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CHARACTERIZATION OF A PROTEASE WITH α- AND β-FIBRINOGENASE ACTIVITY FROM THE WESTERN DIAMONDBACK RATTLESNAKE, *CROTALUS ATROX*

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A metalloprotease from the rattlesnake *Crotalus atrox* venom was isolated and purified from multiple-step chromatographies including anion-exchange chromatography, gel permeation and reversed-phase HPLC. The fraction was shown to be homogeneous as judged by SDS-gel electrophoresis. It also showed a high proteolytic activity against α- and β-chains of fibrinogen molecules. Further characterization of the purified fraction with fribrinogenase activity indicated that it is a single-chain protease with a molecular mass of about 24 kDa and an acidic isoelectric point. It is relatively heat stable up to about 65 °C, inhibited by EDTA, β-mercaptoethanol, but not by phenylmethanesulfonyl fluoride, N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, soybean trypsin inhibitor and aprotinin. Amino acid analysis showed that the enzyme possesses an amino acid composition very similar to some metalloproteases characterized before from the closely related rattlesnake venoms. N-Terminal sequence analysis of the enzyme corroborated some similarity between this enzyme and the reported sequences of these enzymes characterized from the Crotalidae snake family. This study indicated the presence of a novel fibrinogenase (termed Catroxase) with N-terminal sequence different from the metalloprotease with hemorrhagic activity isolated from the same Western diamondback rattlesnake.

The venoms of various snakes have been shown to possess fibrinolytic or fibrinogenolytic activity, notably in the snake families of Crotalidae and Viperidae [1-3]. It is noted that members of the crotalid family possess higher activities and more varieties of different fibrinogenolytic proteases than most species of the Elapidae family [4,5]. In addition to the basic interest in the structural and functional relationships of these enzymes to the important process of fibrin polymerization and clotting process, all these biologically active proteases have been used widely as biochemical agents in the studies of various pathological phenomena of clinical medicine. In view of their potential clinical usefulness, fibrinolytic enzymes isolated from various snake venoms should also be a good source and unique research tool for study of the haemostatic process and the development of potential antithrombotic agents [6-9].

Recently we have re-evaluate the components of venom proteases from Western diamondback rattlesnake *Crotalus atrox* and found that all fractions isolated from the anion-exchange chromatography showed various extents of specific proteolytic activity against α-

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or β-chains of fibrinogen molecules [10]. Characterization of one of the purified fractions with α-fribrinogenase activity indicated that it is a single-chain thrombin-like protease with a molecular mass of about 30 kDa. In this study we have further applied multiple-step chromatographies for the isolation and purification of one novel protease with both α- and β-chains fibrinogenolytic activities and compared its enzymatic properties and partial sequence with some reported fibrinogenases isolated from venoms of the same or closely-related snake species.

MATERIALS AND METHODS

Chemicals, instrumentation, protein isolation and purification

The lyophilized venom powder was obtained from Sigma Chemical Company (St. Louis, MO). The inhibitors for proteases such as soybean trypsin inhibitor, aprotinin, β-mercaptoethanol, phenylmethanesulfonyl fluoride (PMSF), N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) were also from Sigma Chemical Company. Venom components were isolated by anion-exchange chromatography on an open column (2.5 x 15 cm) packed with TSK DEAE-650 (M) (Merck, Darmstadt, Germany) gel suspension. Dissolved venom powder in 0.05 M ammonium bicarbonate, pH 7.5 starting buffer (20-25mg/ml) was applied to TSK DEAE-650 (total 5 ml) open column and then eluted in a linear gradient of 0.1-0.5 M ammonium bicarbonate, followed by 0.5-1.0 M ammonium bicarbonate, pH 8.0 buffers. A gel permeation chromatography on a SynChropak GPC-100 column (SynChrom, Inc., Lafayette, IN, USA) was carried out on a Hitachi's liquid chromatograph with a model L-6200 pump and a variable UV monitor. The reversed-phase HPLC column (4.0 x 300 mm, SynChropak RP-C_{18}, 6.5 μm bead) was used to purify and desalt the fraction isolated from the above gel-permeation chromatography.

Fibrinogenolytic activity

The fibrinogenolytic activity was assayed on SDS-polyacrylamide slab gel (5 % stacking/ 14 % resolving gel) as described [11]. Small vials containing about 5 μg purified fibrinogen in 50 mM Tris-HCl pH 7.7 buffer were incubated with 1-10 μl of various fractions (containing about 0.1-1.0 μg protein) from ion-exchange column at 37 °C for 1 h. After the incubation the digestion was stopped by adding 0.1% SDS/1% β-mercaptoethanol and heated at 90 °C for 3 min. The proteolytic activity was monitored on the Coomassie blue-stained gel after electrophoresis by observing the cleavage patterns of purified fibrinogen chains.

Amino acid and sequence analyses

The amino acid compositions were determined with a Beckman 6300 amino acid analyzer using a single-column system based on conventional ion-exchange chromatography system. The special rapid procedure for the preparation of protein hydrolysates using microwave irradiation before amino acid analysis was essentially according to the previous report [12].

N-Terminal sequence analysis was carried out by automated Edman degradation with a microsequencing sequenator (Model 477A, Applied Biosystems). The lyophilized column fractions each containing about 1-5 nmoles of protein were dissolved in 200 μl of 0.1 % trifluoroacetic acid (TFA) or 0.1 % SDS/0.1 % TFA (1:1 v/v) and 10 μl each for sequence determinations.

Sequence comparison and hydropathy profile

A program analysis of the local hydrophilicity of N-terminal protein segments along their amino acid sequences based on the Kyte-Doolittle hydropathy scale [13] is carried out on the MacVector sequence analysis software for Macintosh computers (International Biotechnologies, Inc., New Haven, CT). However the signs of the values have been reversed in order to plot the hydrophilicity instead of hydrophobicity scale. A window of size N=7 was run along the length of peptide segments; for each window, the hydropathy values of the 7 amino acids were summed and divided by 7 to obtain the average hydrophilicity per residue for the window. Values above the axis denote hydrophilic regions which may be exposed on the outside of the protein molecule whereas those values below the axis indicate hydrophobic regions which tend to be buried inside the protein.

RESULTS AND DISCUSSION

In contrast to a more thorough understanding of the toxin components from another Elapidae family [4,5] (including various cobras), there is still a lack of clear and systematic classification of venom components isolated from rattlesnakes. It is well known that the major components of crotalid snake family contain various types of proteolytic enzymes [14]. However different groups reported disparate proteases in the crotalid venoms. They included crotalase, a thrombin-like enzyme from *Crotalus adamanteus* [15]; hemorrhagic toxins, anticoagulant proteases and kallikrein-like enzymes from *Crotalus atrox* [16-18]. In this study we have adopted a consistent purification protocol in the endeavor to make a

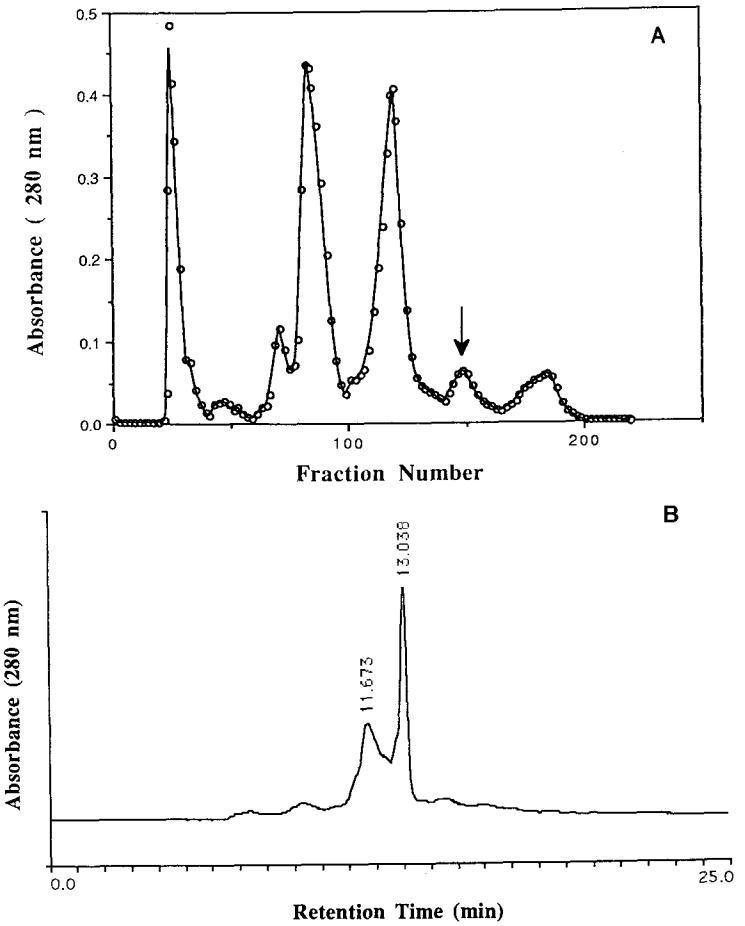

Fig. 1. **(A)** Anion-exchange chromatography on TSK DEAE-650 (M) column of crude venom from rattlesnake, *Crotalus atrox*. About 100-125 mg of lyophilized crude venom dissolved in the starting buffer of 0.05 M ammonium bicarbonate, pH 7.5, was applied to the column equilibrated with the same buffer. Elution was carried out in three steps as described in Materials and Methods. The column eluates (3.5 ml/tube per 4 min) were monitored for absorbance at 280 nm. The arrow indicates the peak collected, lyophilized and used for further purification on GPC-100 HPLC. **(B)** Gel-permeation HPLC of the collected fraction from (A). The lyophilized sample was dissolved in the running buffer of 0.1 M ammonium acetate, pH 6.4. Injection volume was 50 µl each time and eluting fractions detected at 280 nm. The peak of 11.67 min retention time was shown to be pure on SDS-PAGE and possessed strong fibrinogenolytic activity.

systematic comparison and characterization of venom components from *C. atrox* based on their structures and enzymatic activities.

Isolation and purification of a novel fibrinogenase from the rattlesnake, C. atrox

Fig. 1A shows the general elution pattern of the crude venom on TSK DEAE-650 anion-exchange column. The fraction indicated by the arrow showed the greatest fibrinogenolytic activity among all fractions studied. Therefore further purification of this fraction was carried out in order to obtain the purified fraction for sequence analysis. A second purification step of the lyophilized fraction was performed on the GPC-100 gel permeation (**Fig. 1B**) HPLC, resulting in the first peak of about 24 kDa (a retention time of 11.67 min) showing the fibrinogenolytic activity and the last peak (<10 kDa and a retention time of 13.04 min) devoid of activity. A reversed-phase HPLC was last used to desalt the 24 kDa protein which showed one single band under denaturing SDS-gel conditions (Lane 2 of **Fig. 2**). The subunit molecular mass was estimated to be about 24-26 kDa, corroborating that it is a single polypeptide chain without extra subunits. The protein was shown to be a non-glycoprotein by being negative on glycoprotein staining (unpublished results). Since this protein with fibrinogenolytic activity shows different structural and enzymatic properties (*vide infra*) from some reported proteases characterized from the same Crotalidae snake family, we have therefore termed this novel fibrinogenase as **Catroxase** to distinguish it from other similar fibrinogenases.

*Fibrinogenolytic activity of **Catroxase***

According to the guidelines of the International Committee on Thrombosis and Haemostasis [19] fibrinogenolytic enzymes of snake venoms have been defined operationally as α- or β-chain degrading fibrinogenases based on their direct-acting

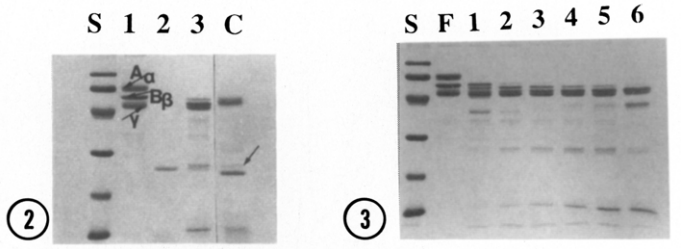

Fig. 2. SDS-gel electrophoresis (SDS-PAGE) of crude venom and purified Catroxase from GPC-100 chromatography under denaturing conditions in the presence of 5 mM dithiothreitol. Lane S, standard proteins used as molecular mass markers (in kDa): phosphorylase b (94), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (30), soybean trypsin inhibitor (20), and lysozyme (14). Lane C, crude venom ; lane 1, control purified fibrinogen with three subunit-chains; lane 2, purified Catroxase; lane 3, fibrinogen incubated with Catroxase for 60 min at 37 °C before stopping the reaction. The gel was stained with Coomassie blue. Note that Catroxase shows pure single band on the gel with a molecular mass of about 24 kDa. By densitometry of lane C, it was estimated that Catroxase (arrow) constituted about 2-3 % of total proteins in the crude venom.

Fig. 3. Time-course study of fibrinogenolytic activity of Catroxase on SDS-PAGE. Electrophoretic conditions were the same as in Fig. 2. Lane S, standard proteins used as molecular mass markers as in Fig. 2; lane F, purified fibrinogen without addition of enzyme; lanes 1-6, fibrinogen incubated with Catroxase at 37 °C for 5, 15, 30, 45, 60 and 120 min, respectively. Note that Catroxase showed specific cleavages on Aα and Bβ chains with γ chain almost intact.

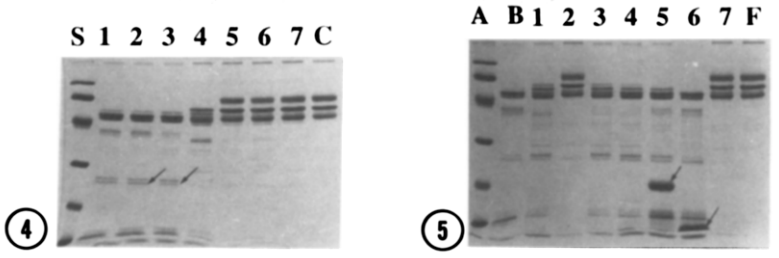

Fig. 4. Thermostability study of fibrinogenolytic activity of Catroxase on SDS-PAGE. Electrophoretic conditions were the same as in Fig. 2. Lane S, standard proteins used as molecular mass markers as in Fig. 2; lane C, purified fibrinogen control without addition of enzyme; lanes 1-7, fibrinogen incubated for 60 min with Catroxase pre-incubated at 37, 45, 55, 65, 75, 85 and 95 °C for 30 min, respectively. The arrow indicates the Catroxase (about 1 μg) used in the assay mixtures.

Fig. 5. Inhibition study of fibrinogenolytic activity of Catroxase on SDS-PAGE. Lane A, standard proteins used as molecular mass markers as in Fig. 2; lane F, purified fibrinogen without addition of enzyme; lane B, fibrinogen incubated with Catroxase; lanes 1-7, mixtures of fibrinogen and Catroxase in the presence of TPCK, 2-mercaptoethanol, TLCK, PMSF, trypsin inhibitor, aprotinin and EDTA, respectively. Arrows in lanes 5 and 6 indicates the added trypsin inhibitor and aprotinin, respectively. Note that Catroxase is only inhibited by 2-mercaptoethanol (lane 2) and EDTA (lane 7).

digestive activity on these fibrinogen subunit chains. It is of interest to note that Catroxase of *C. atrox* venom possessed mixed types of fibrinogenolytic activity when assayed using purified fibrinogen (**Fig. 2**), with both Aα and Bβ chains of fibrinogen are cleaved under identical digestion conditions. **Fig. 3** shows the time-course digestion of fibrinogen by Catroxase, revealing that within 5 min Aα chain has been completely cleaved and about 50 % Bβ chain also disappeared. By 30 min both Aα and Bβ chains of fibrinogen are completely destroyed while γ chain remains intact.

*Thermostability of **Catroxase** and effects of protease inhibitors*

Fig. 4 shows the effect of temperature on the fibrinogenolytic activity of Catroxase. This enzyme shows unusual stability against heating at 65 °C for about 30 min. It is more stable than the α-fibrinogenase we characterized before from this species [10].

We have also studied the effects of various protease inhibitors on the fibrinogenase activity of Catroxase using SDS-gel electrophoresis similar to that described in Figs 2-4. It is found that this enzyme was inhibited by EDTA (Lane 7 of **Fig. 5**), attesting to its being a metalloprotein. The serine protease inhibitors, PMSF, TPCK, TLCK and aprotinin, were all shown to have no effect on the fibrinogenolytic activity of Catroxase, indicating that this protein does not belongs to the serine proteases family as commonly shown for some venom fibrinogenases and thrombin-like proteases [19,20]. The enzyme was also not inhibited by soybean trypsin inhibitor, which distinguished it from trypsin-like proteases. Similar to crotalase from *C. adamanteus*, Catroxase loses its activity in the presence of β-mercaptoethanol. All these properties attest to the unique nature of this special mixed type of α/β fibrinogenase isolated from *C. atrox* as compared to other published fibrinogenases. It is to be emphasized that the present study is the first demonstration of the existence of this protease in this rattlesnake venom despite various studies have been carried out on this

Table 1. Comparison of amino acid compositions of Catroxase and other fibrinogenases from crotalid snakes

Amino acids	Catroxase (this report)	Atroxase (Ref. 21)	H_2 protease (Ref. 23)	J protease (Ref. 24)
1/2Cys	6.8 (7)	12	7	7
Asx	30.5 (31)	27	32	31
Thr	8.7 (9)	9	10	10
Ser	13.3 (14)	23	13	13
Glx	22.7 (23)	23	17	19
Pro	7.7 (8)	6	7	7
Gly	13.6 (14)	9	7	14
Ala	8.1 (8)	7	9	13
Val	10.6 (11)	12	14	9
Met	5.8 (6)	4	8	7
Ile	14.5 (15)	11	12	15
Leu	11.8 (12)	17	19	15
Tyr	12.6 (13)	6	8	9
Phe	7.2 (7)	8	5	6
His	7.5 (8)	8	7	8
Lys	10.4 (10)	8	18	11
Arg	6.8 (7)	11	6	8
Trp	1.6 (2)	3	2	n.d.
Total residues	(205)	(204)	(201)	(202)

Data for Catroxase are expressed as the number of residues per molecule of protein using alanine as the reference to calculate the residues of other amino acids. Values represent the mean of duplicate determinations. The hydrolysis condition is 140°C for 3 h using 6 M HCl or 4 M methanesulfonic acid containing 0.2 % 3-(2-aminoethyl)indole. Values for the other fibrinogenases are taken from references [21], [23] and [24].

crotalid species. As estimated from the percent yields of two chromatographic steps in Fig. 1A and 1B, Catroxase comprises only about 2-3 % of total crude venom protein which may account for the previous neglect of reporting its presence in this venom.

Amino acid composition and partial sequence of Catroxase

The amino acid composition (**Table 1**) of the purified Catroxase of *C. atrox* was determined in order to establish the identity of this novel α/β fibrinogenase. It is found to be somewhat similar to those compositions published for atroxase [21] and toxin Ht-d [22] characterized from the same species and H_2 protease from the Habu snake [23] plus J protease from the snake *Bothrops jararaca* [24]. In general the pair-wise composition comparison (Table 1) showed distinct differences regarding the contents of certain amino

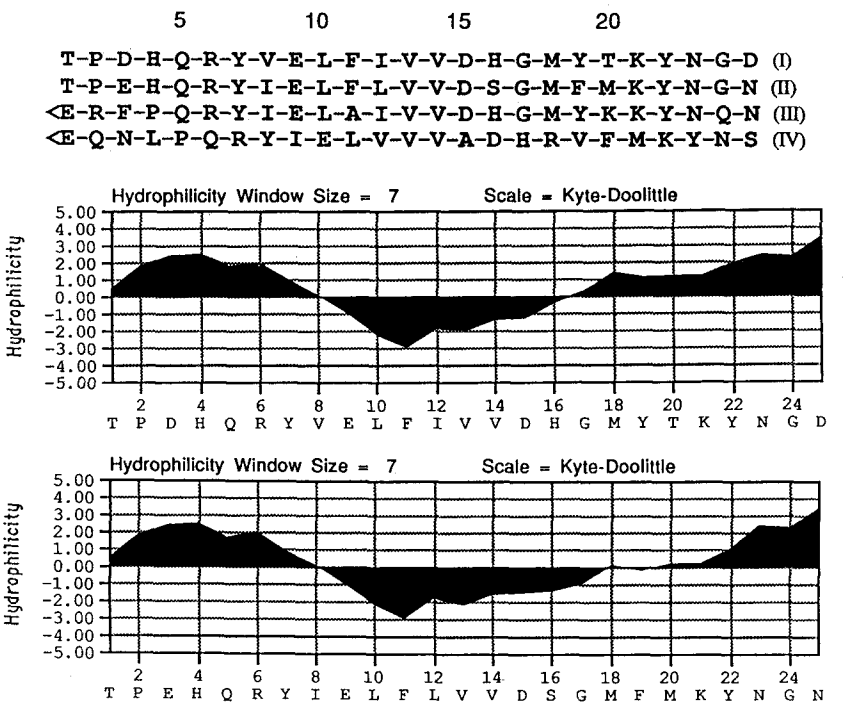

Fig. 6. Comparison of N-terminal sequences of Catroxase (I), J-protease (II), H_2 protease (III) and Ht-d hemorrhagic protease (IV) from crotalid species. The sequences listed for II, III and IV were taken from Refs. [24], [23] and [22],respectively. Amino acid residues are denoted by one-letter symbols and <E indicates a blocked terminal pyroglutamate. Hydropathy profiles for Catroxase (I) and J-protease (II) are shown below the sequences to exemplify the similar distribution of hydrophilic amino acids in the amino-terminal segments between these two proteases.

acids among these similar proteases. For example Catroxase showed different contents of serine, glycine, tyrosine, cysteine and arginine when compared to that of atroxase from *C. atrox*. Further N-terminal sequence comparison (**Fig. 6**) of the partial sequences of the four proteases corroborated some similarity in the primary structure of these proteases and yet revealed the unique unblocked N-terminal sequences of Catroxase and J protease as compared to the blocked N-terminal sequences of the other two. It is of interest to note that the hydropathy profiles of Catroxase and J protease from distant genera indeed show similar distribution of hydrophilic amino acids, which may reflect structural similarity too.

Conclusion and perspectives

It is crucial to point out that Catroxase characterized in this study showed some distinct inhibition properties from those of other published fibrinogenases in the literature [25]. Furthermore the amino acid and sequence comparisons ruled out the possibility that it is identical to atroxase or hemorrhagic toxin Ht-d isolated from the same crotalid species. Judging on the unique and similar N-terminal sequences shared between Catroxase and J protease from the snake *Bothrops jararaca*, it appears that this protease is also present in some more distantly related snake and may play a more general and significant role in

eliciting the fibrinogenolytic activity and the subsequent outcome of blood clotting or platelet aggregation generally associated with rattlesnake bites. Further functional characterization and structure determination of Catroxase may prove essential to understand the physiological role of this novel fibrinogenolytic factor and its possible medical application.

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