Characterization of Cerastobin, a Thrombin-like Enzyme from the Venom of *Cerastes vipera* (Sahara Sand Viper)†

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ABSTRACT: Cerastobin, a thrombin-like enzyme, was isolated from the venom of *Cerastes vipera* (Sahara sand viper) in homogeneous form. Cerastobin had a molecular weight of 38 000 with 348 amino acid residues. It had an isoelectric point of 7.7 (a pH optimum of 7.9 and a temperature optimum of 45 °C). Cerastobin hydrolyzed arginine-containing synthetic substrates such as TAME, BAME, and BAEE, but BAPNA was not hydrolyzed. Cerastobin had thrombin-like activity, producing fibrin from fibrinogen and also hydrolyzing chromogenic substrates for thrombin such as 2AcOH·H-D-CHG-But-Arg-*p*NA (CBS 34.47) and H-D-Phe-Pip-Arg-*p*NA (S-2238). It showed kallikrein-like activity and hydrolyzed kallikrein substrates 2AcOH·H-D-Phe-Gly-Arg-*p*NA (CBS 33.27) and H-D-Pro-Phe-Arg-*p*NA (S-2302). It produced bradykinin from bradykininogen, as uterus contraction was observed. A serine inhibitor, DFP, exerted a pronounced inhibitory effect, suggesting that cerastobin is a serine-type protease. The sequence of 37 residues from the amino-terminal end was investigated. The amino-terminal amino acid was valine as it is in most other thrombin-like enzymes. The amino acid sequence of cerastobin was similar to that of thrombin in some residues and had some homology with that of kallikrein. However, cerastobin showed a high degree of homology to thrombin-like enzymes isolated from various snake venoms. Factor X was partially degraded by cerastobin. It was also found that antithrombin III was degraded by the enzyme. The α and β chains of fibrin monomer were preferentially hydrolyzed by cerastobin, but the γ chain was quite resistant.

It is known that snake venom exerts a profound effect on blood coagulation (Fujikawa et al., 1974; Hofmann & Bon, 1987). Sometimes both coagulant and anticoagulant factors are present in the same venom (Tu, 1977). Recently, thrombin-like enzyme have attained considerable attention because of their potential therapeutic use in myocardial infarction and thrombotic diseases, as these enzymes can act on fibrinogen, leading to defibrinogenation of blood and to a consequent decrease in blood viscosity. So, the flow properties of blood are improved (Ehrly, 1972). Thrombin-like enzymes are also a useful tool for studying the mechanism of conversion of fibrinogen into fibrin (Exner et al., 1983). Thrombin-like enzymes are widely distributed in venoms of the Crotalidae family (Gaffney, 1977); however, they are found in the snake venoms of a few other families (Bajwa et al., 1982). The well-known thrombin-like enzymes are Arvin (ancrod), batroxabin, reptilase, gabonase, crotalase, and many others (Markland & Damus, 1971; Hessel & Blomback, 1971; Pirkle et al., 1986; Itoh et al., 1987).

Like thrombin, these enzymes from snake venoms can hydrolyze synthetic substrates at bonds involving the carbonyl group of basic amino acids like arginine esters. Markland and Damus (1971) characterized from *Crotalus adamenteus* venom a thrombin-like enzyme that shows a high specificity toward arginine esters.

The present paper reports the results of purification and characterization of cerastobin, which showed arginine esterase and thrombin-like activity, from the venom of *Cerastes vipera* found in North African deserts. The enzyme formed fibrin clots but differed from Arvin or crotalase in many aspects.

Materials and Methods

The crude venom was milked from mature *C. vipera* at the serpentarium of the Biochemistry Department, Ain Shams Medical School, Cairo, Egypt, and immediately frozen and lyophilized. Sephadex G-100 and G-200 and DEAE-Sephadex A-50 were obtained from Pharmacia; the chromogenic substrates CBS 34.47, CBS 33.27, CBS 33.08, and CBS 30.41 were obtained from Diagnostica Stago (France). Other synthetic peptides, S-2238, S-2302, and S-2222, were purchased from Kabi Diagnostica (Stockholm, Sweden). TAME,[1] BAME, BAEE, BAPNA, DMSO, benzamidine, trasylol, soybean trypsin inhibitor, EDTA, fibrinogen (human and bovine), factor X, factor X activating enzyme (*Vipera russelli*), distearoyl-L-α-phosphatidylcholine, ADP, and collagen were from Sigma (St. Louis, MO). The animals (white Swiss rats) were obtained from the Pharmacology Department, Ain Shams Medical School. Other chemicals used were analytical grade from commercial sources.

Isolation Procedure. Cerastobin was isolated by a combination of gel filtration and ion-exchange chromatography

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[1] Abbreviations: BAEE, N^{α}-benzoyl-L-arginine ethyl ester hydrochloride; BAME, N^{α}-benzoyl-L-arginine methyl ester; BAPNA, N^{α}-benzoyl-DL-arginine *p*-nitroanalide; But, aminobutyric acid; CHA, cyclohexylalanyl; CHG, cyclohexylglutamyl; DFP, diisopropyl fluorophosphate; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HPLC, high-performance liquid chromatography; Pip, piperidyl; PITC, phenyl isothiocyanate; PNA, *p*-nitroaniline; PPP, platelet-poor plasma; PRP, platelet-rich plasma; SDS, sodium dodecyl sulfate; TAME, N^{α}-*p*-tosyl-L-arginine methyl ester.

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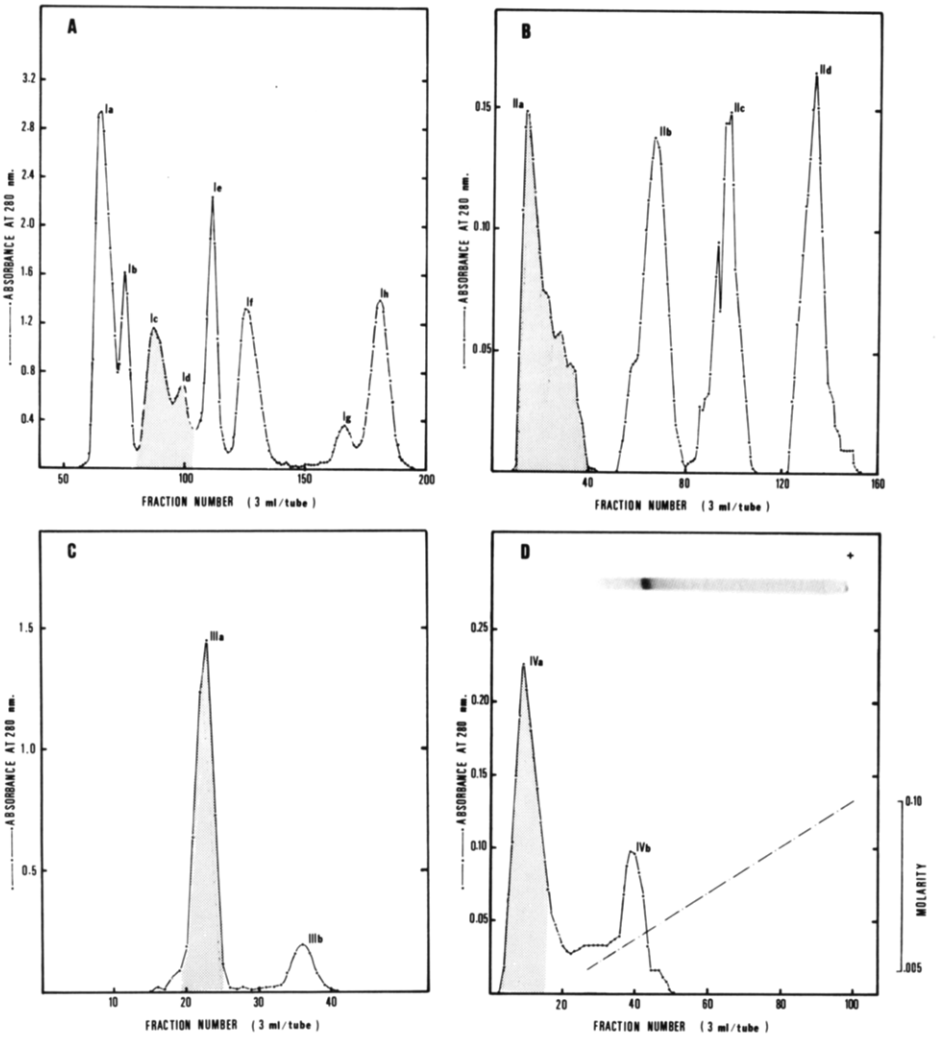

FIGURE 1: Scheme of isolation of cerastobin from the venom of *C. vipera*. The isolation procedure is described under Materials and Methods. (A) Separation on G-100. (B) Separation on DEAE-Sephadex A-50. (C) Separation on G-200. (D) Separation on DEAE-Sephadex A-50 and a homogeneous band for the final fraction.

at 4 °C (Figure 1). First, 250 mg of the crude venom was dissolved in 2 mL of 20 mM ammonium acetate buffer, pH 4.6, and the insoluble material was removed by centrifugation (2000 g) for 30 min at 4 °C. The supernatant was separated on a Sephadex G-100 superfine column (2.5 × 100 cm). The column was equilibrated with and the venom was eluted by the 20 mM ammonium acetate buffer, pH 6.8. Fractions C and D, which contained arginine esterase activity, were further fractionated on a DEAE-Sephadex A-50 column (1.6 × 20 cm) eluted in a stepwise gradient by phosphate buffer, pH 6.8, at 0.01, 0.1, and 0.3 M (120 mL of each buffer strength), and finally the column was washed by 0.5 M NaCl. Fraction IIa, which showed the esterase activity, was dialyzed against distilled water, lyophilized, and further fractionated on a Sephadex G-200 superfine column (1.6 × 90 cm) eluted by 20 mM ammonium acetate buffer, pH 4.6. Fraction IIIa, having the esterase activity, was lyophilized, dissolved in 1 mL of 5 mM phosphate buffer, pH 7.5, applied to another DEAE-Sephadex A-50 column (1.6 × 20 cm), and eluted by phosphate buffer, pH 7.5, in a linear gradient from 5 to 100 mM starting from tube 25. The enzyme fraction was subjected to a disc gel electrophoresis; 50 μg of cerastobin was applied on top of a 10% polyacrylamide gel. Tris–glycine (50 mM), pH 8.3, was used as running buffer. Protein in the gel was stained with 0.25% Coomassie blue R-250 in 7.5% acetic acid containing 5% methanol, for 2 h, and then destained in 7.5% acetic acid. The sample gave a single band, indicating the homogeneity of the preparation. Homogeneity was also confirmed by isoelectric focusing and Ouchterlony immunodiffusion.

Enzyme Activity Assay. Arginine esterase activity was assayed with TAME as a substrate. The reaction mixture contained 0.1 mL of the enzyme, 0.8 mL of 0.4 M Tris-HCl buffer, pH 8.1, and 0.1 mL of 100 mM TAME. This mixture was incubated at 37 °C for 15 min, and then the colorimetric assay was completed according to the hydroxamate method of Robert (1958). This method was also used to detect the effect of temperature, pH, cations, anions, and various inhibitors on cerastobin hydrolysis of TAME as well as to determine the effect of cerastobin on other substrates such as BAME and BAEE. Arginine amidase activity was determined with BAPNA as a substrate by a modification of the method of Erlanger et al. (1961). BAPNA (43.5 mg) was dissolved in 1 mL of DMSO and brought to 100 mL with 60 mM phosphate buffer, pH 7.5. Between 5 and 100 μL of enzyme solution was added to 2 mL of the substrate solution, and the change in absorbance at 410 nm was recorded. For the study of the inhibitors' effects, cerastobin (3 μg) was incubated with each inhibitor for 15 min. Then the hydrolysis of TAME was measured. DFP was dissolved in 0.1 M Tris buffer, pH 8.1, and incubated with the enzyme.

Molecular Weight. The molecular weight was determined by SDS–polyacrylamide gel electrophoresis. Protein standards used were α-lactalbumin (14 000), soybean trypsin inhibitor

(20 100), carbonic anhydrase (30 000), ovalbumin (43 000), bovine albumin (67 000), and phosphorylase B (94 000). The enzyme and the standards were treated with SDS, reduced with β-mercaptoethanol for 3 min at 100 °C, and then run on a 10% polyacrylamide gel.

Amino Acid Composition. Amino acid composition analyses were carried out on HPLC equipment (Beckman, Model 345) involving precolumn derivatization of amino acids using phenyl isothiocyanate (PITC) followed by reverse-phase chromatography for the quantitative determination of protein hydrolysates according to the method of Heinrickson (1984) and using HPLC methanol instead of acetonitrile as the based mobile phase. Tryptophan was spectrophotometrically determined according to the method of Edelhoch (1967).

Amino Acid Sequence. The purified cerastobin was reduced and alkylated to make carboxymethylated enzyme by following the method of Crestfield as described by Elzinga (1970).

Amino acid sequence was determined directly on an Applied Biosystems 470A protein sequencer and PTH analyzer, on-line PTH-amino acid separation system 120A.

Carbohydrate Content. Neutral sugar content was estimated according to the method of Dubois et al. (1956).

Assay of Thrombin-like Activity. Thrombin-like activity of cerastobin was tested by several methods. The first method used chromogenic substrates CBS-34.47 and S-2238 following the procedure of Guichaoua and Martinoli (1982).

Both substrates contain the *p*-nitroaniline chromogenic group next to an arginine residue. When the arginine bond is split, *p*-nitroaniline is released, and the increase in absorbance can be followed. The second method tested the effect of the enzyme on 1% fibrinogen (human and bovine) dissolved in 50 mM imidazole–10 mM NaCl buffer, pH 7.4, both by determining fibrin clot in a test tube (Ware et al., 1967) and by detecting on an aggregometer the change in light transmission that accompanied the conversion of fibrinogen into fibrin [this is a modification of the turbidimetric method of Mosesson et al. (1967)]. As fibrin was formed, the turbidity increased with a concurrent decrease in transmission. In the third method, fibrinogenase activity was detected by incubating an equal volume of the fibrinogen (1000 μg) and cerastobin (10 μg) at 37 °C. Denaturing agents (10 M urea, 4% SDS, and 4% β-mercaptoethanol) (0.1 mL) were added to the fibrin that was produced from fibrinogen by the action of thrombin or cerastobin. The mixture was incubated at 37 °C overnight. Aliquots (100 μL) were applied to the top of a gradient SDS–polyacrylamide slab gel (7–17%), and the electrophoretic separation was continued according to the method of Laemmli (1970).

Assay for Coagulant Activity. The coagulant activities of cerastobin were determined on normal human citrated plasma (1 part of 3.8% sodium citrate and 9 parts of blood) (Biggs & MacFarlene, 1962). The test was done by adding 0.1 mL of plasma, 0.1 mL (1:100) of phospholipid, 0.1 mL of cerastobin (10 μg), and 0.1 mL of 25 mM calcium chloride and calculating the clotting time.

Effect on Factor X. The effect on factor X was measured by the amidolytic activity of factor Xa according to the method described by Hofmann and Bon (1987). This method uses S-2222, the structure of which is Bz-Ile-Glu-Gly-Arg-*p*NA. When the substrate is hydrolyzed, *p*-nitroaniline is released, and this can be followed spectrophotometrically.

Factor X, 160 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 concentration of cerastobin (10 μg). Aliquots (50 μL) were removed at different incubation times at 25 °C, 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-2222 by recording the formation of *p*-nitroaniline at 405 nm. Parallel with the determination of the amidolytic activity, aliquots from the incubation mixture were taken and immediately frozen to −18 °C after addition of an equal amount of 13 mM EGTA. After lyophilization, their peptide composition was analyzed by SDS–polyacrylamide slab gel electrophoresis (7–17%) in the presence of 4% SDS and 2% β-mercaptoethanol. A control test using *V. russelli*, a well-known factor X activator, was run in parallel with the test.

Kininogenase Activity. The chromogenic substrates CBS-33.07 and S-2302 were used to check the kallikrein-like action of cerastobin. The kininogenase activity of the enzyme was also tested on isolated gravid rat uterus according to the procedure of Kitchen (1984).

Fibrinolytic Activity. The plasmin-like activity of cerastobin was determined by using chromogenic substrates CBS-33.08 and CBS-30.41. The fibrinolytic effect was tested by studying the peptide release from nonpolymerized fibrin (resulting from the addition of 0.1 mL of "10 units/mL" thrombin to 0.1 mL of 1% fibrinogen in imidazole–saline buffer). One-tenth milliliter of cerastobin (10 μg) or plasmin (0.6 units/mL) was added to the clot, and the mixture was incubated to 37 °C. After the incubation time, 0.1 mL of denaturing solvent (10 M urea, 4% SDS, and 4% β-mercaptoethanol) was added, and the mixture was left at 37 °C overnight. Then 10 μL of the mixture was separated by a gradient SDS–polyacrylamide gel (7–17%).

Interaction between Cerastobin and Antithrombin III. The hydrolytic effect of cerastobin on antithrombin III was tested by incubating the cerastobin (10 μg) with antithrombin III (1 unit) at 37 °C. At different intervals, aliquots were taken and the antithrombin III effect on thrombin was detected by the turbidimetric method. Also, 0.1 mL of the mixture was denatured by 0.1 mL of solvent containing 10 M urea, 4% SDS, and 4% β-mercaptoethanol and then separated by a gradient SDS–polyacrylamide slab gel (7–17%).

Effect on Platelet Aggregation. Human blood was mixed with 3.8% sodium citrate (9:1 v/v) in a siliconized glass or plastic tube. Platelet-rich plasma (PRP) was obtained by centrifuging the citrated blood at 100 g for 10 min. Platelet-poor plasma (PPP) was collected after centrifuging the citrated blood at 500 g for another 10–15 min. A mixture of PRP and PPP was prepared so as to adjust the platelet count to be 250 000/mm^3 as detected by hemocytometer. The effect of cerastobin on platelet aggregation was detected according to the turbidimetric method of Born and Cross (1963) and O'Brien (1962) by monitoring the change in light transmission in an aggregometer, Model Chrono-Log.

Results

Isolation. Cerastobin was isolated by a combination of gel filbration and DEAE ion-exchange chromatography (Figure 1). Homogeneity of the final fraction in disc gel electrophoresis is shown in Figure 1D. The purification of enzyme activity was 11.7-fold, while the yield was 9.3% (Table I).

Chemical Properties. The isoelectric point of cerastobin as determined by isoelectric focusing was 7.7. The arginine esterase activity showed a pH optimum of 7.9 and a temperature optimum of 45 °C; beyond 45 °C the activity dropped sharply. Carbohydrate content determined by Dubois et al.'s method was negative, indicating there are no carbohydrates in the purified cerastobin. Substrate specificities were investigated by using a variety of substrates (Table II). Of the

Table I: Recovery of Proteins and Arginine Esterase Activity from *C. vipera* Venom

fractionation step	recovery (mg)	sp act. × 10^3	act. increase (*x*-fold)	yield (%)
crude venom	250	9.52		100
Sephadex G-100, superfine	36.80	21.10	2.21	32.62
DEAE-Sephadex A-50	7.40	46.00	4.83	14.39
Sephadex G-200	4.60	71.05	7.46	13.78
DEAE-Sephadex A-50	1.90	112.0	11.7	9.30

Table II: Substrate Specificity of Cerastobin

substrate	sp act. (units/mg) crude venom	cerastobin
N^{α}-*p*-tosyl-L-arginine methyl ester (TAME)	9.52	82.9
N^{α}-benzoyl-L-arginine methyl ester (BAME)	5.21	37.6
N^{α}-benzoyl-L-arginine ethyl ester hydrochloride (BAEE)	4.08	30.6
N^{α}-benzoyl-DL-arginine *p*-nitroanalide (BAPNA)	zero	zero
azocoll	8.70	5.20
2AcOH·H-D-CHG-But-Arg-*p*NA (CBS 34.47)	8.40	13.3
H-D-Phe-Pip-Arg-*p*NA (S-2238)	11.5	14.2
2AcOH·H-D-Phe-Gly-Arg-*p*NA (CBS 33.27)	3.34	2.10
H-D-Pro-Phe-Arg-*p*NA (S-2302)	6.00	5.20
2AcOH·H-D-Nle-CHA-Arg-*p*NA (CBS 33.08)	1.40	1.20
2AcOH·H-D-But-CHA-Lys-*p*NA (CBS 33.08)	1.40	1.20
2AcOH·H-D-But-CHA-Lys-*p*NA (CBS 30.41)	1.60	0.18

Table III: Amino Acid Content of Cerastobin

amino acid	calcd value	nearest integer	amino acid	calcd value	nearest integer
Asx	33.75	34	Met	15.00	15
Glx	25.13	25	Val	15.10	15
Ser	29.73	30	$^1/_2$-Cys[a]	3.83	4
Gly	35.76	36	Ile	27.31	27
His	9.63	10	Leu	21.77	22
Thr	27.89	28	Lys	18.25	18
Ala	16.22	16	Phe	4.84	5
Arg	21.38	21	Trp[b]	6.37	6
Pro	28.05	28			
Tyr	7.96	8	total residues		348

[a] Half-cysteine was confirmed by analysis of reduced carboxymethylated sample. [b] Tryptophan was determined spectrophotometrically.

synthetic substrates containing arginine bonds that were tested, TAME, BAME, and BAEE were cleaved, but BAPNA was not cleaved. Michaelis–Menten constants K_m were measured for the enzyme reactions with substrates TAME, BAME, and BAEE. They were 1.47×10^{-3}, 2.0×10^{-3}, and 2.2×10^{-3} M, respectively. Cerastobin hydrolyzed the protein azocoll, but its activity was lower than that of crude venom. Chromogenic substrates for thrombin (CBS 34.47 and S-2238) were hydrolyzed. Kallikrein substrates (CBS 33.27 and S-2302) and plasmin substrates (CBS 33.08 and CBS 30.41) were also hydrolyzed by cerastobin, but the specific activities were less than those of crude venoms.

The degree of inhibition by a number of inhibitors was examined. Benzamidine, which inhibits trypsin by interacting with the aspartic acid residue of trypsin, had a partial inhibitory effect. However, a serine inhibitor, DFP, exerted a profound inhibitory effect. This suggests strongly that cerastobin is a serine-type protease.

Trasylol is well known for inactivating trypsin, kallikrein, chymotrypsin, and plasmin. It showed no effect on cerastobin, suggesting that cerastobin is distinctly different from these enzymes. This was further confirmed by the fact that soybean trypsin inhibitor had no effect on cerastobin. A chelating agent, EDTA, did not affect the hydrolysis of TAME, indicating that the enzyme is probably not a metalloenzyme. Iodoacetamide inhibited the enzyme activity completely, suggesting that the sulfhydryl may be essential for the hydrolytic activity.

The amino acid composition of the purified enzyme after the HCl hydrolysis is shown in Table III. The enzyme has 348 amino acid residues with a molecular weight of 38 000 as calculated by SDS–polyacrylamide gel electrophoresis. The sequence of 37 residues from the amino-terminal end, identified from PTH-amino acids, is shown in Table IV. The amino-terminal amino acid was found to be valine, as it is in most other thrombin-like enzymes.

Thrombin-like Enzyme. Thrombin-like enzyme activity was found by three independent methods. When the hydrolysis of chromogenic substrates CBS 34.47 and S-2238 was followed, both substrates were hydrolyzed by crude venom as well as by purified enzyme.

When the formation of fibrin from fibrinogen was followed on an aggregometer, the rate of transmission decrease was enhanced as the amount of the enzyme increased. Fibrin clot formation was also clearly visualized in a test tube.

Figure 2 shows the acrylamide gel electrophoresis of the products of hydrolysis of fibrinogen by cerastobin. The fibrinogen molecule consists of three chains, Aα, Bβ, and γ. Since fibrinogen is the dimer of these chains, it is often expressed as $(A\alpha,B\beta,\gamma)_2$. Thrombin is known to hydrolyze Aα and Bβ chains by releasing fibrinopeptides A and B, and fibrinogen becomes fibrin monomer $\alpha\beta\gamma$. The enzyme was an Aα and Bβ type of enzyme hydrolyzing the Aα chain of fibrinogen readily but the Bβ chain slowly. In the absence

Table IV: Partial Amino Acid Sequence of Cerastobin, Other Venom Thrombin-like Enzymes, Kallikrein, and Thrombin

	Residue no. 5 10 15 20 25 30 35	Reference
Cerastobin	V I G G A E C N I N E H R S I V L L Y (...) S S R L F G H (...) T L I N K E W V L	Present paper
Gabonase	V V G G A E G K I D G H E C L A L L Y	Pirkle et al., 1986
Crotalase	V I G G D E C N I N E H R F L V A L Y	Pirkle et al., 1981
Batroxabin	V I G G D E C D I N E H P F L A F M Y Y S P R Y F C G M T L I N Q E W V L	Itoh et al., 1987
Kallikrein	V V G G Y N C E M H S Q P W Q V A V Y Y F G E Y L C G G V L I D P S W V I	Swift et al., 1982
Thrombin	I V E G S N A E I G M S P W Q V M L F R K S P Q E L L C G A S L I S N R W V L	Butkowski et al., 1977

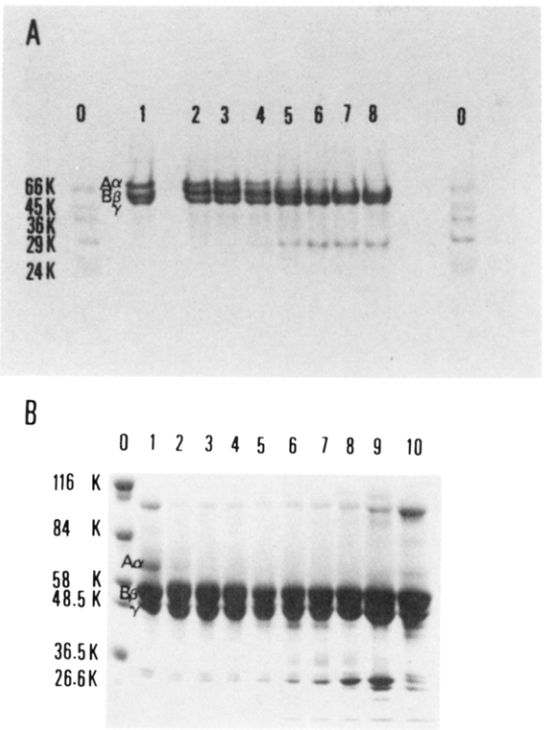

FIGURE 2: Hydrolysis of (A) bovine fibrinogen and (B) human fibrinogen by cerastobin. Samples were reduced prior to application to the SDS gels. Lane 0, molecular weight standard; lane 1, reduced fibrinogen incubated with 1 unit of thrombin for 15 min. Reduced fibrinogen was incubated with cerastobin with the following conditions: A2, 15 min; A3, 30 min; A4, 1 h; A5, 3 h; A6, 6 h; A7, 12 h; A8, 24 h; B2, 15 min; B3, 30 min; B4, 1 h; B5, 3 h; B6, 6 h; B7, 12 h; B8, 24 h; B9, 48 h; and B10, reduced human fibrinogen incubated with 1 unit of thrombin for 48 h.

of enzyme, fibrinogen cleavage was very minimal in both the 24- and 48-h samples. All three units of fibrinogen Aα, Bβ, and γ were preserved.

Effect on Blood Coagulation. Cerastobin promotes blood coagulation. It clotted the citrated plasma in 53 s after addition of 0.1 mL of 25 mM $CaCl_2$ and in 136 s in the absence of added Ca^{2+}.

A chromogenic substrate, S-2222, was hydrolyzed by cerastobin in the absence and in the presence of phospholipids (Figure 3A,B). Degradation products of factor X by *V. russelli* venom and cerastobin were compared on SDS electrophoresis (Figure 3C). *V. russelli* venom showed a main degradation product of 36 kdaltons. Factor X consists of two polypeptide chains with molecular weights of 25 000 and 50 000, respectively. Under reduced conditions, the 50K and 25K bands of heavy and light chains from factor X were visible (Figure 3). The band of 70K is apparently due to a contaminant present in factor X. As factor X was incubated with cerastobin, the 50K chain was hydrolyzed and the degradation product of 36 kdaltons was produced. However, cerastobin showed the main degradation product to be 26 kdaltons. Therefore, cerastobin degrades factor X differently from *V. russelli* venom, which is known for the activation of factor X.

Cerastobin partially neutralized antithrombin, which was degraded by the enzyme (Figure 4A, lane 3). The degradation product was about 59 000 daltons and had less activity toward thrombin, as detected by the turbidimetric method (Figure 4B). When antithrombin was incubated with cerastobin for 10 min, the amount of antithrombin degradation was not large. This means that more thrombin could be neutralized by the antithrombin and less fibrinogen converted to fibrin (Figure 4B, part a). But when more antithrombin was degraded by

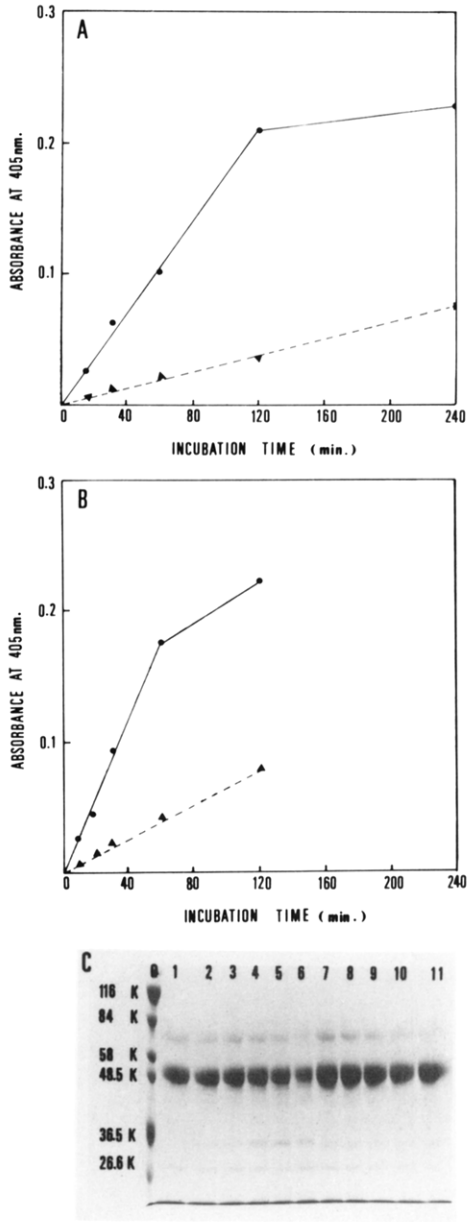

FIGURE 3: Effect of cerastobin on blood coagulation factor X as measured with chromogenic substrate S-2222 (A) in the absence of phospholipids and (B) in the presence of phospholipids. (▲) Cerastobin; (●) *V. russelli* venoms as the positive control. (C) Peptides released from factor X by incubating with cerastobin and *V. russelli* venom. Lane 0, standard molecular weight proteins; lane 1, human factor X; lanes 2–6, factor X incubated with *V. russelli* venom for 10, 30, 60, 120, and 240 min, respectively; lanes 7–11, factor X incubated with cerastobin for 10, 30, 60, 120, and 240 min, respectively.

cerastobin, the remaining antithrombin should neutralize less thrombin. Hence thrombin should convert fibrinogen to fibrin faster. This was exactly the case, as shown in Figure 4B, part b.

The effect of cerastobin on nonpolymerized fibrin (fibrin monomer) is shown in Figure 5. Since fibrin was made without factor XIII, fibrin is monomeric without γ–γ dimer formation. Column 1 is reduced fibrin showing the α, β, and γ chains. As fibrin monomer was incubated with plasmin, α, β, and γ were hydrolyzed and degradation products at 40 000 daltons appeared (Figure 5, lanes 2–4). When cerastobin was incubated with fibrin monomer, α and β chains were preferentially hydrolyzed (Figure 5, lanes 5–10). The γ chain was fairly resistant, but after prolonged incubation (48 h), it started to show some hydrolysis (Figure 5, lane 10). The incubation

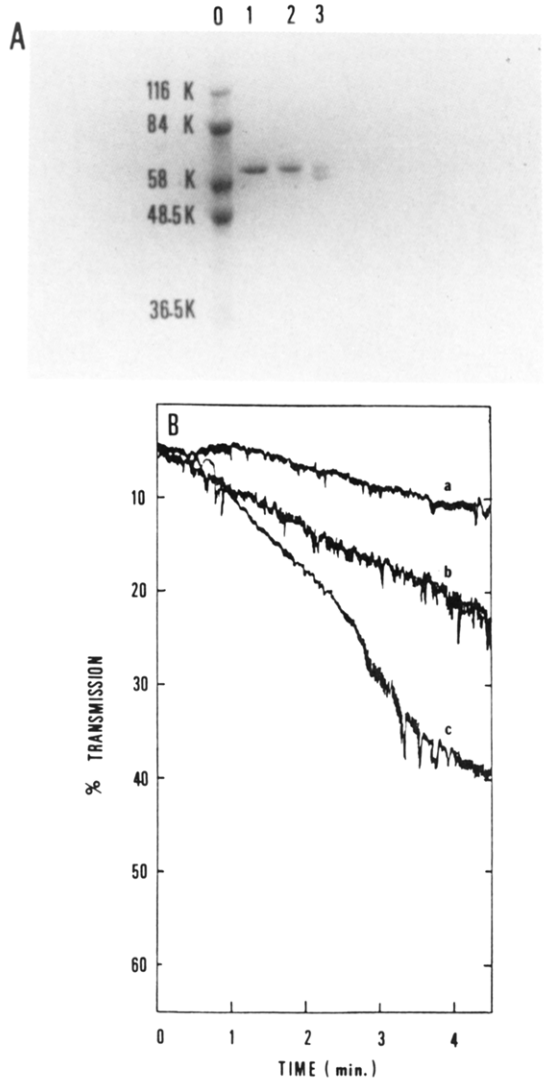

FIGURE 4: Effect of cerastobin on antithrombin III determined by (A) electrophoresis and (B) a turbidimetric method. (A) The samples in lanes 1, 2, and 3 are as follows: lane 1, antithrombin (1 unit) (control); lane 2, antithrombin (1 unit) + 10 μg of cerastobin incubated at 37 °C for 10 min; lane 3, antithrombin (1 unit) + 10 μg of cerastobin incubated at 37 °C for 120 min. (B) First, 1 unit of antithrombin III was incubated with 10 μg of cerastobin. Then, at the indicated times, the mixture was mixed with 1 unit of thrombin, and the conversion of fibrinogen to fibrin was measured by the turbidimetric method. (a) Antithrombin III preincubated with cerastobin for 10 min. (b) Antithrombin preincubated with cerastobin for 120 min. (c) Control: 1 unit of thrombin and 10 μg of cerastobin without antithrombin. N.B. Decreasing transmission indicates increasing amount of clot.

mixture after 48 h showed three degradation products (Figure 5, lane 10, arrows a–c). The degradation products shown by arrows a and b in Figure 5 may be similar to those produced by plasmin; the 26 000-dalton product shown by arrow c was quite distinct and was not shown in the degradation products produced by plasmin. This indicates that cerastobin is different from plasmin, although synthetic chromogenic substrates CBS 33.08 and CBS 30.41 (Table II) were hydrolyzed by both plasmin and cerastobin.

Platelet Aggregation. The platelet is the initiation site of blood coagulation by an intrinsic mechanism, and it is known that snake venoms have a pronounced effect on platelet aggregation (Brinkhous et al., 1981; Longenecker & Longenecker, 1984; Teng et al., 1984). Crude venom (60 μg) of *C. vipera* itself caused platelet aggregation, as can be seen in Figure 6A. When 6 μg (Figure 6B) and 30 μg (Figure 6C)

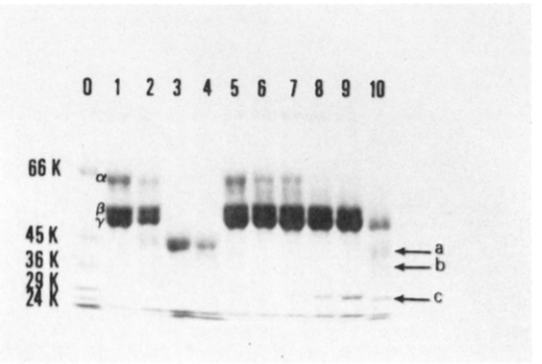

FIGURE 5: Hydrolysis of monomeric fibrin by (1) thrombin, (2–4) plasmin, and (5–10) cerastobin. Incubation times: (1) 15 min; (2) 30 min; (3) 60 min; (4) 24 h; (5) 30 min; (6) 60 min; (7) 3 h; (8) 12 h; (9) 24 h; (10) 48 h. Standard molecular weight pattern is shown in lane 0. Fragments a–c are clearly shown.

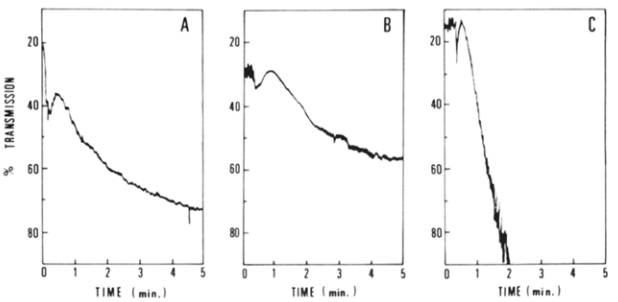

FIGURE 6: Platelet aggregation by (A) 60 μg of crude venom, (B) 6 μg of cerastobin, and (C) 30 μg of cerastobin. Amount used was 250 000 platelet particles/mm^3, and the aggregation was followed turbidimetrically by using a platelet aggregometer, Model Chronolog. Decreasing transmission corresponds to increasing aggregation.

of cerastobin were added, platelet aggregation was also observed. The aggregation was more pronounced with the higher amount of cerastobin. The degree of aggregation was 50, 53.7, and 100% for 60 μg of crude venom, 6 μg of cerastobin, and 30 μg of cerastobin, respectively. From this experiment, it is clear that cerastobin has a potent platelet aggregatory effect.

Kallikrein-like Activity. Kallikrein is known to act on bradykininogen to produce a hypotensive agent, bradykinin. Two independent methods, a biochemical and a physiological one, indicated that cerastobin had kallikrein-like activity. Chromogenic substrates for kallikrein CBS 33.27 and S-2302 (Table II) were hydrolyzed by cerastobin. Kallikrein activity is also frequently detected by contraction of isolated rat gravid uterus due to released bradykinin. Cerastobin also caused an increase in uterine contraction. After the uterus preparation was washed by Tyrode solution, atropine was added to block the nerve ending. Addition of cerastobin after the sympathetic nerve ending blocking caused further contraction of the uterus. This indicated that the increase in contraction was a direct effect of bradykinin produced by cerastobin and not due to the nerve transmitter acetylcholine.

Discussion

A number of thrombin-like enzymes were isolated from snake venoms. These enzymes behave like thrombin by producing fibrin from fibrinogen. However, some important differences were observed. Notably, crotalase, a thrombin-like enzyme from *C. adamanteus* venom, was found to be similar to kallikrein. This property is distinctly different from thrombin. Therefore, it is of interest to compare cerastobin with thrombin, kallikrein, and other snake thrombin-like enzymes.

Comparison to Thrombin. Cerastobin was found to have many properties in common with those of thrombin. Like thrombin, cerastobin was found to be a serine protease acting on both αA and βB chains of fibrinogen. They both hydrolyzed BAEE and TAME and caused the aggregation of platelets. But differences were also found. The N-terminal amino acid of thrombin is isoleucine, but for cerastobin it is valine. Thrombin is a glycoprotein, but cerastobin is not.

Comparison to Kallikrein. There are some reports showing that snake venom thrombin-like enzymes have some similarity to kallikrein. Indeed, this was also the case for cerastobin. Cerastobin hydrolyzed chromogenic substrates for kallikrein. Both cerastobin and kallikrein released bradykinin and caused uterine contraction. Moreover, the N-terminal amino acid of both cerastobin and kallikrein is valine. They are both serine proteases and can be inhibited by DFP. However, there is a big difference in molecular weight; cerastobin is 38 000, whereas kallikrein is 99 800.

Comparison of amino acid sequences of cerastobin and kallikrein shows 11 out of 37 residues are identical. On the other hand, some residues are also similar to that of thrombin (Table IV).

Comparison to Other Snake Venom Thrombin-like Enzymes. Several thrombin-like enzymes were isolated from the venoms of *Agkistrodon rhodostroma* (Hatton, 1973; Nolan et al., 1976), *C. adamanteus* (Markland & Damus, 1971; Markland, 1976), *Bothrops atrox* (Holleman & Weiss, 1976; Stocker & Barlow, 1976), and *Bitis gabonica* (Niljoen et al., 1979; Pirkle et al., 1986). Although the molecular weights are roughly similar (31 400–40 000), there are many differences in other properties such as isoelectric point and enzyme activities on various blood clotting factors.

There are remarkable similarities in amino acid sequences between cerastobin and other thrombin-like enzymes (Table IV). The amino terminal is valine for all thrombin-like enzymes including cerastobin. The homology within snake thrombin-like enzyme is much higher compared to those of kallikrein and thrombin. High homology is reasonable because all these enzymes were isolated from snake venoms. Moreover, they all convert fibrinogen to fibrin.

Effect of Cerastobin on Blood Coagulation. Snake venoms have been shown to affect many blood coagulation factors. Cerastobin's main action is the conversion of fibrinogen to fibrin and the degradation of factor X. It is worthwhile to review other snake venom enzymes that affect factor X. Factor X activating enzyme was isolated from the venom of *V. russelli* (Di Scipio et al., 1977) and *B. atrox* (Hofmann & Bon, 1987). The molecular weight of the enzyme from the first venom was 59 000, while the enzyme from *B. atrox* venom consists of two polypeptide units with molecular weights of 59 000 and 14 000. Cerastobin shows a molecular weight of only 38 000, so it is different from the factor X activating enzyme isolated from either *V. russelli* or *B. atrox* venoms. Cerastobin's main action on factor X seems to be its degradation.

Registry No. TAME, 901-47-3; BAME, 967-88-4; BAEE, 971-21-1; cerastobin, 37259-58-8; factor X, 9001-29-0; antithrombin, 9000-94-6.

References

Bajwa, S., Russell, F. E., & Markland, F. S. (1982) *Proc. West. Pharmacol. Soc. 25*, 353.

Biggs, R., & MacFarlene, R. G. (1962) *Human Blood Coagulation and Its Disorders*, 3rd ed., Blackwell Scientific Publications, Oxford.

Born, G. V. P., & Cross, M. J. (1963) *J. Physiol. (London) 168*, 178.

Brinkhous, K. M., Read, M. S., Reddick, P. L., & Griggs, T. R. (1981) *Ann. N.Y. Acad. Sci. 370*, 191.

Butkowski, R. J., Elion, J., Downing, M. R., & Mann, K. G. (1977) *J. Biol. Chem. 252*, 4942.

Di Scipio, R. G., Hermadson, M. A., & Davie, E. W. (1977) *Biochemistry 16*, 5053.

Dubois, M., Gillies, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350.

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

Ehrly, A. M. (1972) 18th International Congress of Angiology, Rio de Janeiro.

Elzinga, M. (1970) *Biochemistry 9*, 1365.

Erlanger, B. F., Kokowsky, N., & Cohen, W. (1961) *Arch. Biochem. Biophys. 95*, 271.

Exner, T., Richard, K. A., & Kronenberg, H. (1983) *Thromb. Res. 31*, 489.

Fujikawa, K., Legaz, M. E., Kato, H., & Davie, E. W. (1974) *Biochemistry 13*, 4508.

Gaffney, P. J. (1977) *Thrombos. Haemostasis 38*, 562.

Guichaoua, J. F., & Martinoli, J. L. (1982) *Ann. Biol. Clin. (Paris) 40*, 471.

Hatton, M. W. C. (1973) *Biochem. J. 131*, 799.

Heinrickson, C. L., & Meredith, S. C. (1984) *Anal. Biochem. 136*, 65.

Hessel, B., & Blomback, M. (1971) *FEBS Lett. 18*, 318.

Hofmann, H., & Bon, C. (1987) *Biochemistry 26*, 780.

Hollemon, W. H., & Weiss, L. J. (1976) *J. Biol. Chem. 251*, 1663.

Itoh, N., Tanaka, N., Mihashi, S., & Yamashina, I. (1987) *J. Biol. Chem. 262*, 3132.

Kitchin, I. (1984) in *Textbook of in Vitro Practical Pharmacology*, pp 33–39, Blackwell Scientific Publications, Oxford.

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

Longenecker, G. L., & Longenecker, H. E. (1984) *J. Toxicol., Toxin Rev. 3*, 223.

Markland, F. S. (1976) *Methods Enzymol. 45*, 223.

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

Mosesson, M. W., Alkjaersing, N., Sweet, B., & Sherry, S. (1967) *Biochemistry 6*, 3279.

Nolan, D., Hall, L. S., & Barlow, G. H. (1976) *Methods Enzymol. 45*, 205.

O'Brien, J. R. (1962) *J. Clin. Pathol. 115*, 452.

Pirkle, H., Markland, F. S., Theodor, I., Baumgartner, R., Bajwa, S. S., & Kirkakossian, H. (1981) *Biochem. Biophys. Res. Commun. 99*, 715.

Pirkle, H., Theodor, I., Miyada, D., & Simmons, G. (1986) *J. Biol. Chem. 261*, 8830.

Robert, P. S. (1958) *J. Biol. Chem. 232*, 285.

Stocker, K., & Barlow, G. H. (1976) *Methods Enzymol. 45*, 214.

Swift, G. H., Dagorn, J. C., Ashley, P. L., Scott, W., Cummings, S. W., & MacDonald, R. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7263.

Teng, C. M., Chen, Y. H., & Ouyang, C. (1984) *Biochim. Biophys. Acta 772*, 393.

Tu, A. T. (1977) in *Venoms: Chemistry and Molecular Biology*, pp 1–21, Wiley, New York.

Viljoen, C. C., Meehan, C. M., & Botes, D. P. (1969) *Toxicon 17*, 145.

Ware, A. G., Guest, M. M., & Seegers, W. H. (1947) *Arch. Biochem. Biophys. 13*, 231.