg of α_2-macroglobulin. Proteolytic activity was determined by using 0.1% DMC or 1% casein as previously described.

Proteolytic Specificity. Equal volumes of oxidized B chain, 1 mg/mL, were incubated with atroxase, 20 μg/mL, at 37 °C in 5 mM imidazole–saline buffer (1 volume of buffer/9 volumes of saline), pH 7.4. At various time intervals, 0.2-mL aliquots were withdrawn from the digestion mixture, and the reaction was stopped by heating at 100 °C for 3 min. The samples were chromatographed by RP-HPLC (Beckman, C_8 ultrapore). A linear gradient from 5% to 75% CH_3CN in water, containing 0.1% TFA, was applied for a duration of 27 min at a flow rate of 1 mL/min. Peptide fragments were collected, hydrolyzed in 6 M HCl for 24 h at 110 °C, and analyzed for amino acids.

Degradation of Fibrinogen, Fibrin, and Thrombin. Fibrinogenolytic activity was measured by incubating 1 mL of a 2% human fibrinogen solution with 1 mL of atroxase or crude venom at 37 °C in 5 mM imidazole–saline buffer (1:9), pH 7.4. At various time intervals, 0.2 mL of the incubation mixture was withdrawn and assayed for clottable fibrinogen (Ouyang & Huang, 1976). Specific fibrinogenase activity was calculated by dividing the rate of fibrinogen destruction (milligrams per minute) by the amount of the fibrinogenase within the reaction.

Specific cleavage of fibrinogen was shown on 10% polyacrylamide gels. At various time intervals, 0.1 mL of the above incubation mixture was withdrawn and added to 0.1 mL of denaturing solution (10 M urea, 4% SDS, and 4% 2-mercaptoethanol). The samples were reduced and denatured overnight at 37 °C before being electrophoresed by SDS-PAGE.

Low molecular weight fibrinogen degradation products produced by atroxase, plasmin, or thrombin hydrolysis were compared by using RP-HPLC. Fibrinogen, 4 mL at 5 mg/mL in 5 mM Tris buffer, pH 7.2, was incubated at 37 °C with 1 mL of either atroxase (50 μg/mL), plasmin (0.04 unit/mL), or thrombin (20 units/mL). At various time intervals, aliquots of the incubates containing atroxase or plasmin were placed in a boiling water bath for 3 min and then filtered by using an Amicon ultrafiltration cell (CF 25), molecular weight cutoff 25 000. Samples containing thrombin were centrifuged at 250*g* for 15 min, and the supernatant was filtered. Filtered samples were chromatographed by RP-HPLC (Beckman, C_8 ultrapore).

Fibrinogen degradation in plasma was measured at 37 °C by incubating either 10, 20, 30, or 50 μg of atroxase with 1 mL of a 1:5 dilution of fresh citrated human plasma in imidazole–saline buffer (1:9), pH 7.4. At various times, 0.2-mL aliquots were rapidly added to 0.1 mL of thrombin, 100 units/mL, and the clotting times were measured at 37 °C. The fibrinogen concentration of each aliquot was determined from a calibration curve prepared from a fibrinogen reference (Clauss, 1957).

Fibrin hydrolysis was demonstrated by SDS–PAGE using 10% polyacrylamide gels. Thrombin, 0.1 mL containing 10 units/mL, was added to 0.1 mL of a 1% fibrinogen solution in 5 mL imidazole–saline buffer (1:9), pH 7.4. The fibrin clot was allowed to form for 1 h at room temperature. After 1 h, 0.1 mL of atroxase or plasmin was added to the clot and incubated at 37 °C for various time intervals. The reaction was stopped by the addition of 0.3 mL of denaturing solution, incubated overnight, and run on SDS–polyacrylamide gels.

Fibrinolytic activity was measured by using the fibrin plate technique of Astrup and Müllertz (1952). Protease samples (25 μL) were carefully placed on the fibrin surface using a Hamilton syringe and incubated at 37 °C for 24 h.

Antithrombin activity, a measure of the destruction rate of thrombin, was measured by using the thrombin-specific chromogenic substrate S-2238. Atroxase, 1 mL of either 25, 50, or 100 μg/mL in 50 mM Tris buffer, pH 8.4, containing 175 mM NaCl, was incubated with 3.5 nkat (1 kat of thrombin will hydrolyze 1 mol of S-2238/s) of thrombin at 37 °C. After a 15-min incubation, thrombin activity was determined by using S-2238 (Abildgaard et al., 1977).

Platelet Aggregation. Blood from healthy volunteers was collected in 3.8% trisodium citrate (9 volumes of blood/1 volume of citrate) and immediately centrifuged at room temperature for 10 min at 250*g* to obtain PRP. Centrifugation of the original blood sample at 1500*g* for 10 min gave PPP. Platelet aggregation was recorded with a Payton aggregometer (Model 1010) at 37 °C using siliconized glass tubes. Changes in light transmission of the platelet suspensions were recorded under continuous stirring at 900 rpm.

The reaction mixture consisted of 450 μL of PRP which was preincubated for 1 min at 37 °C prior to the addition of 50 μL of either 1 mg/mL crude venom, 1 mg/mL atroxase, or 0.2 mM ADP. The maximum aggregation response obtained from the addition of ADP, an aggregation agonist, was given a value of 100% aggregation.

Hemorrhagic, Capillary Permeability Increasing, and Lethal Activities. Hemorrhagic activity was tested by subcutaneously injecting Swiss Webster mice (20–22 g) with the test samples dissolved in 0.1 mL of 0.9% saline (Kondo et al., 1960). The MHD was defined as the least amount of protein that caused a hemorrhagic reaction 5 mm in diameter 6 h after injection (Bjarnason & Tu, 1978).

CPI activity was tested by intravenously injecting 0.1 mL of saline containing 1% Evans blue dye into Swiss Webster mice (Miles & Wilhelm, 1955). Test samples in 0.1 mL of saline solution were then injected intradermally in the dorsal area. After 1 h, the skins were removed and inspected for dye released into the surrounding tissue resulting from CPI activity. CPI activity was expressed as the cross-sectional area of the dye spot.

Lethal toxicity was tested by intravenously injecting Swiss Webster mice (20–22 g) with test samples containing doses up to 25 μg protein/g body weight in 0.1 mL of saline. The mice were observed for 24 h after injection.

Results

Purification and Homogeneity. Isolation of atroxase was achieved by using a three-step fractionation (Figure 1a–c). Chromatography of crude venom using a column of DEAE-cellulose yielded four major fractions (Figure 1a). Protein samples from fractions A1, B1, and C1 rendered fibrinogen

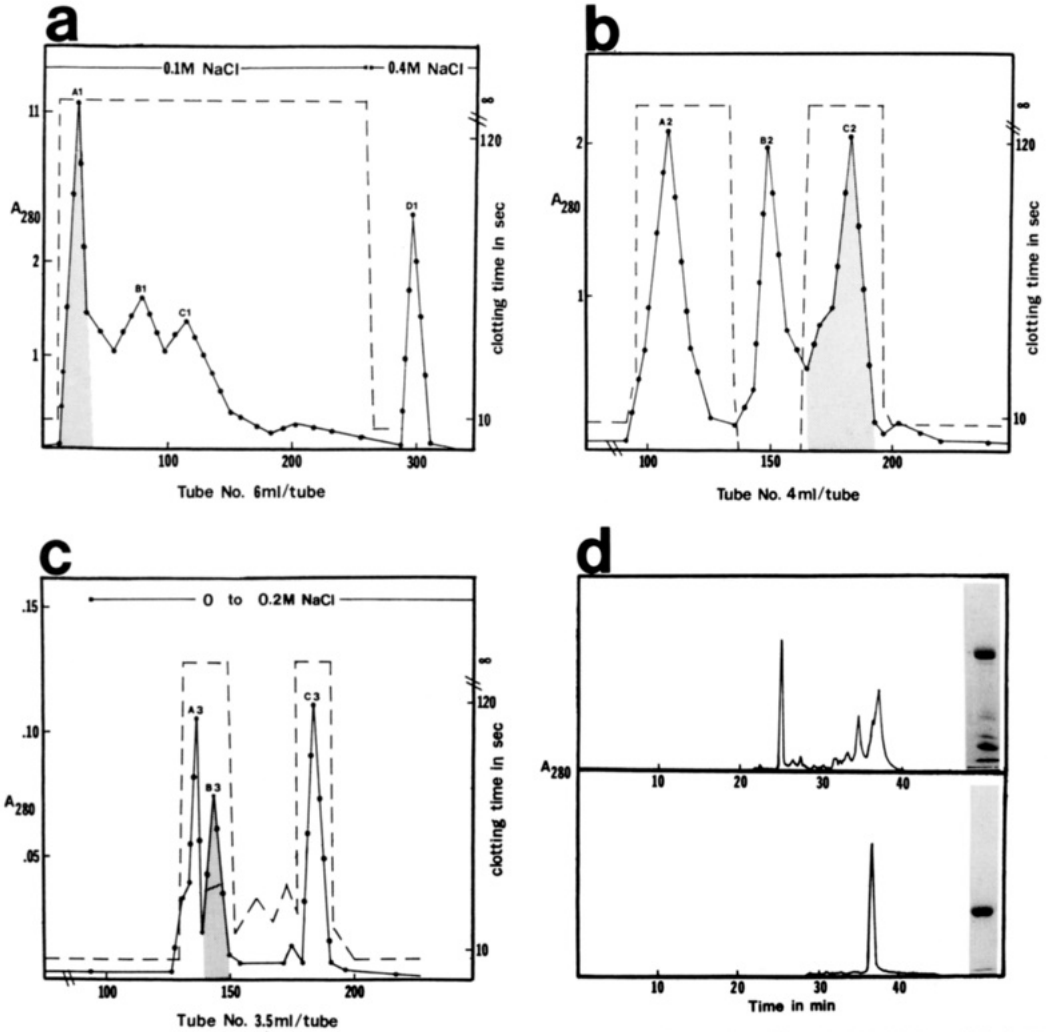


FIGURE 1: Fractionation of *C. atrox* venom. Purification of atroxase was accomplished by using (a) DEAE-cellulose, (b) G-75 superfine resin, and (c) CM-cellulose. The elution profiles were monitored by A_{280} (—). Anticoagulant activity, based on fibrinogen destruction, was shown as clotting time (---). Shaded fractions represent those which were collected and further chromatographed in the preceding step. Fraction B3 contained purified atroxase. (d) RP-HPLC and SDS–PAGE (insert) of crude venom (upper trace) and atroxase (lower trace).

incoagulable and lysed fibrin when tested on fibrin plates. Cross-sectional diameters of the lysed zones on the fibrin plates using 2 mg/mL protein samples from each fraction were approximately 1000 mm^2 for fraction A1, 450 mm^2 for fraction B1, and 125 mm^2 for C1.

Further chromatography of fraction A1 using a column of Sephadex G-75 resin resulted in three protein fractions, A2, B2, and C2 (Figure 1b). Fractions A2 and C2 rendered purified fibrinogen incoagulable. Incubation of fibrinogen with protein samples from fraction B2, however, resulted in clot formation before the addition of thrombin. Fibrinolytic activity, when tested on fibrin plates, was approximately 3 times greater in fraction C2 when compared to fraction A2.

Further chromatography of fraction C2 using a column of CM-cellulose gave three protein fractions, A3, B3, and C3 (Figure 1c). All fractions hydrolyzed fibrinogen to an incoagulable form. When tested for hemorrhagic activity, fraction A3 contained an MHD of approximately 25 μg, and fraction C3 contained an MHD of 3 μg, while B3 showed no hemorrhagic activity at doses up to 50 μg. Fibrinolytic activity using 50 μg/mL B3 resulted in a 196 mm^2 lysed zone, while at the same concentration fibrinolysis by crude venom could not be detected. From 2 g of crude venom, 15 mg of protein within fraction B3 was obtained.

Atroxase, contained within fraction B3, eluted as a single symmetrical peak from RP-HPLC and was found to be electrophoretically homogeneous by SDS–PAGE (Figure 1d). SDS–PAGE analysis, under reduced conditions, gave a calculated molecular weight of 23 500. Isoelectric focusing studies found atroxase to be a basic protein with a p*I* of 9.6.

Chemical Composition. The amino acid composition of atroxase is shown in Table I. The composition was calculated on the basis of the observed molecular weight from SDS–PAGE. From atroxase's basic p*I*, it is expected to contain a large ratio of basic to acidic amino acids. The composition, however, shows that only 19 out of a possible 69 charged residues are lysine and arginine. This suggests that many of the detected Asx and Glx residues within the protein exist as asparagine and glutamine. Before hydrolysis, the samples were carboxymethylated in the absence of reducing agents to detect any free sulfhydryl groups as (carboxymethyl)cysteine. Amino acid analysis showed no (carboxymethyl)cysteine residues, indicating no free sulfhydryls. After hydrolysis, dithiothreitol was used to reduce cystine. Half-cystines were then reacted with sodium tetrathionate, forming sulfocysteine. Twelve sulfocysteine groups per mole of protein were detected, suggesting six disulfide bridges present in the intact protein. Hydrolyzing the protein with a nonoxidizing sulfonic acid showed tryptophan to be present at three residues per protein molecule. This correlated well with the spectrophotometric determination of tryptophan using the method of Edelhoch (1967).

Table I: Amino Acid Composition of Atroxase

amino acid	hydrolysis time (h) 24	48	72	min residues based on Met	residues/protein molecule	nearest integer
Asx	10.67	9.67	10.66	6.72	27.01	27
Thr	3.79	3.25	3.55	2.29	9.21	9
Ser[b]	8.32	7.22	7.20	5.71	22.95	23
Glx	8.96	8.68	8.86	5.74	23.08	23
Pro	2.42	2.56	2.07	1.53	6.15	6
Gly	3.40	3.32	3.41	2.20	8.84	9
Ala	2.66	2.51	2.73	1.71	6.87	7
Val[b]	4.05	4.19	4.33	2.90	11.66	12
Met[b]	1.50	1.44	1.42	1.00	4.02	4
Ile	4.32	3.78	4.46	2.72	10.93	11
Leu	6.80	6.01	6.68	4.23	17.01	17
Tyr	2.28	2.07	2.38	1.46	5.87	6
Phe	3.30	2.99	3.20	2.05	8.24	8
His	2.92	2.94	2.96	1.91	7.68	8
Lys	3.05	3.06	3.04	1.98	7.96	8
Trp[a,b]	1.05	0.83	0.75	0.79	3.18	3
Arg	4.36	4.36	4.36	2.83	11.38	11
Cys-SO_3	4.30	5.20	3.67	2.85	11.47	12
NH_4	8.08	9.33	9.35	6.08		
						total 206

[a] Tryptophan content was confirmed by the spectroscopic method of Edelhoch (1967). [b] The hydrolysis values for Trp, Ser, and Met were extrapolated to time zero. The hydrolysis value for Val was extrapolated to infinite time.

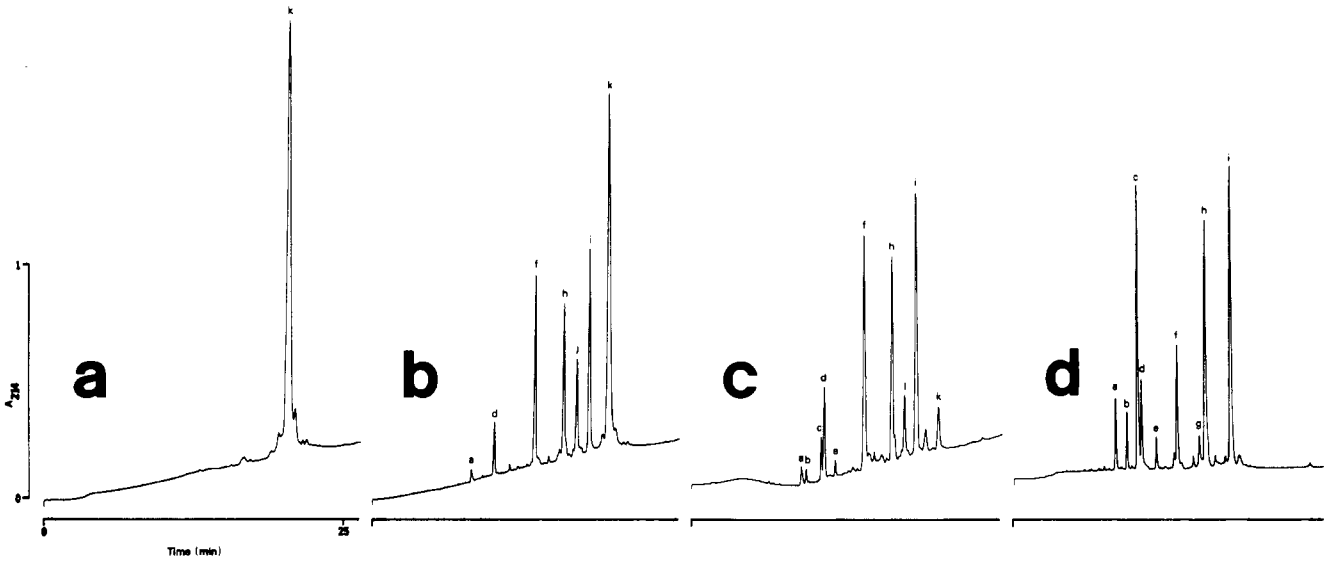


FIGURE 2: RP-HPLC profiles of an insulin B chain digestion by atroxase. Chromatography of an incubate containing 1 mg of insulin with 20 μg of atroxase was performed at times (a) 0, (b) 30, (c) 60, and (d) 120 min. After complete hydrolysis of insulin B chain (as seen from the disappearance of fraction k), fragments a–i were collected and their amino acid compositions determined.

A total of 19 metals were tested for binding to atroxase. Of these, three were found in significant quantities. Zinc and potassium both formed a 1:1 complex with atroxase. Calcium was determined to be present at about 1 mol of calcium/3 mol of atroxase.

Chemical analysis for neutral sugar found atroxase to contain no carbohydrate moiety.

Proteolytic Activity. The specific activity of atroxase, using DMC as substrate, was 127.7 units/mg compared to 39.1 units/mg for crude venom. Maximal proteolytic activity occurred at a temperature of 55 °C and a pH of 9.0. Activity dropped sharply at more alkaline conditions or higher temperatures, with a complete loss of activity occurring at pH 11 or at 70 °C. Catalytic activity was not enhanced in the presence of added Zn^{2+}; however, 2 mM Zn^{2+} protected the enzyme from inhibition by 0.5 mM EDTA, when added at the same time as the EDTA.

The effect of metal chelation on proteolytic activity suggested atroxase to be a metalloprotease. Hydrolysis of both fibrin and DMC was completely inhibited by a 10 mM sample of either EDTA or 1,10-phenanthroline. Aprotinin, PMSF, and soybean trypsin inhibitor showed no inhibition of proteolytic activity.

While most plasma protease inhibitors are enzymatically inactivated by proteases within *C. atrox* venom, α_2-macroglobulin is the only plasma protease inhibitor known to decrease proteolytic activity among several crotalid crude venoms (Kress, 1988). Preincubations of 38, 75, 150, and 300 μg of α_2-macroglobulin with atroxase for 30 min resulted in activity decreases of 3%, 11%, 30%, and 56%, respectively. Preincubations of atroxase with α_2-macroglobulin using shorter or longer time periods showed no significant change in the degree of inhibition.

Substrate Specificity. The progressive cleavage of oxidized B chain of insulin was monitored by HPLC (Figure 2). Cleavage sites were determined from the amino acid composition of the degradation products observed after 2 h of digestion. Nine fragments, a–i, resulted from five cleavage points

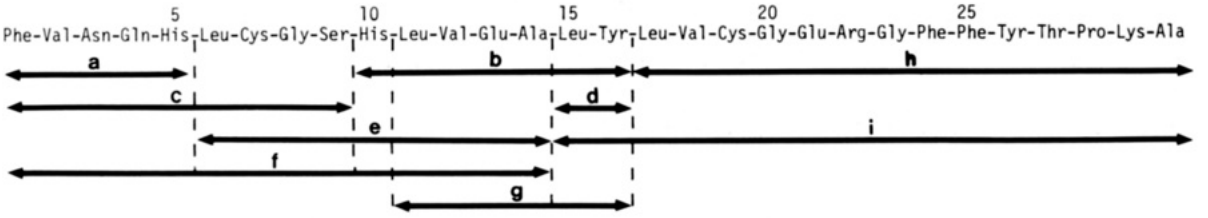


FIGURE 3: Digestion fragments of oxidized insulin B chain. Fragments a–i represent those fragments in the order they appeared from RP-HPLC after a 120-min digest. Fragments were identified by comparing their amino acid compositions to the known sequence. Dashed lines indicate cleavage sites.

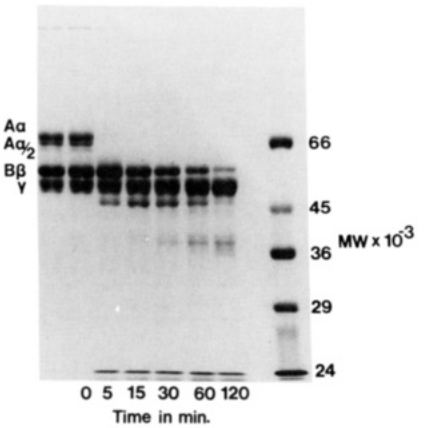


FIGURE 4: SDS–PAGE analysis of reduced human fibrinogen after digestion by atroxase. Fibrinogen consists of three polypeptide chains Aα or an incomplete Aα chain, Aα/2 (M_r 66 000 and 64 000), Bβ (M_r 54 000), and γ (M_r 48 000). Lanes 2–7 depict 2% fibrinogen samples after incubation with 10 μg of atroxase for the specified times. Lane 1 was a control consisting of fibrinogen incubated for 120 min without protease. Lane 8 contained molecular weight markers.

(Figure 3). Four of the hydrolyzed bonds occurred on the N-terminal side of Leu-6, -11, -15, and -17. The fifth cleavage site occurred at Ser_9-His_{10}.

Effect on Blood Coagulation Components. Both crude venom and atroxase contained fibrinogenolytic activity. The amount of clottable fibrinogen remaining after incubation with atroxase or crude venom was determined at 0, 5, 10, 15, 60, and 120 min. Using 20 μg of protein/mL in the test solutions, it was found that crude venom had a specific fibrinogenase activity of 7.00 mg of fibrinogen min^{-1} (mg of protein)$^{-1}$ with atroxase having a specific activity of 41.67 mg of fibrinogen min^{-1} (mg of protein)$^{-1}$.

Proteolytic activity by 30 μg/mL atroxase resulted in a 90% decrease in plasma fibrinogen levels after a 30-min incubation with a 1:5 plasma dilution. Protease concentrations greater than 30 μg/mL rendered the plasma incoagulable within 30 min, while no significant decreases in fibrinogen levels occurred at protease concentrations of 10 μg/mL or less even after prolonged incubation. This was expected to be caused by the inactivation of atroxase by α_2-macroglobulin, whereas protease concentrations above 10 μg/mL appeared to exhaust the plasma protease inhibitor, thereby allowing degradation of plasma fibrinogen.

SDS–PAGE analysis showed atroxase to be an Aα, Bβ fibrinogenase with the Aα chains being degraded within 5 min followed by a slower degradation of the Bβ chains (Figure 4). The fibrinogenase appeared to have little or no effect on the γ chain after 2 h of hydrolysis. High molecular weight degradation products first appeared with molecular weights of about 47 000 and 24 000. After 15 min, several new bands appeared with molecular weights around 37 000, while the Bβ chain and the 47 000 molecular weight degradation product became further hydrolyzed. These high molecular weight

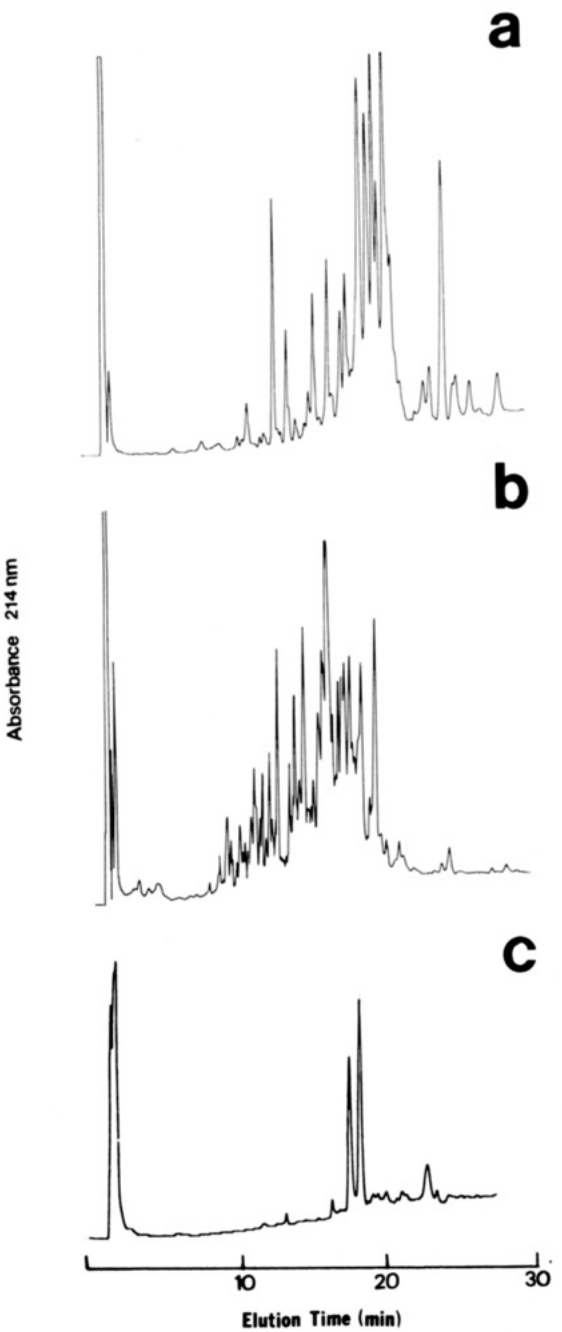


FIGURE 5: Analysis of fibrinopeptides using RP-HPLC. Each chromatogram represents fibrinogen cleavage products after 60 min of hydrolysis by (a) 50 μg of atroxase, (b) 0.04 unit of plasmin, or (c) 20 units of thrombin. Chromatography was performed by using a linear gradient from 5% to 75% acetonitrile in water, containing 0.05% TFA, for a duration of 30 min and a flow rate of 1 mL/min.

bands indicate different cleavage sites from those of thrombin. Cleavage of fibrinogen by thrombin results in α and β chains with molecular weights of 62 000 and 54 000, respectively.

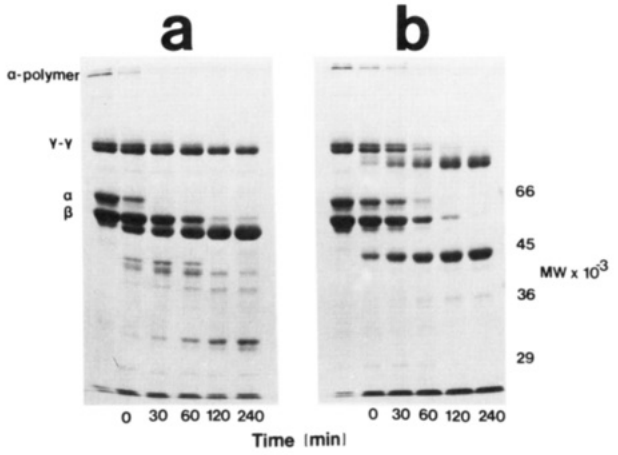


FIGURE 6: SDS–PAGE analysis of reduced fibrin. Fibrin was hydrolyzed by (a) 50 μg/mL atroxase or (b) 0.1 unit/mL plasmin for the specified time periods. The first lane in each gel represents reduced fibrin without protease. Fibrin consisted of α-polymers, unpolymerized α and β chains, and γ dimers.

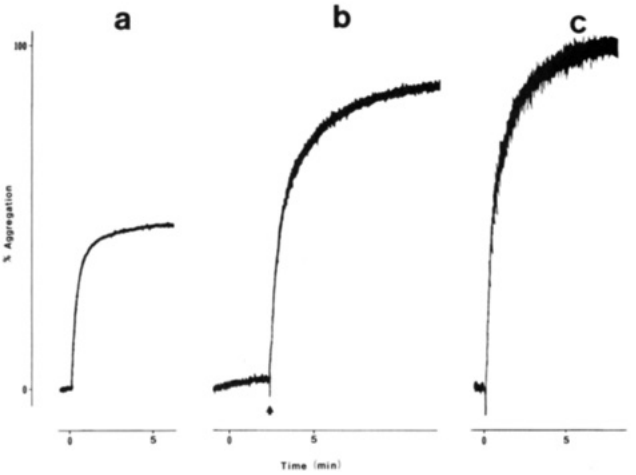


FIGURE 7: Effect of crude venom and atroxase on platelet aggregation. (a) Platelet aggregation induced by 50 μL of crude venom (1 mg/mL). (b) Platelet aggregation in the presence of 50 μL of atroxase (1 mg/mL) with 50 μL of ADP (0.2 mM) being added after 2.5 min (arrow). (c) Induced platelet aggregation using 50 μL of 0.2 mM ADP, without protease, was considered 100% aggregation.

Separation of low molecular weight degradation products from an incubate of fibrinogen with atroxase resulted in several fibrinopeptide fractions, indicating extensive hydrolysis of the molecule (Figure 5a). The nonspecific action of atroxase on fibrinogen was comparable to a nonspecific serine protease, plasmin (Figure 5b), while thrombin, a specific serine protease, released only two major peptide products corresponding to fibrinopeptides A and B (Figure 5c).

Hydrolysis of fibrin by atroxase resulted in the degradation of α-polymer, α and β chains, while the γ–γ chains appeared unaffected within the time tested (Figure 6a). Fibrin hydrolysis with plasmin readily degraded α-polymer, α, β, and γ–γ chains (Figure 6b).

Atroxase was unable to hydrolyze S-2238, a synthetic substrate specific for thrombin. Further incubations of 25, 50, and 100 μg/mL atroxase with 3.5 nkat of thrombin had no effect on the ability of thrombin to hydrolyze its substrate, indicating that atroxase contained no antithrombin activity.

Platelet aggregation in the presence of crude venom or atroxase was compared to the aggregation response induced by ADP. Addition of crude venom to PRP induced aggregation with a response approximately half of that obtained by ADP (Figure 7a). Atroxase did not induce platelet aggregation. The possibility that atroxase may inhibit platelet aggregation was tested by adding ADP to an incubate of PRP with atroxase. Addition of ADP after a 2.5-min incubation of PRP with atroxase resulted in platelet aggregation approximately 90% of that obtained by ADP alone (Figure 7b).

Biological Activities. Atroxase, at dosages up to 50 μg, showed no hemorrhagic or CPI activity when injected intradermally into Swiss Webster mice. Low lethality was also demonstrated as all mice survived the highest intravenously injected dose of 20 μg/g. In comparison, crude venom contained an MHD of 2.0 μg and an LD_{50} of 3.5 μg/g. This indicates atroxase to be a nontoxic venom component.

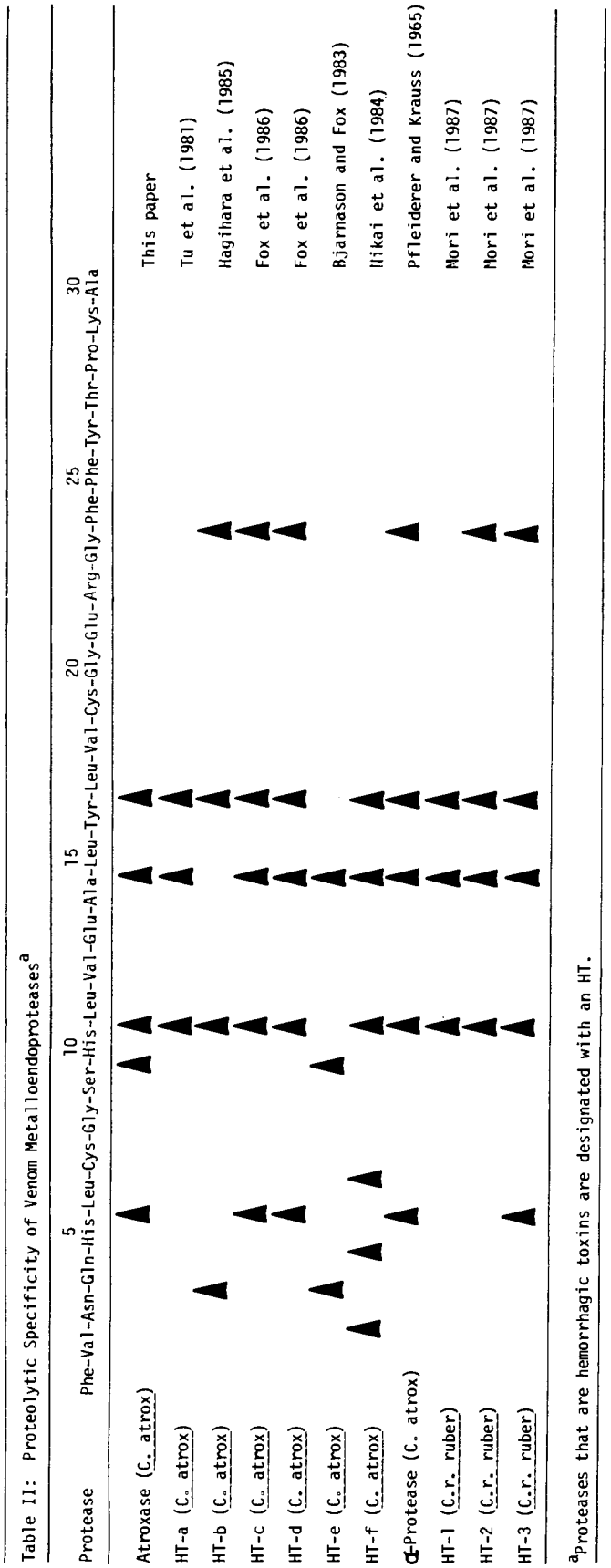
Discussion

Biologically active proteases are commonly found in the venoms from Crotalidae and Viperidae families (Tu, 1982). Fractionation of *C. atrox* venom showed that it contains several proteolytic fractions. Most proteolytic fractions were capable of rendering fibrinogen incoagulable, while fraction B2 converted fibrinogen into fibrin, or a fibrin derivative, resulting in clot formation. Procoagulant activity, although uncommon in venom from adult species of *C. atrox*, has been reported within juvenile venoms (Reid & Theakston, 1978). In this investigation, a metalloendoprotease was purified that is capable of promoting anticoagulation by the extensive hydrolysis of the Aα and Bβ chains of fibrinogen and the α-polymer, α, and β chains of fibrin. Atroxase was nonhemorrhagic, exhibited low lethality, and did not cause edema. Other metalloendoproteases found within the same venom include seven hemorrhagic toxins, HT-*a*, -*b*, -*c*, -*d*, -*e* (Bjarnason & Tu, 1978), -*f* (Nikai et al., 1984), and -*g* (Nikai et al., 1985), two anticoagulants, proteases I and IV (Pandya & Budzynski, 1984), and α-protease (Kruzel & Kress, 1985).

The variety of metalloproteases found within *C. atrox* venom make it necessary to compare atroxase, molecular weight 23 500 and p*I* 9.6, to enzymes with similar characteristics. Comparisons between HT-*b*, the only known basic hemorrhagic toxin, and atroxase show both to contain similar molecular weights and no free sulfhydryls or carbohydrate moiety, and both are Aα, Bβ fibrinogenases. HT-*b* can, however, be readily distinguished from atroxase by their biological effects. Atroxase, even at dosages of 50 μg, does not cause hemorrhage, unlike HT-*b* with an MHD of 3 μg. α-Protease, like atroxase, is a nonhemorrhagic metalloprotease. α-Protease has a basic p*I* of 8.15, 1.5 pH units lower than that of atroxase, and an amino acid content of 232 residues compared to 206 residues within atroxase. Proteases I and IV are anticoagulant proteases with molecular weights of 20 500 and 43 000, respectively. Protease IV was found to be an acidic glycoprotein while protease I was found to be a nonglycosylated basic protein. Protease I obtains optimal proteolytic activity at 37 °C and a pH of 7, compared to atroxase with an optimal proteolytic activity at 55 °C and a pH of 9.0. Unfortunately, the hemorrhagic activity was not determined for protease I; therefore, it is difficult to differentiate the enzyme from a similar protease, HT-*b*. Because very little information is known about the fibrinolytic enzyme fractions reported by Bajwa et al. (1980), a comparison is very difficult.

Metalloproteases within *C. atrox* venom and other venoms appear to have very similar proteolytic specificity. With insulin B chain as substrate, hemorrhagic toxins show a preference to cleave on the N-terminal side of Leu (Table II). This specificity was also observed among the nonhemorrhagic proteases of atroxase and α-protease. It is interesting that both hemorrhagic and nonhemorrhagic proteases within snake

Table II: Proteolytic Specificity of Venom Metalloendoproteases[a]



[a] Proteases that are hemorrhagic toxins are designated with an HT.

venom can be similar in both chemical compositions and proteolytic specificity. In future studies, it will be of interest to find out the determining factor among these zinc proteases that results in hemorrhage.

Nontoxic fibrinolytic enzymes found within *C. atrox* venom may be of considerable importance because of their potential therapeutic value (Bajwa et al., 1980). Thrombolytic agents such as streptokinase (Sherry, 1954), urokinase (Sobel et al., 1952), and more recently tissue-type plasminogen activator (Bachman, 1984) promote rapid dissolution of thrombi by activating the body's natural fibrinolytic system, the conversion of plasminogen to plasmin. It is possible that venom fibrinases may likewise lyse thrombi, however, through the direct proteolysis on fibrin occlusions.

References

Abildgaard, U., Lie, M., & Odegard, O. (1977) *Thromb. Res. 11*, 549–553.

Astrup, T., & Müllertz, S. (1952) *Arch. Biochem. Biophys. 40*, 346–351.

Bachmann, F., Egbert, I. R., & Kruithof, E. K. O. (1984) *Semin. Thromb. Hemostasis 10*, 6–17.

Bajwa, S. S., Markland, F. S., & Russell, F. E. (1980) *Toxicon 18*, 285–290.

Bajwa, S. S., Kirakossian, H., Reddy, K. N. N., & Markland, F. S. (1982) *Toxicon 20*, 427–432.

Bjarnason, J. B., & Tu, A. T. (1978) *Biochemistry 17*, 3395–3404.

Bjarnason, J. B., & Fox, W. (1983) *Biochemistry 22*, 3770–3778.

Civello, D. J., Moran, J. B., & Geren, C. R. (1983b) *Biochemistry 22*, 755–762.

Clauss, A. (1957) *Acta Haematol. 17*, 237.

Daoud, E., Tu, A. T., & El-Asmar, M. F. (1986) *Thromb. Res. 42*, 52–62.

Denson, K. W. E. (1969) *Toxicon 7*, 5–11.

Denson, K. W. E., Russell, F. E., Almagro, D., & Bishop, R. C. (1972) *Toxicon 10*, 557–562.

Devi, A., Banerjee, S., & Copley, A. L. (1972) *Toxicon 10*, 563–573.

Edelhoch, H. (1967) *Biochemistry 6*, 1948–1954.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Esnouf, M. P., & Tunnah, G. W. (1967) *Br. J. Haematol. 13*, 581–590.

Fox, J. W., Cambell, R., Beggerly, L., & Bjarnason, J. B. (1986) *Eur. J. Biochem. 156*, 65–72.

Hagihara, S., Komori, Y., & Tu, A. T. (1985) *Comp. Biochem. Physiol., C: Comp. Pharmacol. Toxicol. 82C*, 21–27.

Komori, Y., Hagihara, S., & Tu, A. T. (1985) *Biochim. Biophys. Acta 829*, 127–130.

Kondo, H., Kondo, S., Ikezawa, H., Murata, R., & Ohsaka, A. (1960) *Jpn. J. Med. Sci. Biol. 13*, 43–51.

Kress, L. F. (1988) in *Hemostasis and Animal Venoms: The Action of Snake Venom Metalloproteinases on Plasma Proteinase Inhibitors* (Pirkle, H., & Markland, F. S., Eds.) Marcel Dekker, New York.

Kruzel, M., & Kress, L. F. (1985) *Anal. Biochem. 151*, 471–478.

Kunitz, M. (1947) *J. Gen. Physiol. 30*, 291–310.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lin, Y., Means, G. E., & Freeney, R. E. (1969) *J. Biol. Chem. 244*, 789–793.

Markland, F. S., & Damus, P. S. (1971) *J. Biol. Chem. 246*, 6460–6473.

Markland, F. S., & Pirkle, H. (1977) in *Chemistry & Biology of Thrombin* (Lundblad, R. L., Fenton, J. W., & Mann,

K. G., Eds.) pp 71–89, Ann Arbor Science, Ann Arbor, MI.
Miles, A. A., & Wilhelm, D. L. (1955) *Br. J. Exp. Pathol. 36*, 71–75.
Moran, J. B., & Geren, C. R. (1981) *Biochim. Biophys. Acta 659*, 161–168.
Mori, N., Nikai, T., Sugihara, H., & Tu, A. T. (1987) *Arch. Biochem. Biophys. 253*, 108–121.
Morris, D. L. (1948) *Science (Washington, D.C.) 107*, 254–255.
Nikai, T., Mori, N., Kishida, M., Sugihara, H., & Tu, A. T. (1984) *Arch. Biochem. Biophys. 231*, 309–319.
Nikai, T., Mori, N., Kishida, M., Tsuboi, M., & Sugihara, H. (1985) *Am. J. Trop. Med. Hyg. 34*, 1167–1172.
Ouyang, C., & Yang, F. Y. (1974) *Biochim. Biophys. Acta 351*, 354–363.
Ouyang, C., & Huang, T. (1976) *Biochim. Biophys. Acta 439*, 146–153.
Ouyang, C., Hong, I. S., & Teng, C. M. (1971) *Thromb. Diath. Haemorrh. 26*, 224–234.
Pandya, B. V., & Budzynski, A. Z. (1984) *Biochemistry 23*, 460–470.
Pfleiderer, G., & Krauss, A. (1965) *Biochem. Z. 342*, 85–94.
Read, S. M., & Northcote, D. H. (1981) *Anal. Biochem. 116*, 53–64.
Reid, H. A., & Theakston, R. D. G. (1978) *Am. J. Trop. Med. Hyg. 27*, 1053–1057.
Richmond, V. L., Denis, R. S., & Cohen, E. (1985) *Anal. Biochem. 145*, 343–350.
Righetti, P., & Drysdale, J. W. (1971) *Biochim. Biophys. Acta 236*, 17–28.
Sapru, Z. Z., Tu, A. T., & Bailey, G. S. (1983) *Biochim. Biophys. Acta 747*, 225–231.
Sherry, S. (1954) *J. Clin. Invest. 33*, 1054–1063.
Shu, Y. Y., Moran, J. B., & Geren, L. R. (1983) *Biochim. Biophys. Acta 748*, 236–244.
Simpson, R. J., Neuberger, M. R., & Liu, T. Y. (1976) *J. Biol. Chem. 25*, 1936–1940.
Sobel, G. W., Mohler, S. R., Jones, N. W., Dowdy, A. B. C., & Guest, M. M. (1952) *Am. J. Physiol. 171*, 786–769.
Spackman, D. H., Stein, W. N., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.
Tu, A. T. (1982) in *Rattlesnake Venoms: Their Actions and Treatment* (Tu, A. T., Ed.) pp 163–209, Marcel Dekker, New York.
Tu, A. T., Nikai, T., & Baker, J. O. (1981) *Biochemistry 20*, 7004–7009.
Yang, C. O., & Teng, C. M. (1976) *Biochim. Biophys. Acta 420*, 298–308.

Stereochemistry of Phospho Group Transfer Catalyzed by a Mutant Alkaline Phosphatase

John E. Butler-Ransohoff,[‡] Debra A. Kendall,[‡] Sally Freeman,[§] Jeremy R. Knowles,[§] and E. T. Kaiser*,[‡]

Laboratory of Bioorganic Chemistry and Biochemistry, Rockefeller University, New York, New York 10021, and Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138

Received December 30, 1987; Revised Manuscript Received February 18, 1988

ABSTRACT: The stereochemical course of the phospho group transfer catalyzed by mutant (S102C) alkaline phosphatase from *Escherichia coli* was investigated by using ^{31}P nuclear magnetic resonance spectroscopy. Transphosphorylation from 4-nitrophenyl (R_P)-[^{16}O,^{17}O,^{18}O]phosphate to (*S*)-propane-1,2-diol occurs with overall retention of configuration at phosphorus. This result is consistent with the view that the hydrolysis of substrates by this mutant enzyme proceeds by way of a covalent phosphoenzyme intermediate in the same manner as the wild-type alkaline phosphatase.

Bacterial alkaline phosphatase is present in the periplasmic space of *Escherichia coli* (*E. coli*) and catalyzes the nonspecific hydrolysis of a wide range of phosphate monoesters (Reid & Wilson, 1971). The mechanism of the enzymatic reaction has been thoroughly studied, and the formation of a phosphorylated enzyme intermediate is now widely accepted as an essential step in the reaction pathway. At low pH, the rate-limiting step of the enzymatic reaction is the dephosphorylation of the covalent intermediate (Reid & Wilson, 1971; Coleman & Gettins, 1983), the accumulation of which has allowed its identification as the phosphate monoester of Ser-102 (Schwartz et al., 1963). At high pH, however, the rate-limiting step of the reaction is the release of noncovalently bound product phosphate from the active site of the enzyme. Even at high pH the overall stereochemical course of the reaction catalyzed by bacterial alkaline phosphatase is known to be retention of configuration at phosphorus (Jones et al., 1978), and this finding is consistent with the necessary formation of the phosphoenzyme intermediate.

We have reported the use of site-directed mutagenesis to replace the active-site hydroxyl group with a thiol by changing Ser-102 to Cys. The resulting thiol enzyme [S102C, previously referred to as thiol alkaline phosphatase (TAP)] was found to catalyze the hydrolysis of a variety of phosphate monoesters (Ghosh et al., 1986), though the V_{max} observed for the mutant enzyme was found to be dependent upon the pK_a of the substrate's leaving group. This observation suggests that, unlike the behavior of the wild-type enzyme (Hall & Williams, 1986), the rate-determining step of the reaction catalyzed by the mutant S102C phosphatase is the phosphorylation of the active-site thiol group rather than either dephosphorylation or product release. Further evidence was provided by the fact that the V_{max} values of the S102C alkaline phosphatase are independent of the concentration of tris(hydroxymethyl)-aminomethane (Tris) in the reaction buffer. Tris acts as an alternate phospho group acceptor from the wild-type phosphoenzyme, and higher concentrations of Tris increase the rate

[‡] Rockefeller University.
[§] Harvard University.

0006-2960/88/0427-4777$01.50/0