A Novel Venombin B from *Agkistrodon contortrix contortrix*: Evidence for Recognition Properties in the Surface around the Primary Specificity Pocket Different from Thrombin†

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ABSTRACT: A novel thrombin-like enzyme (named contortrixobin) has been purified to homogeneity from the venom of *Agkistrodon contortrix contortrix* by affinity chromatography on arginine−Sepharose, anionic exchange chromatography, and HPLC. The complete amino acid sequence has been determined by Edman degradation and by mass spectral analysis of peptides generated by enzymatic cleavage. A microheterogeneity at the level of residue 234 has been detected, as demonstrated by peptides differing for the occurrence of Pro234 (∼85%) or Asp234 (∼15%). Contortrixobin (i) has six disulfide bonds whose sequence positions have been determined by mass spectrometry and (ii) does not contain carbohydrates in its structure. As expected, the 234 residue sequence of contortrixobin exhibits strong homology with snake venom serine proteases acting on either fibrinogen or other blood coagulation components. The interaction of contortrixobin with chromogenic substrates indicates a higher specificity for arginine over lysine in the primary subsite and a faster attack to ester than amides. The hydrolytic activity of contortrixobin is strongly inhibited by diisopropyl fluorophosphate and to a less extent by phenylmethylsulfonyl fluoride, benzamidine, and 4′,6-diamidino-2-phenylindole; hirudin (a specific α-thrombin inhibitor) as well as basic pancreatic trypsin inhibitor has a small effect on contortrixobin's catalytic properties. Contortrixobin (i) preferentially releases fibrinopeptide B from human fibrinogen, (ii) activates blood coagulation Factors V and XIII with a rate 250−500-fold lower than human α-thrombin, and (iii) does not induce thrombocyte aggregation, intracytoplasmatic calcium ion increase in platelets, and activation of Factor VIII. Evidence for biorecognition properties different from thrombin is also reported.

Snake venoms, particularly those belonging to the Crotalidae and Viperidae families, are known to contain proteases which strongly affect blood coagulation and the hemostatic system (*1*, *2*). A number of these enzymes are termed TLE,[1] because of their ability to operate just like the most familiar of the many actions of thrombin (*3*), i.e., the capacity to interact directly with fibrinogen molecules, causing them to polymerize into fibrin fibers. However, in some cases, snake thrombin-like proteases express more than one of the thrombin functions or also activate other coagulation factors (*1*, *2*), even though the mechanisms by which they work may differ from those of the mammalian enzymes (*4*).

Previous investigations indicated that most TLEs in snake venom are characterized by release, either selectively or in combination, of FPA and/or FPB (*5*). Aberrant fibrin monomers (i.e., fibrin units lacking only one type of fibrinopeptide instead of both, like those produced by thrombin attack) form clots that in plasma are not cross-linked by Factor XIII (*6*), because many TLEs do not recognize Factor XIII. Consequently, such abnormal fibrin polymers are easily dispersible and more susceptible to plasmin proteolysis (*7*). Therefore, after injecting intravenously, these TLEs can cause a rapid defibrinogenation. In fact, their proteolytic effect on fibrinogen (different from that accomplished by α-thrombin) but not on Factor XIII produces the formation of non-cross-linked fibrin monomers that are rapidly removed from the blood circulation either

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[1] Abbreviations: BPTI, basic pancreatic trypsin inhibitor; DAPA, dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)amide; DAPI, 4′,6-diamidino-2-phenylindole; E-64, L-*trans*-epoxysuccinylleucylamido(4-guanidino)-butane; EDTA, ethylenediaminetetraacetate; ESMS, electrospray mass spectrometry; FPA, fibrinopeptide A; FPB, fibrinopeptide B; HPLC, high-performance liquid chromatography; MALDIMS, matrix-assisted laser desorption ionization mass spectrometry; -ONp, *p*-nitrophenyl ester; Pip, pipecolyl; PMSF, phenylmethanesulfonyl fluoride; -pNA, *p*-nitroanilide; PPACK, D-Phe-Pro-Arg chloromethyl ketone; SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TFA, trifluoroacetic acid; TLCK, *N*-α-*p*-tosyllysine chloromethyl ketone; TLE, thrombin-like enzyme; Z-, carbobenzyloxy-.

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by fibrinolysis or via the reticulo-endothelial system (*8*). Because of these functional properties, many TLEs act in vitro as procoagulants converting fibrinogen to fibrin, while in vivo they cause a benign defibrinogenation (*9, 10*). Accordingly, due to the ability to lower the circulating fibrinogen concentration, some TLEs appear to be an appropriate alternative to heparin in thrombotic diseases (*6*), and may be regarded in some cases as the first-choice anticoagulant (*11*).

In view of the potential clinical application of these fibrinogenases [or venombins, according to a recent proposal (*5*)], we fractionated the venom from the Southern copperhead snake (*Agkistrodon contortrix contortrix*) from which several components acting on blood coagulation have already been isolated and characterized (*12*−*15*), including phospholipase A_2 (*16*) and a TLE that preferentially releases FPB (*17, 18*). In the present paper, we describe the purification and the biochemical characterization of contortrixobin, a novel thrombin-like protease from *Agkistrodon contortrix contortrix* venom with preferential activity upon β-chains of human fibrinogen.

MATERIALS AND METHODS

Materials. Lyophilized crude venom from *Agkistrodon contortrix contortrix*, human prothrombin, some chromogenic substrates (*N*-*p*-tosyl-Gly-L-Pro-L-Lys-*p*-nitroanilide, *N*-*p*-tosyl-Gly-L-Pro-L-Arg-*p*-nitroanilide, D-Phe-L-pipecolyl-L-Arg-*p*-nitroanilide, Z-Arg-*p*-nitroanilide, Z-Lys-*p*-nitrophenyl ester, and Z-Ala-*p*-nitrophenyl ester), bovine β-trypsin, aprotinin (or BPTI), hirudin, *N*-α-*p*-tosyllysine chloromethyl ketone, benzamidine, HEPES, and MOPS were purchased from Sigma Italia (Milano, Italy). Human thrombin, human coagulation Factor V, human coagulation Factor XIII, and some chromogenic substrates (H-Sar-L-Pro-L-Arg-*p*-nitroanilide and benzoyl-D-Phe-L-Val-L-Arg-*p*-nitroanilide) were obtained from Calbiochem-Novabiochem Co. (Inalco, Milano, Italy). Bovine coagulation Factor Xa, Factor V deficient plasma, Factor VIII deficient plasma, synthetic phospholipid micelles, recombinant tissue factor (RecombiPlastin), synthetic phospholipids, and colloidal silicon (SynthAsil) were from Ortho Clinical Diagnostics (Milano, Italy). Recombinant Factor VIII (Recombinate) was supplied by Baxter Spa (Roma, Italy). Dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)-amide was purchased from Hematological Technologies Inc. (Essex Junction, VT). D-Phe-Pro-Arg chloromethyl ketone was purchased from Bachem (Bubendorf, Switzerland). 4-(2-Aminoethyl)benzene sulfonylfluoride (Pefabloc) was obtained from Pentapharm AG (Basel, Switzerland). Endoproteinase Asp-N, pepsin, chymotrypsin (all sequencing grade), and phenylmethanesulfonyl fluoride were from Roche Molecular Biochemicals (Roche Diagnostics, Monza, MI, Italy). Trypsin (code TRTCPK) was obtained from Worthington Biochemical Co. (D. B. A. Italia, Segrate, MI, Italy). 4′,6-Diamidino-2-phenylindole was purchased from Fluka (Sigma Aldrich Italia, Milano, Italy). Iodo[2-^{14}C]acetate was from Radiochemical Center (Amersham Italia, Milano, Italy). Endoprotease GluC was from Cooper Biomedicals (Freehold, NJ). Guanidinium chloride (recrystallized from methanol) was supplied from Merck (Bracco, Milano, Italy). The liquid chromatography solvents, HPLC grade, were from Carlo Erba Reagenti (Milano, Italy); sequencing-grade chemicals were from Perkin-Elmer−Applied Biosystems (Warrington, U.K.). Eglin *c* was a kind gift of Ciba-Geigy AG (Basel, Switzerland). All other reagents were of analytical grade. Z-L-Arg-*p*-nitrophenyl ester was synthesized according to a previous procedure (*19*).

Purification of Contortrixobin. Contortrixobin was purified from lyophilized crude venom by a three-step procedure. Venom was fractionated by affinity chromatography on an arginine−Sepharose 4B (Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy) column (1.5 cm × 12 cm) in 50 mM Tris-HCl, 30 mM NaCl, pH 8.0, competitively eluting with 0.15 M guanidine hydrochloride in the same buffer. The eluate fractions containing the clotting activity (measured in terms of visible clot formation in a mixture consisting of 400 μL of human thrombin-free plasma and 100 μL of tested fraction) were dialyzed against 20 mM Tris-HCl, pH 8.0, concentrated by ultrafiltration, and then applied to a DEAE-Sepharose fast flow (Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy) column (2 cm × 25 cm) equilibrated with 20 mM Tris-HCl, pH 8.0, and eluted with a linear concentration gradient of NaCl (0−0.5 M). The clotting activity was observed only in the fractions eluted with 0.16 M NaCl. The fractions were pooled and dialyzed against deionized water, and a portion of the material was further purified by HPLC on a Resource Q (Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy) column equilibrated with 20 mM Bis-tris (pH 6.5). The bound enzyme was eluted with a NaCl linear gradient (0−0.5 M) at a flow rate of 2 mL/min. The elution profile was monitored at 280 nm. The fractions of the major protein component were pooled, dialyzed against distilled water, and then concentrated by ultrafiltration. Clotting activity was present only in the major peak, which showed a single band on SDS−15% PAGE (*20*); 1.2−2 mg of pure contortrixobin was isolated from 1 g of crude venom.

Protein Alkylation and Enzymatic Hydrolysis. The purified protein (6 mg) was carboxymethylated with iodo[2-^{14}C]-acetate as described previously (*21*). Aliquots (2 mg) of the carboxymethylated contortrixobin were suspended in 0.5 mL of 0.1 M ammonium bicarbonate, pH 7.5, and incubated at 37 °C after addition of 50 μg of trypsin for 3 h or 2 μg of Asp-N endoproteinase for 18 h. The last sample of protein (2 mg) was dissolved in 0.5 mL of 5% (v/v) formic acid, and incubated with pepsin (40 μg) at 25 °C for 5 min.

Peptide Purification. The enzymatic digests were purified by using a Beckman Gold chromatography system on a macroporous reverse-phase column (Aquapore RP-300, 4.6 mm × 250 mm, 7 μm, Brownlee Labs) eluted with a linear gradient of 0−35% acetonitrile in 0.2% (v/v) trifluoroacetic acid, at a flow rate of 1 mL/min. Elution of the peptides was monitored using a diode array detector (Beckman model 168) at 220 and 280 nm. Further purification of impure fractions was achieved by reverse-phase HPLC on a C18 column (4.6 mm × 150 mm, 3 μm; Supelco) eluted with the same solvent system as above.

Sequence Analysis. The amino acid sequence of peptide samples was determined by automated Edman degradation using Applied Biosystem model 475A, 476A, or 477A sequencers. Samples (0.2−1 nmol) were loaded onto poly(vinylidene difluoride) membranes (ProBlot, Applied Biosystem), coated with 2 μL of Polybrene (100 mg/mL, 50% methanol), and run with a Blott-cartridge using an optimized gas-phase fast program. N-Terminal sequence analysis of the

protein was performed on samples electrotransferred on ProBlot membranes after SDS−PAGE (*22*) using a liquid-phase fast program.

Amino Acid Analysis. The amino acid composition of the carboxymethylated protein (0.1 mg) was determined after hydrolysis with 6 M HCl, containing 0.1% phenol, at 110 °C for 24, 48, and 72 h. Amino acid analyses were performed using a Pharmacia 4151 Alpha Plus instrument.

Mass Spectrometry Analysis. Intact proteins were submitted to ESMS analysis, using a BIO-Q triple quadrupole mass spectrometer (Micromass, Manchester, U.K.). Samples were dissolved in 1% (v/v) acetic acid, and 2−10 μL was injected into the mass spectrometer at a flow rate of 10 μL/min. The quadrupole was scanned from *m*/*z* 500 to 1800 at 10 s/scan, and the spectra were acquired and elaborated using the MASSLYNX software. Calibration was performed by the multiply charged ions from a separate injection of myoglobin (molecular mass 16 951.5 Da). All mass values are reported as average masses.

Matrix-assisted laser desorption ionization (MALDI) mass spectra were recorded using a Voyager DE MALDI-TOF mass spectrometer (Perkin-Elmer−Perseptive Biosystem); a mixture of analyte solution, α-cyano-4-hydroxycinnamic acid, and bovine insulin was applied to the sample plate and dried. Mass calibration was performed using the molecular ions from bovine insulin at 5734.54 Da and the matrix at 379.06 Da as internal standards. Raw data were analyzed by using computer software provided by the manufacturer and are reported as average masses.

Peptide Nomenclature. Peptide were numbered retrospectively according to their location in the sequence, starting from the N-terminus. Tryptic peptides were designated with T, Asp-N endoproteinase peptides with D, peptic peptides with P.

Determination of Disulfide Bonds. Assay for free thiol groups, measured by a standard method (*23*), was negative in contortrixobin. Native contortrixobin (0.15 mg) was suspended in 0.2 mL of 0.1 M ammonium bicarbonate, pH 6.5, and incubated at 37 °C for 18 h after addition of chymotrypsin or trypsin (E:S ratio = 1:50). The digests were lyophilized and analyzed by MALDIMS. Assignments of the recorded mass values to disulfide-containing peptides were performed on the basis of their molecular mass as previously described and confirmed following a step of manual Edman degradation (*24*).

Structure Comparison. A search of the Swiss-Prot database was performed, and pairwise and multiple sequence alignments were carried out with the programs FASTA, BESTFIT, and PILEUP, respectively, from the Genetic Computer Group sequence analysis software package (GCG, version 8), using a Vax/VMS system (*25*).

Hydrolytic Properties As Measured with Synthetic Substrates. The catalytic behavior of contortrixobin, in comparison with that of human α-thrombin, was investigated by monitoring the steady-state hydrolysis of synthetic single amino acid amides or esters (*26*) and tripeptide amides (*27*), measuring the increase in absorbance at 405 nm due to release of *p*-nitroaniline or *p*-nitrophenol by using a Jasco V 500 spectrophotometer. Assays were performed in polystyrene cuvettes at 20 °C. Molar concentrations of the enzymes were measured by active-site titration with 4-methylumbelliferyl *p*-guanidinobenzoate (*28*). Amidolytic and esterolytic activities were determined in 50 mM phosphate buffer, 0.1 M NaCl, pH 7.4 (or pH 6.8), at 20 °C [0.1% poly(ethylene glycol) 8000 (w/v) was added when thrombin was used]. The concentration of released *p*-nitroaniline was measured using the appropriate extinction coefficient [ϵ (M, 1 cm) = 9920] at 405 nm. The contortrixobin-catalyzed hydrolysis of esters was monitored at 405 nm at pH 7.4 [ϵ (M, 1 cm) = 8900] and at 360 nm at pH 6.8 [ϵ (M, 1 cm) = 4500]. The data were fitted to the Michaelis−Menten equation by a nonlinear regression analysis program (GraFit, Erithacus software, London, U.K.).

Determination of the Equilibrium Dissociation Constant for Synthetic Inhibitors. Values of the inhibition constants (K_i) for benzamidine and DAPI binding to contortrixobin, human α-thrombin, and bovine β-trypsin were determined in 50 mM phosphate buffer, 0.1 M NaCl, pH 7.4, at 20 °C [0.1% poly(ethylene glycol) 8000 (w/v) was added when thrombin was used], by the inhibitory effect on the proteinases-catalyzed hydrolysis of Z-ArgONp. The *p*-nitrophenol release was monitored at 405 nm as reported above. The K_i values were obtained with a graphical method (*29*), and an average error value of ±7% was evaluated. Contortrixobin was also incubated with 1200- and 600-fold molar excesses of TLCK, PPACK, PMSF, and 4-(2-aminoethyl)benzene sulfonylfluoride in 50 mM phosphate buffer, 0.1 M NaCl, pH 7.4, at 20 °C. At appropriate intervals (5 and 30 min), aliquots were withdrawn, and their hydrolytic activities were assayed with Z-Arg-ONp as substrate.

Determination of the Equilibrium Dissociation Constant for Protein Inhibitors. Values of the equilibrium constant for BPTI binding to contortrixobin and human α-thrombin were determined from the evaluation of the inhibitory effect of BTPI on the enzymatic hydrolysis of tosyl-Gly-L-Pro-L-Arg-pNA by titration of the proteinase with the inhibitor and measuring the residual activity (*30*). Hirudin binding to contortrixobin and human α-thrombin was determined from the effect of the inhibitor (at one inhibitor concentration, 1.0×10^{-6} M) on the apparent steady-state velocity of the enzyme hydrolysis of tosyl-Gly-L-Pro-L-Arg-pNA (*31*). Data analysis was carried out on a computer employing an interactive nonlinear least-squares curve-fitting procedure according to the Marquart algorithm. The standard deviation on the fitted parameters was obtained by a systematic search of the parameter space, allowing only one parameter to vary for any single search.

Analysis of Fibrinopeptides Released by Contortrixobin. Commercial human fibrinogen of high purity and activity was purchased from Calbiochem−Novabiochem Co. (Inalco, Milano, Italy) and further purified and tested for activity as previously described (*32*). The purity of the fibrinogen preparation was tested by HPLC and SDS−PAGE (10−25% gradient gels run under both reducing and nonreducing conditions) and found to greater than 99%. The fibrinogen concentrations [ϵ (1 mg/mL, 1 cm) = 1.506 at 280 nm and molar mass of 340 kDa] used in the kinetics experiments were in the range of 2−100 μM. The contortrixobin concentration, determined spectrophotometrically, was 0.01 NIH unit/mL ($\sim 10^{-10}$ M). After addition of contortrixobin, hydrolytic attack was left to proceed for various time intervals, and each reaction solution was then quenched by addition of 1 M HCl [to 10% (v/v) final concentration]. Zero-time points were obtained by adding HCl before addition of

the enzyme. The maximum release of fibrinopeptides was determined by adding to a separate sample of fibrinogen α-thrombin at high concentration (40 NIH units/mL) and allowing the reaction to proceed for 24 h (a period much longer than the time intervals at which the rates of hydrolysis were measured). In the kinetic experiments, the rates were measured over a time period of 1−1.5 h. No clots were observed in the kinetic runs, although intermediate polymers were expected to have formed. On the other hand, in the experiments to obtain the infinite-time points, clots were present. Immediately after the reaction was stopped by addition of HCl, all protein material was precipitated by addition of TFA [1% (v/v) final concentration]. These samples were then centrifuged at 13 000 rpm for 10 min at 4 °C. After centrifugation, the supernatant, containing the fibrinopeptide(s), was analyzed by using a reverse-phase C18 column (4.6 mm × 250 mm Vydac, Hesperia, CA). Linear gradient elution (0−95%) of acetonitrile/2-propanol 4:1 (v/v) in 0.1% TFA was performed for 45 min at a flow rate of 0.8 mL/min, the absorbance at 220 nm being monitored. Eluates corresponding to the peptide peaks were lyophilized; the powder so obtained was first hydrolyzed with 6 M HCl and then analyzed for amino acid composition. The areas under the peaks in the chromatograms of the kinetic samples corresponding to FPA and FPB were determined and converted to concentration of fibrinopeptide by using calibration curves. The initial rates of release of fibrinopeptides A and B at each concentration of fibrinogen were then determined from the slope of the least-squares line through a plot of (moles of peptide released per liter) vs time. Only those points for which the concentration of substrate hydrolyzed was less than 25% of the initial substrate concentration were used in the determination of initial velocities. Appropriate control experiments were performed to ensure that there were no losses of fibrinopeptides during the entire procedure.

Factor V Activation. The commercial preparation of Factor V was gel-filtered through a DG10 column (Bio-Rad Laboratories, Roma, Italy), equilibrated in 10 mM HEPES, 0.15 M NaCl, 0.1% PEG 6000, pH 7.50. Its concentration was determined spectrophotometrically, at 280 nm by using ϵ(1% w/v, 1 cm) = 9.6 and an equivalent mass of 300 kDa (*33*). The effect of contortrixobin on Factor V activation was determined by prothrombinase assays (*34*). The results were expressed as moles of thrombin produced per second per mole of Factor Va. The kinetic analysis of such data showed that the process of Factor Va production followed a single-exponential reaction. Thus, the experimental data were fitted to the following relation:

$$\mathrm{Va}(t) = \mathrm{Va}(\mathrm{max})[1 - \exp(-k_{\mathrm{obs}}t)]$$

where Va(t) and Va(max) are the Factor Va produced at time t and the maximum Factor Va production, respectively. An apparent $k_{\mathrm{cat}}/K_{\mathrm{m}}$ was calculated by dividing the k_{obs} value by the enzyme concentration (contortrixobin or thrombin).

Clotting Factor Va and Factor VIIIa Activities. Factor Va and Factor VIIIa activities were measured as previously detailed (*35*). The activity of 3.5 μM contortrixobin was assayed and compared to that of 10 nM α-thrombin.

Factor XIII Activation. The specific activity of human coagulation Factor XIII, as certified by the manufacturer, was equal to 40 units/mg, and its molar mass was equal to 340 kDa. The preparation was gel-filtered by desalting DG10 columns (Bio-Rad Laboratories, Roma, Italy) equilibrated in 10 mM HEPES, 0.15 M NaCl, 0.1% PEG 6000, pH 7.50. The concentration of *ab* protomeric units of Factor XIII was determined spectrophotometrically, at 280 nm, by using ϵ (1%, 1 cm) = 13.8 and an equivalent mass of 170 kDa (*36*). The rates of activation peptide release by human α-thrombin and contortrixobin were carried out at 25 °C, under experimental conditions where the concentration of Factor XIII was less than K_{m} values previously reported for its hydrolysis by α-thrombin (ranging from 80 to 100 μM) (*37*). The detection of activation peptide was determined at 214 nm, while the concentration of the eluted peptides was measured from the peak area calibrated against a reference activation peptide concentration curve, obtained by exaustive hydrolysis of 100 nM Factor XIII by 50 nM α-thrombin at 37 °C for 60 min.

Assay for Aggregation and Intracytoplasmic Ca^{2+} Increase in Stimulated Gel-Filtered Platelets. Platelet-rich plasma, obtained from healthy donors, was gel-filtered on Sepharose 2B columns and eluted in 20 mM HEPES, 135 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.2% serum bovine albumin (w/v), pH 7.4. The platelet count was adjusted to 2×10^5/μL. Gel-filtered platelets were stimulated by α-thrombin used over a concentration ranging from 0.15 to 50 nM and by contortrixobin from 50 nM to 6.4 μM at 37 °C. Aggregometric responses were evaluated by measuring the initial slope of the aggregometric curve and expressed as % (AU)/min.

Measurements of intraplatelet Ca^{2+} concentration were performed by using the fluorescent dye for Ca^{2+}, fura-2-acetoxymethyl ester, as previously detailed (*38*). Fura-2-acetoxymethyl ester-loaded platelets were stimulated with human α-thrombin concentrations ranging from 0.15 to 40 nM and contortrixobin concentrations ranging from 50 nM to 6 μM.

RESULTS

Purification and Amino Acid Sequence Determination. Contortrixobin was purified from the venom of *Agkistrodon contortrix contortrix* on arginine−Sepharose and DEAE-Sepharose columns (Figure 1), and isolated as a single peak by HPLC, affording a unique species (for details, see Materials and Methods). From the mobility of contortrixobin relative to that of reference proteins on SDS−PAGE (Figure 2), its molar mass was estimated to be 26 000 g/mol. The protein band on SDS−PAGE was not stained with Schiff's periodate reagent, suggesting that no carbohydrate is bound to contortrixobin. On analytical isoelectric focusing, the purified protein showed two bands (ratio ∼6:1), the main one with p*I* equal to 5.54 and the minor one with p*I* equal to 5.40 (data not shown). The contortrixobin extinction coefficient [ϵ (1%, 1 cm)] was 13.4 at 280 nm.

The ESMS analysis of native contortrixobin (Figure 3) demonstrated the presence of two major components, suggesting the occurrence of isoform phenomena (see below); this sample was used for exhaustive molecular characterization.

The complete primary structure of contortrixobin is reported in Figure 4. The sequence was deduced following

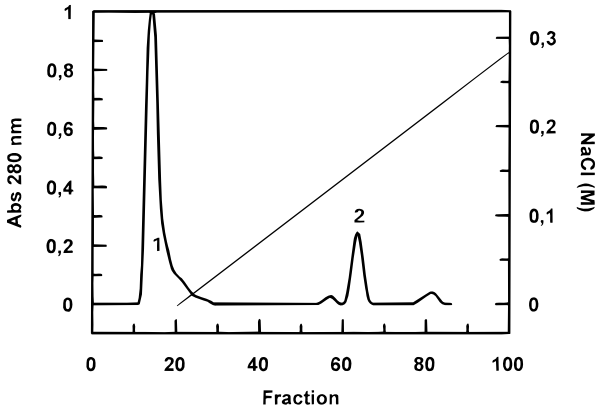

FIGURE 1: Chromatographic pattern of elution from the DEAE-Sepharose column, i.e., the second step of contortrixobin purification from *Agkistrodon contortrix contortrix* venom. Clotting activity was observed both in peak 1 (clot formation in the range of hours) and in peak 2 (clot formation within minutes). Fractions 61−66 (corresponding to peak 2) were used for further purification of contortrixobin by HPLC.

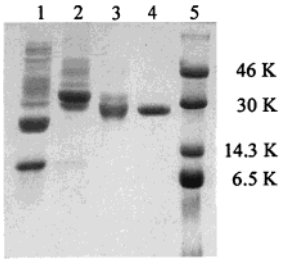

FIGURE 2: SDS−PAGE of proteins present in southern copperhead venom at various stages of purification. Lane 1, crude venom; lane 2, fraction from arginine−Sepharose 4B affinity column; lane 3, fraction from DEAE-Sepharose column; lane 4, fraction from HPLC; lane 5, protein standards (Amersham) with molecular masses expressed in kDa (46 = ovalbumin; 30 = carbonic anhydrase; 21.5 = trypsin inhibitor; 14.3 = lysozyme; 6.5 = aprotinin).

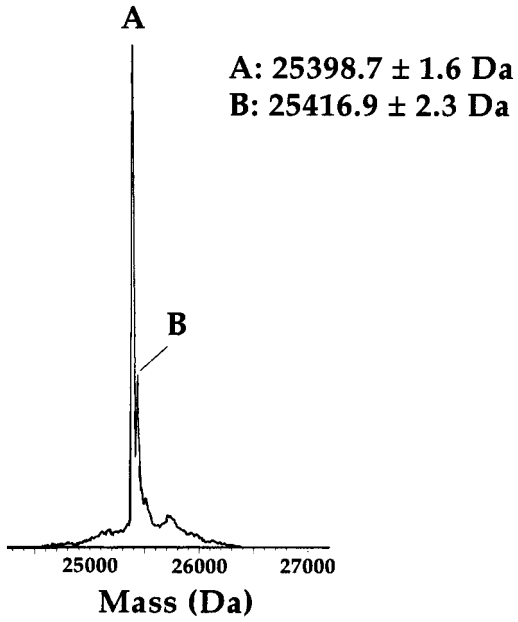

FIGURE 3: Transformed ESMS spectrum of HPLC-purified contortrixobin. For details, see text.

the isolation and identification of an almost complete set of peptic peptides, which were ordered with the help of overlapping peptides produced by β-trypsin and Asp-N endoproteinase. The peptic peptides as well as the Asp-N endoproteinase and tryptic peptides, used to reconstruct the contortrixobin sequence, are shown in the figure. N-terminal analysis of the protein by automated Edman degradation was also used. A microheterogeneity was detected at the level of residue 234. In fact, peptides differing in the occurrence of Pro234 or Asp234 were identified. The reported sequence of contortrixobin was unambiguous, (i) being based on replicate or overlapping sequence analysis and (ii) being consistent with the molecular mass, measured by MALDIMS, and/or the composition of the peptides derived from corresponding regions. Therefore, the theoretical mass value of the enzyme isoforms (25 400.7 and 25 418.8 Da, respectively), calculated on the basis of the amino acid sequence reported and assuming all cysteine residues involved in disulfide bridges, was in perfect agreement with that determined by ESMS (Figure 3). Furthermore, mass spectrometric analysis ruled out the presence of additional posttranslational glycosylation phenomena observed in most of the other TLEs from snake venoms, confirming what was observed by periodate staining.

Determination of Disulfide Bonds. Disulfide bridges occurring within contortrixobin were identified by extensive use of enzymatic hydrolysis combined with mass spectrometric analysis of the unfractionated peptide mixtures. Native contortrixobin was digested with chymotrypsin, and the resulting peptide mixture was directly analyzed by MALDIMS, producing the spectrum shown in Figure 5A. A series of signals was assigned to S−S-bridged peptides on the basis of their unique mass values before and after a single step of manual Edman degradation and their disappearance following incubation with dithiothreitol (data not shown). The signal at *m*/*z* 3046.8 was interpreted as arising from the peptide (142−169) whose mass value was 2 Da lower than the expected one, thus suggesting the occurrence of an intramolecular disulfide bond between Cys150 and Cys165. This signal was accompanied by a satellite peak 18 Da higher associated with the same peptide hydrolyzed at Tyr161. Moreover, the signals at *m*/*z* 1784.1 and 1965.3 were assigned to the peptide pairs (146−154)+(162−169) and (146−158)+(162−169), respectively, thus confirming the occurrence of the S−S bridge Cys150−Cys165. A series of related signals detected at *m*/*z* 1858.2, 2376.4, and 4332.0 were assigned to the fragments (25−30)+(36−47), (20−30)+(36−47), and (20−59), respectively, all joined by a disulfide bond involving Cys26 and Cys42. Similarly, the signals at *m*/*z* 2033.5 and 2559.9, attributed to the peptide pairs (106−119)+(185−190) and (106−119)+(179−190), led to the assignment of the disulfide Cys118−Cys186. In addition, the MALDI spectrum showed the occurrence of two peaks at *m*/*z* 2411.6 and 4229.2 corresponding to the peptide fragments (170−178)+(197−210) and (170−178)+-(191−219) linked by an S−S bridge between Cys176 and Cys201. Finally, a series of signals at *m*/*z* 2850.6, 3230.3, 3531.9, and 3646.7 were associated with the peptide (124−141) linked to shortened forms of the N-terminal peptides (1−9), (1−12), (1−14), and (1−15), respectively, by the disulfide Cys7−Cys139. The assignment of the remaining cysteine pairings in the enzyme was obtained by an independent digestion of the native protein with trypsin. Cys74 and Cys232 were involved in a peptide cluster presenting high molecular mass (data not shown), and

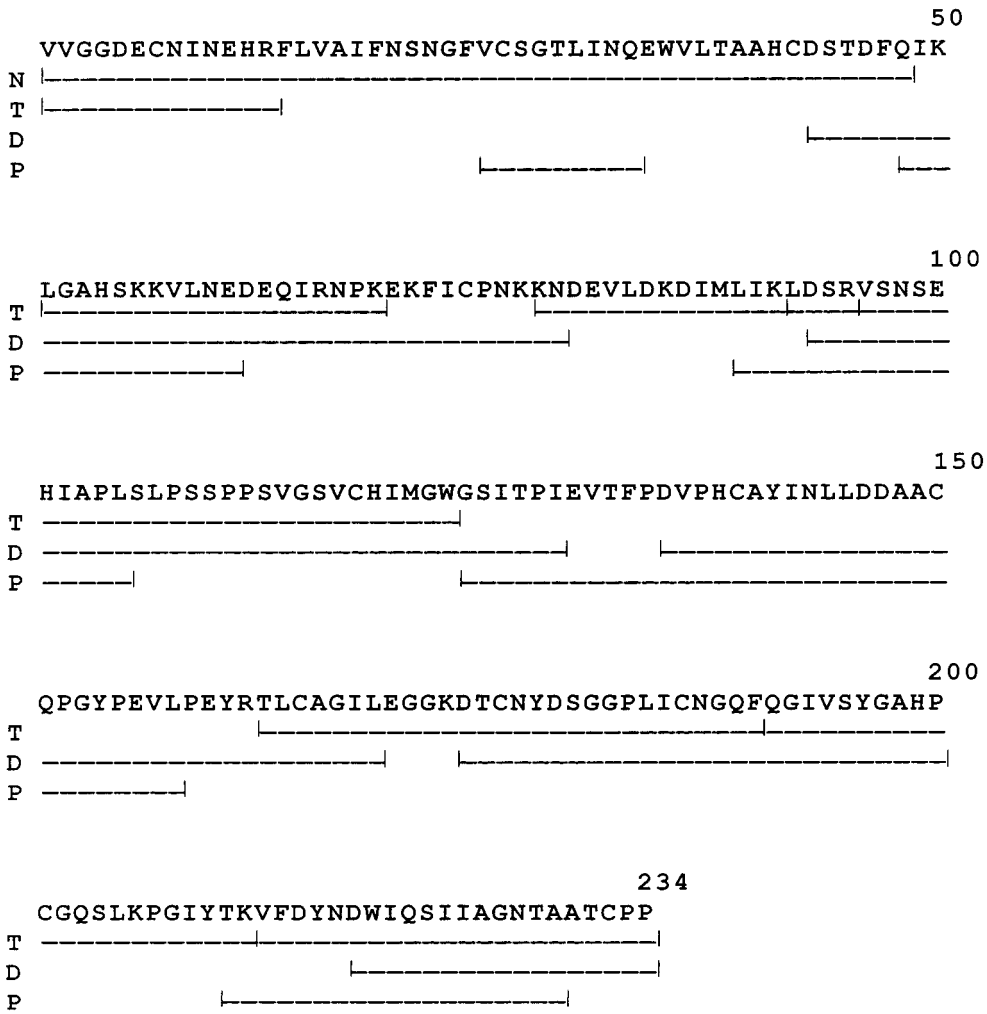

FIGURE 4: Amino acid sequence of contortrixobin. Horizontal lines correspond to continuous sequences determined by automated Edman degradation. Short vertical lines indicate ends of sequenced peptides and satisfactory identification. N, amino-terminal sequence of intact S-alkylated molecule; P, pepsin-digested peptide; T, trypsin-digested peptide; D, Asp-N endoproteinase-digested peptide.

therefore the peptide mixture was subdigested with endoprotease GluC and endoprotease AspN and further subjected to MALDIMS analysis. The spectrum obtained confirmed the previous S−S pairing assignment and led to the identification of the remaining disulfide bridge. In fact, as shown in Figure 5B, a series of related signals was detected at *m*/*z* 2605.9, 2720.4, 2862.8, 2977.1, 3244.2, and 3502.0 that were associated with the peptide pairs (72−78)+(218−234), (72−78)+(217−234), (70−78)+(218−234), (70−78)+(213−234), (72−78)+(213−234), and (72−78)+(211−234), all joined by an S−S bridge involving Cys74 and Cys232. All signals were accompanied by satellite peaks 18 Da higher associated with the same peptides where the amino acid replacement Pro234→Asp occurred. These assignments were verified by MALDIMS analysis of the peptide mixture following a single step of Edman degradation.

Therefore, this approach allowed the determination of the complete pattern of disulfide pairings in contortrixobin as Cys7−Cys139, Cys26−Cys42, Cys74−Cys232, Cys118−Cys186, Cys150−Cys165, and Cys176−Cys201 [or, according to the chymotrypsinogen numbering (*39*), Cys22−Cys157, Cys42−Cys58, Cys91−Cys250, Cys136−Cys201, Cys168−Cys182, and Cys191−Cys220; see also Figure 8, where the comparison of contortrixobin with other TLEs is reported].

Hydrolytic Activities on Synthetic Substrates. Contortrixobin displayed hydrolytic activity on both *p*-nitroanilides and *p*-nitrophenyl esters. Table 1 shows the catalytic efficiency (as measured by k_c/K_m) toward single amino acid substrates with an arginyl or lysyl residue in position P1 (nomenclature according to *40*). The data indicate that the esterase activity of contortrixobin toward arginyl substrates is 4 orders of magnitude higher than amidase activity. Moreover, the preference at pH 6.8 of contortrixobin for arginine over lysine in the primary subsite by a factor of 2.6 is clearly provided by the higher values of the second-order rate constant k_c/K_m, the simplest single parameter for assessing the specificity of an enzyme for competing substrates (*29*). It appears relevant that Z-Lys-pNA was not hydrolyzed by contortrixobin to any detectable extent even at 50 μM enzyme concentration. As expected for all trypsin-like serine proteases, Z-Ala-ONp is the less sensitive ester to the snake enzyme attack, possibly due the shortness and the absence of positive charge in the side chain of this substrate.

The large increase in k_c/K_m values (20−500-fold) for the tripeptide anilide substrates, as compared to Z-Arg-pNA (Table 2), gives evidence of the significant influence of residues in P2 and in P3 on the hydrolysis rate of the Arg−NH bond. The functional weight of these secondary subsites on substrate recognition was highlighted from the results

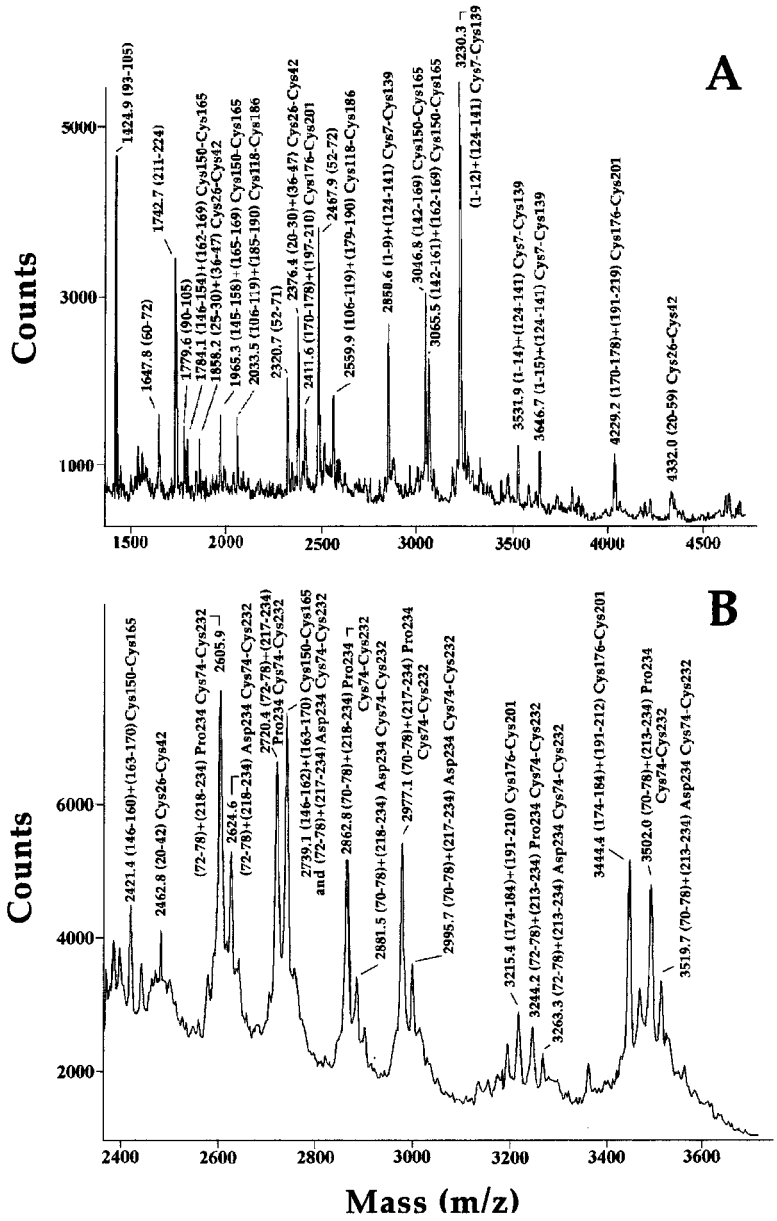

FIGURE 5: Panel A: MALDI mass spectrum of native contortrixobin following chymotrypsin hydrolysis. Signals corresponding to the disulfide-bridged peptides are indicated. The assignment to the corresponding peptide pairs and the cysteinyl residues involved in the S−S link are reported. Panel B: MALDI spectrum of the tryptic digest of native contortrixobin following hydrolysis with endoproteinase AspN and endoproteinase GluC. Signals corresponding to the disulfide-bridged peptides are indicated. The assignment to the corresponding peptide pairs and the cysteinyl residues involved in the S−S link are reported

Table 1: Values of Steady-State Parameters for the Contortrixobin-Catalyzed Hydrolysis of Esters and Amides, in 50 mM Phosphate Buffer, 0.1 M NaCl at 20 °C

substrate	pH	K_m (μM)	k_c (s^{-1})	k_c/K_m (M^{-1} s^{-1})
Z-Arg-ONp	7.4	4.6	12	2.6×10^6
Z-Arg-ONp	6.8	2.7	1.5	5.5×10^5
Z-Lys-ONp	6.8	1.95	0.41	2.1×10^5
Z-Ala-ONp	7.4	53.4	0.1	1.9×10^3
Z-Arg-pNA	7.4	44.6	0.02	3.5×10^2
Z-Lys-pNA	7.4	nd[a]	nd[a]	nd[a]

[a] Not detectable (see text).

obtained for the tripeptidyl anilide with lysine in position P1 that showed a relatively high value of k_c/K_m (Table 2), as compared to single amino acid anilide which did not form a productive bond with contortrixobin (see Table 1). Values of the second-order rate constant for human α-thrombin, in good agreement with those reported in the literature (e.g., *41*) once differences in experimental conditions are taken into consideration, are given for comparison in Table 2. It is well-known that the best *p*-nitroanilide substrates for α-thrombin have a proline in the P2 position (*41*); similarly, good substrates for contortrixobin are tripeptidyl anilides with a bulky nonpolar residue at the same position. In addition, it is interesting to notice that the substitution of tosylglycine with methylglycine (sarcosine) in position P3 does not reduce contortrixobin's specificity, as occurs with thrombin (Table 2). This would imply that (i) in contrast with the human protease the venombin does not need a bulky aromatic substituent in the P3 position to optimize catalytic interaction with the substrates, and (ii) the contortrixobin subsite S3 is structurally different from that of thrombin. Finally, the experimental data (Table 2) strongly suggest that contor-

Table 2: Values of Steady-State Parameters for the Hydrolysis of Various Tripeptidyl Anilides Catalyzed by Contortrixobin and Human α-Thrombin (in 50 mM Phosphate Buffer, 0.1 M NaCl, pH 7.4 at 20 °C)

enzyme	substrate	K_m (μM)	k_c (s^{-1})	k_c/K_m ($M^{-1}\ s^{-1}$)
contortrixobin	Bz-Phe-Val-Arg-pNA	18.7	1.1	5.9×10^4
contortrixobin[a]	Bz-Phe-Val-Arg-pNA	11.0	0.6	5.5×10^4
α-thrombin	Bz-Phe-Val-Arg-pNA	551.9	11.1	2.0×10^4
contortrixobin	Phe-Pip-Arg-pNA	411.2	2.8	6.8×10^3
α-thrombin	Phe-Pip-Arg-pNA	1.4	31.4	2.2×10^7
contortrixobin	Tosyl-Gly-Pro-Arg-pNA	18.7	2.1	1.1×10^5
α-thrombin	Tosyl-Gly-Pro-Arg-pNA	4.2	47.8	1.1×10^7
contortrixobin	Sar-Pro-Arg-pNA	28.7	5.6	1.9×10^5
α-thrombin	Sar-Pro-Arg-pNA	107.2	51.7	4.8×10^5
contortrixobin	Tosyl-Gly-Pro-Lys-pNA	105.2	0.1	1.3×10^3
α-thrombin	Tosyl-Gly-Pro-Lys-pNA	40.3	21.6	5.4×10^5

[a] In 50 mM Tris-HCl, 0.1 M KCl, pH 7.4.

Table 3: Equilibrium Constants of Some Serine Proteases with Small Synthetic Inhibitors (in 50 mM Phosphate Buffer, 0.1 M NaCl, pH 7.4)

enzyme	benzamidine K_i (μM)	DAPI K_i (μM)
contortrixobin	140	9.1
human α-thrombin	250	3.6
bovine β-trypsin	36	5.3

trixobin would not be affected by a sodium ion like thrombin, because in the presence of K^+ the k_c/K_m value for Bz-Phe-Val-Arg-pNA hydrolysis is very close to that obtained in the presence of Na^+. This is not an unexpected result, due to the presence of a proline at position 207 (see Figure 4) in the venombin, corresponding to position 225 according to the chymotrypsinogen numbering (*39*). Such a residue in fact is a direct predictor for the lack of allosteric regulation in contortrixobin (*42*). Analysis of the complete set of sequences of serine proteases of the chymotrypsin family shows that residue 225 is, remarkably, either proline or tyrosine in 47 out of a total of 55 different proteases (*42*). When the catalytic activity of a number of these serine proteases was studied, those with Tyr225 (such as thrombin) or Phe225 were found to discriminate among monovalent cations and showed maximal catalytic activity in the presence of sodium ions. On the other hand, proteases with Pro225, such as trypsin or contortrixobin, show loss of discrimination among monovalent cations and accordingly no allosteric regulation.

Studies on Reversible Synthetic Inhibitors. Since the arginyl residue is preferentially bound by contortrixobin with respect to the lysyl residue (see Table 1), as observed also for trypsin (*43*) and thrombin, a similarity in amino acid components as well as in the overall conformation of the primary specificity pocket of these three proteinases is expected. To shed more light on such structural aspects, the equilibrium constants of contortrixobin with benzamidine and DAPI were determined in comparison with those of human α-thrombin and bovine β-trypsin. The choice fell on these inhibitors, since benzamidine is considered an accurate structural analogue for arginine (*44*), and DAPI is a benzamidine derivative, whose hydrogen in the para-position is substituted with 6-amidinoindole (in other words, DAPI is a long molecule with two positively charged heads at the ends). The results reported in Table 3 indicate that: (i) the affinity of benzamidine for trypsin was higher than that observed for the inhibitor binding to contortrixobin and thrombin [in line with the evidence that the inhibitor molecule finds a much more hydrophobic surrounding in thrombin than in trypsin (*39*)]; and (ii) the equilibrium constants for DAPI were similar for all three proteases. The inhibition patterns were strictly competitive, and the complex formation conformed to simple equilibria, as indicated by the unitary value of the Hill coefficient ($n = 1.00 \pm 0.02$). The functional data (Table 3) suggested that the association of benzamidine with the S1 subsite is more hampered by the structural characteristics of the specificity pocket of contortrixobin than by those of trypsin, but less disturbed than in thrombin. In particular, since two of the supposed structural determinants of specificity are identical in all proteases (i.e., Gly216 and Gly226 present in the binding cavity, corresponding to residues 197 and 208 in Figure 4), the guanidinium group of benzamidine is expected to fill the base of the substrate binding cleft similarly in all three enzymes, and therefore the differences in interactions are expected to be mainly localized in the region facing the inhibitor aromatic ring. The similar affinity of DAPI for the three proteases is supposed to be determined by the binding to the S1 subsite through its 6-amidinindole group [even though the association with the S1 pocket can occur for a fraction of molecules also through the benzamidine moiety, as suggested from crystallographic data on trypsin (*44*)]. Therefore, additional interactions of the second DAPI's guanidinium group with glutamyl or aspartyl residues on the protein surface are precluded, since it behaves as a planar molecule, not capable of the required conformational readjustments (*44*).

Effects of Irreversible Synthetic Inhibitors and Protein Inhibitors. The effects of various inhibitors, specific for different classes of proteases, were preliminarily investigated. The esterolytic activity of contortrixobin was affected neither by a metal chelator (EDTA) nor by a thiol alkylating agent (E-64), while the other chemicals studied inhibited the venombin from 37% to 100%. In particular, under the experimental conditions chosen, the enzymatic activity was decreased by reagents that (i) reduce disulfide bonds (i.e., dithiothreitol, 64.5%; and β-mercaptoethanol, 41.4%) or (ii) are specific for serine proteases (diisopropyl fluorophosphate, 100%; PMSF, 73.5%; and TLCK, 37.7%). The observations indicate that contortrixobin is neither a metalloproteinase nor a cysteine proteinase, but suggest that it is a serine proteinase with disulfide bonds essential for its activity.

Since these inhibitory results were reminiscent of those obtained with thrombin, it appeared of interest to investigate the influence of protein inhibitors of human proteases on the venombin hydrolytic activity: human antithrombin III, BPTI, hirudin, and eglin *c*. Antithrombin III (4.5 μM) was ineffective at pH 7.4 and 20 °C on contortrixobin (50 nM), both in the presence (1.5 μM) and in the absence of heparin. On the other hand, BPTI [homologous to a low molecular mass Kunitz-type protease inhibitor (*45*) present in human plasma at 0.16 μM concentration] associated with contortrixobin with an apparent affinity ($K = 9.2 \times 10^{-4}$ M at pH 7.4 and 20 °C) very similar in value to that measured with human α-thrombin under similar experimental conditions ($K = 8.6 \times 10^{-4}$ M). The absence of an antithrombin III effect on contortrixobin activity was further investigated. Since no structural modification was observed on the inhibitor molecule even after 30 min incubation with the venombin at

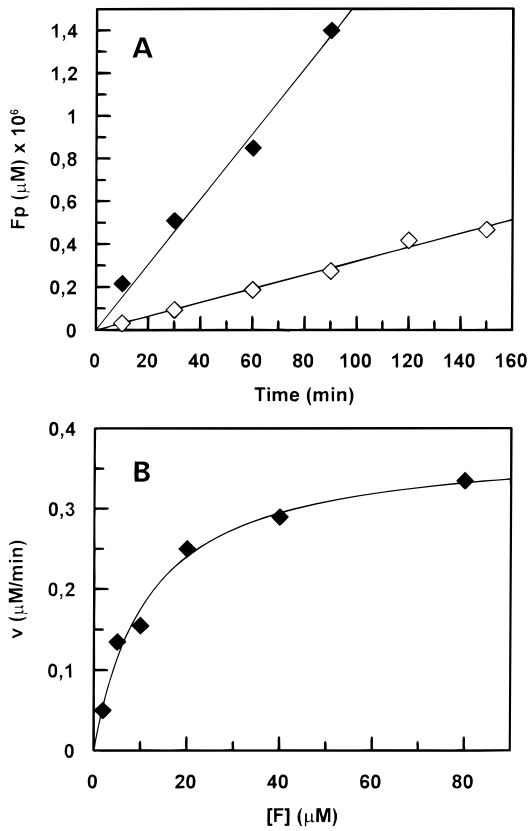

FIGURE 6: Panel A: contortrixobin-mediated release of FPB (◆) and FPA (◇) from human fibrinogen (2.5 μM) in 50 mM phosphate buffer, 0.1 M NaCl, pH 7.4 at 37 °C. The venombin concentration was 100 nM. The best fit of the data yielded a value of initial rate equal to 14.1 pmol/min for FPB and 3.2 pmol/min for FPA. Fp means fibrinopeptide. Panel B: Michaelis−Menten curve for the release of FPB from human fibrinogen ([F], concentration expressed in terms of β-chains) at pH 7.4 and 37 °C. The contortrixobin concentration was 100 nM.

pH 7.4 and 37 °C (as measured by amino-terminal sequence determination; data not reported), the ineffectiveness of antithrombin III was attributed to the impossibility of docking between contortrixobin and the protein inhibitor. Finally, hirudin interacted with very low affinity with contortrixobin (at 100 nM contortrixobin and 1 μM hirudin, only 8% of venombin catalytic activity was inhibited, a value to be compared with $K_a \cong 10^{13}$ M^{-1} for thrombin), while eglin *c* did not associate at all neither with contortrixobin nor with thrombin. Last, contortrixobin covalently attached to Sepharose beads did not abolish its fibrinogenase activity following incubation for 1 h with human plasma (data not reported). These last results suggested that human blood does not present the capability of blocking contortrixobin's catalytic properties.

Release of Fibrinopeptides from Human Fibrinogen. The time course HPLC analysis of supernatants after incubation with contortrixobin at pH 7.4 and 37 °C indicated a predominant occurrence of FPB with respect to FPA in each sample collected in the first 2 h. Apart from fibrinopeptides, no other peptides or single amino acids were detected. The last finding was confirmed by Edman analysis where only fibrin(ogen) amino termini (Tyr, Ala, and Gly) were found in the proportions corresponding to the amount of the released fibrinopeptides. Figure 6A depicts a time course of contortrixobin-catalyzed release of FPB and FPA from human fibrinogen upon limited proteolysis at α- and β-chains. The time dependencies indicate that cleavage of FPB is 4.4 times faster than that of FPA and that the kinetics for the release of both fibrinopeptides are identical in shape. Since the release of FPB could be analyzed as a single kinetic process, steady-state parameters (k_{cat} and K_m) were obtained by fitting the dependence of the initial rate of hydrolysis of β-chains on fibrinogen concentration (Figure 6B). Comparison of data obtained under similar experimental conditions for contortrixobin ($k_{cat} = 6.3 \times 10^{-2}$ s^{-1} and $K_m = 11.3$ μM) and thrombin ($k_{cat} = 38$ s^{-1} and $K_m = 9.7$ μM) revealed that the snake enzyme is 700 times less efficient (as measured by the specificity second-order constant) than human α-thrombin in terms of FPB release from human fibrinogen. It has to be mentioned that the efficiency of the two proteases was measured on different substrates: the action of contortrixobin referred to FPB cleavage from intact fibrinogen, whereas that of thrombin was accomplished on fibrin I monomers (i.e., fibrinogen lacking FPA). Estimation of thrombin's FPB release from intact fibrinogen usually is at least 10-fold smaller than that measured for fibrin I (*46*).

Research of Other Biological Properties. A number of thrombin-like and non-thrombin-like proteases from snake venoms presenting significant sequence similarities (*47*−*51*) show different substrate specificities. In particular, some of them activate coagulation plasma factors rather than act on fibrinogen; others exhibit key cellular bioregulation functions.

To check if contortrixobin could be considered as a multifunctional serine protease like thrombin (*52*, *39*), its action on Factor V, Factor VIII, Factor XIII, and thrombocytes was investigated. Contortrixobin activated human Factors V and XIII with a time course corresponding for more than 90% of the process to a single-exponential event under pseudo-first-order conditions (see Figure 7A,B), thus indicating that such effects can be analyzed as simple kinetic processes from which the following values (in M^{-1} s^{-1}) of the second-order rate constants at pH 7.5 and 37 °C have been calculated: activation by contortrixobin: Factor V = 1.9×10^4 and Factor XIII = 6.4×10^2; activation by human α-thrombin: Factor V = 9.3×10^6 and Factor XIII = 1.5×10^5. Comparison with thrombin indicates that the venombin acts on these clotting factors with a rate 250−500-fold lower than the human protease. No activity of contortrixobin on Factor VIII, thrombocyte aggregation, and platelet calcium ion influx was observed (data not reported).

DISCUSSION

The venom of *Agkistrodon contortrix contortrix* is a valuable source of enzymes acting on the coagulation process. In the present work, isolation and characterization of a new fibrinogen-clotting serine protease that preferentially releases FPB, named contortrixobin, are reported. Two other fibrin-promoting enzymes, purified from the same species and like contortrixobin acting with preference on the fibrinogen β-chains, were previously described (*12*, *17*, *18*). However, differences in molar masses (25.5 kDa vs 68 kDa) and the absence (in contortrixobin) and presence (in previously isolated venombins B) of carbohydrate moieties make sure that the enzyme described in this work is indeed a novel protease. In particular, the absence of N-glycosylated residues is a rare characteristic for TLEs, which contortrixobin shares

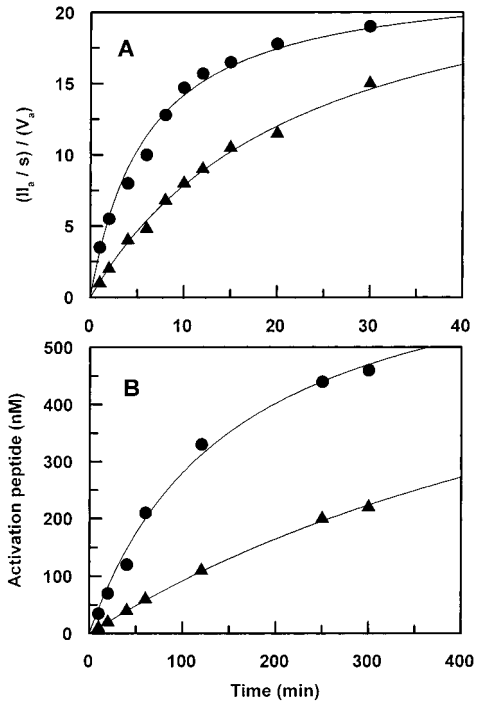

FIGURE 7: Panel A: activation of human Factor V (0.6 μM) by 50 nM contortrixobin (▲) and 0.25 nM human α-thrombin (●) in 10 mM HEPES, 0.15 M NaCl, 0.1% PEG (w/v), pH 7.5 at 37 °C. Panel B: activation of human Factor XIII (0.5 μM) by 50 nM contortrixobin (▲) and 1 nM human α-thrombin (●) in 10 mM HEPES, 0.15 M NaCl, 0.1% PEG (w/v), pH 7.5 at 37 °C. For details, see Materials and Methods.

up to date only with flavoxobin (*53*). This feature selects contortrixobin as an interesting protein for industrial production of recombinant agents useful for research, diagnostic, and medicinal uses. As expected, these 68 kDa venombins B were adsorbed on the arginine−Sepharose column, but eluted from DEAE-Sepharose column within fraction 1 (see Figure 1). In the same chromatographic peak, an additional venombin was also isolated with a fibrinogen-clotting activity 5−10-fold lower than that of contortrixobin, p*I* equal to 9.1, and molar mass of 30 kDa: this latter characteristic is in common with a previously observed (*12)* fibrin-promoting component of the Southern copperhead snake venom, but itself is not enough to let one infer that both proteases correspond to one protein.

Once aligned as shown in Figure 8 [hereinafter only the chymotrypsinogen numbering (*39*) is used)], contortrixobin and the other proteases compared appear to differ in chain length and in sequence (see Table 4). In line with functional considerations based on inhibitory effect and activity on synthetic substrates, the constituents of catalytic sites found in mammalian trypsins are present in the corresponding positions of contortrixobin. These include the components of the catalytic triad (His57, Asp102, and Ser195), residues Val16 and Asp194 (known to form a salt bridge stabilizing the catalytic site), and Asp189 (located at the bottom of the primary specificity pocket). The number of half-cystine residues and their positions in the sequence of contortrixobin coincide with those of all TLEs whose complete primary structure is known (see, also, Figure 8). The absence of free thiols in venombins led to the presumption that all 12 cysteines are linked by the way of disulfide bonds. These pairings were confirmed experimentally for the first time on bilineobin by standard methods (*60*) and in the present work on contortrixobin by a careful mass spectrometric investigation.

The sequence homology observed among the serine proteases in snake venoms is, however, apparently not related to their preferential specificity toward the α-chains or the β-chains of fibrinogen as well as toward other biological activities (see Table 4). This evidence suggests that complete understanding of the interactions between these biomolecules (e.g., protease and fibrinogen or other coagulation factors) cannot be reduced to the description of sequence similarities between TLEs and thrombin. The molecular basis of protease specificity has been investigated intensively, but still remains not completely understood. In particular, experimental observations as a whole suggest that the determinants of protease biorecognition may be quite subtle and involve the contribution of many factors rather than few critical sites (*66*, *67*). In other words, the assumption that the structure of a single protease−inhibitor (or −substrate) complex is sufficient to define the available subsites of an enzyme that has a unique binding site and a uniquely defined mode for ligand binding is not realistic (*68*). The following considerations are therefore in keeping. Thrombin has an extensive substrate recognition surface made up of a multiplicity of areas (*39*), many characteristics of which are poorly represented in contortrixobin: (i) some of the features of the so-called thrombin canyon-like cleft, such as the 60A−60D insertion, are completely absent in contortrixobin; (ii) of the 7 negatively charged residues of thrombin lining the primary specificity pocket, only 2 (Glu186B and Asp189) are present in contortrixobin; (iii) the hydrophobic cage near the catalytic center in thrombin (the so-called apolar binding site) is formed by 5 residues, only 1 of which (Leu99) is shared with contortrixobin; (iv) only 2 (Lys67 and Lys73) of the 10 positively charged residues constituting the thrombin fibrinogen recognition exosite are present also in contortrixobin. Therefore, the contortrixonin ability in substrate (or protein inhibitor) recognition and binding is expected to depend on association sites and/or geometries different from those operative in thrombin, as already proposed for crotalase (*54*). Such a possibility finds strong support in the evidence that both contortrixobin and thrombin form complexes with BPTI with similarly low affinity ($K \sim 10^3$ M^{-1}), suggesting a restricted access of the protein inhibitor to both catalytic centers. No doubt that the structural landscape around the primary specificity pocket of the two proteases is different and therefore their molecular mechanisms for biorecognition are expected to be diverse. Thus, attempts (*39*) to dock BPTI to thrombin's active site with identical position and orientation as observed for the trypsin(ogen) complexes resulted in a collision of residues of the inhibitor binding region with part of the rigid loop around Trp60D of thrombin, a structural feature that is absent in contortrixobin (see also Figure 8). This structural expectation finds experimental support in the evidence that deletion of the 60-insertion loop in thrombin resulted in a 10^3-fold higher affinity for BPTI (*69*). On the other hand, the low association constant of contortrixobin for BPTI should depend on very different structural bases, because of the absence in the venombin of the two rims typical of thrombin's canyon-like cleft. Therefore, the mechanism of the protease−inhibitor interaction in contortrixobin could be more reminiscent of the BPTI association

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                 16              30      36A       45          55   60  60D
thrombin         IVEGSDAEIG MSPWQVMLFR KSPQELLCGA SLISDRWVLT AAHCLLYPPW
crotalase        VIGGDECNIN EHRFLVALYD YWSQLFLCGG TLINNEWVLT AAHC......
calobin          VIGGDECNIN EHRFLVALYN SRSRTLFCGG TLINQEWVLT AAHC......
batroxobin       VIGGDECDIN EHPFLAFMY. .YSPRYFCGM TLINQEWVLT AAHC......
bothrombin       VIGGDECDIN EHPFLAFMY. .YSPQYFCGM TLINQEWVLT AAHC......
ancrod           VIGGDECNIN EHRFLVAVYE GTNWTFICGG VLIHPEWVIT AEHC......
flavoxobin       VIGGDECNIN EHPFLVALYD AWSGRFLCGG TLINPEWVLT AAHC......
mutobin          VIGGDECNIN EHRFLVALYD GLSGTFLCGG TLINQEWVLT AQHC......
kn-bj2           IIGGRFCDIN EHRSLALVKY GN...FQCSG TLINQEWVLS AAHC......
bilineobin       IIGGDECNIN EHRFLVALYD VWSGSFLCGG TLINQEWVLT AAHC......
halystase        IIGGDECNIN EHRFLVALYT PRSRTLFCGG TLINQEWVLT AAHC......
pallabin         IIGGDECNIN EHRFLVALYT SRT..LFCGG TLINQEWVLT AAHC......
tm-vig           VIGGDECNIN EHPFLVLVYY ...DDYQCGG TLLNEEWVLT AAHC......
contortrixobin   VVGGDECNIN EHRFLVAIFN ..SNGFVCSG TLINQEWVLT AAHC......
pa-bj            VVGGRPCKIN VHRSLVLLYN ..SSSLLCSG TLINQEWVLT AAHC......
tsv-pa           VFGGDECNIN EHRSLVVLFN ..SNGFLCGG TLINQDWVVT AAHC......
acc-c            VIGGDECNIN EHRFLALVYA ...NGSLCGG TLINQEWVLT ARHC......
rvv-v            VVGGDECNIN EHPFLVALYT STSSTIHCGG ALINREWVLT AAHC......
trypsin          IVGGYTCGAN TVPYQVSL.. .NSGYHFCGG SLINSQWVVS AAHC......
                    *                         *    **   *    * **

                 60E 60I  65          75  77A    84          93    97A  102
thrombin         DKNHIENDLL VRIGKHSRTR YERNIEKISM LEKIY.IHPR YNWRENLDRD
crotalase        DRTH....IL IYVGVHDRSV QFDKEQRRFP KEKYFFDCSN ..NFTKWDKD
calobin          ERKN....FR IKLGIHSKKV PNEDEQTRVP KEK..FFCLS SKNYTLWDKD
batroxobin       NRRF....MR IHLGKHAGSV ANYDEVVRYP KEK..FICPN KKKNVITDKD
bothrombin       DKTY....MR IYLGIHTRSV ANDDEVIRYP KEK..FICPN KKKNVITDKD
ancrod           ARRR....MN LVFGMHRKSE KFDDEQERYP KKRYFIRCNK ..TRTSWDED
flavoxobin       DSKN....FK MKLGAHSKKV LNEDEQIRNP KEK..FICPN KKNDEVLDKD
mutobin          NRSL....MN IYLGMHNKNV KFDDEQRRYP KKKYFFRCNK ..NFTKWDED
kn-bj2           DGEK....MK IHLGVHSKKV PNKDKQTRVA KEKF..FCLS SKNYTKWDKD
bilineobin       NMSN....IY IYLGMHNQSV QFDDEERRYP KEKYLFRCSK ..NFTKWDKD
halystase        DRKN....FR IKLGMHSKKV PNKDEQTRVP KEKFF..CLS SKNYTLWDKD
pallabin         NMED....IQ IKLGMHSKKV PNEDEQKRVP KEKFF..CLS SKNYTLWDKD
tm-vig           NGKD....ME IYLGVHSKKV PNKDVQRRVP KEKFF..CDS SKTYTKWNKD
contortrixobin   DSTD....FQ IKLGAHSKKV LNEDEQIRNP KEK..FICPN KKNDEVLDKD
pa-bj            DSKN....FK MKLGVHSIKI RNKNERTRHP KEK..FICPN RKKDDVLDKD
tsv-pa           DSNN....FQ LLFGVHSKKI LNEDEQTRDP KEK..FFCPN RKKDDEVDKD
acc-c            DRGN....MR IYLGMHNLKV LNKDALRRFP KEK..YFCLN TRNDTIWDKD
rvv-v            DRRN....IR IKLGMHSKNI RNEDEQIRVP RGKY..FCLN TKFPNGLDKD
trypsin          YKSG....IQ VRLGEDNINV VEGNEQF.IS ASKSIV.HPS YN.SNTLNND
                             *                                      *

                 103         113         123    129A 131        140       149
thrombin         IALMKLKKPV AFSDYIHPVC LPDRETAASL LQAGYKGRVT GWGNLKETWT
crotalase        IMLIRLNKPV SYSEHIAPLS LPSSPPIV... ..GSVCRAM GWGQ.....T
calobin          IMLIRLDSPV SNSEHIAPLS LPSSPPSV... ..GSVCRIM GWGR.....I
batroxobin       IMLIRLDRPV KNSEHIAPLS LPSNPPSV... ..GSVCRIM GWGA.....I
bothrombin       IMLIRLNRPV KNSTHIAPIS LPSNPPSV... ..GSVCRIM GWGA.....I
ancrod           IMLIRLNKPV NNSEHIAPLS LPSNPPIV... ..GSDCRVM GWGS.....I
flavoxobin       IMLIKLDSPV SYSEHIAPLS LPSSPPSV... ..GSVCRIM GWGS.....I
mutobin          IRL...NRPV RFSAHIEPLS LPSNPPSE... ..DSVCRVM GWGQ.....I
kn-bj2           IMLIRLDSPV KNSAHIAPIS LPSSPPIV... ..GSVCRIM GWGT.....I
bilineobin       IMLIRLNKPV RNSEHIAPLS LPSSPPIV... ..GSVCRVM GWGT.....I
halystase        IMLIRLDSPV KNSTHIEPFS LPSSPPSV... ..GSVCRIM GWGR.....I
pallabin         IMLIRLDSPV KNSAHIAPLS LPSSPPSV... ..GSVCRTM GWGR.....I
tm-vig           IMLIRLDRPV RKSAHIAPLS LPSSPPSV... ..GSVCRVM GWGT.....I
contortrixobin   IMLIKLDSRV SNSEHIAPLS LPSSPPSV... ..GSVCHIM GWGS.....I
pa-bj            IMLIRLNRPV SNSEHIAPLS LPSSPPSV... ..GSVCYVM GWGK.....I
tsv-pa           IMLIKLDSSV SNSEHIAPLS LPSSPPSV... ..GSVCRIM GWGK.....T
acc-c            IMLIRLNRPV RNSAHIAPLS LPSNPPSV... ..GSVCRIM GWGT.....I
rvv-v            IMLIRLRRPV TYSTHIAPVS LPSRSRGV... ..GSRCRIM GWGK.....I
trypsin          IMLIKLKSAA SLNSRVASIS LPTSCASA... ..GTQCLIS GWGNTKSS.G
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                149A  151  155        165      174  175   181  184A  186D
thrombin        ANVGKGQPSV LQVVNLPIVE RPVCKDSTRI ...RITDNMF CAGYKPDEGK
crotalase       TSPQETLPDV PHCANINLLD YEVCRTAHPQ FRLPATSRTL CAGVL..EGG
calobin         SPTKETYPDV PHCANINLLE YEMCRAPYPE FGLPATSRTL CAGIL..EGG
batroxobin      TTSEDTYPDV PHCANINLFN NTVCREAYNG ..LPA..KTL CAGVL..QGG
bothrombin      TTSEDTYPDV PHCANINLFN NTVCREAYN. .GLPA..KTL CAGVL..QGG
ancrod          NRRIDVLSDE PRCANINLHN FTMCHGLFR. .KMPKKGRVL CAGDL..RGR
flavoxobin      TPVEETFPDV PHCANINLLD DVECKPGYPE .LLPEY.RTL CAGVL..QGG
mutobin         TSPPETLPDV PHCANINLFN YTVCRGAYPR .MPT...KVL CAGVL..EGG
kn-bj2          STSKVILSDV PHCANINLLN YTVCRAAYPE .LPAT.SRTL CAGIL..QGG
bilineobin      TSPNETLPDV PRCVNINLFN YTVCRGVFP. .RLPERSRIL CAGVL..EGG
halystase       SPTEETFPDV PHCVNINLLE YEMCRAPYPE FELPATSRTL CAGIL..EGG
pallabin        SSTKETYPDV PHCVNINLLE YEMCRAPYPE FELPATSRTL CAGIL..EGG
tm-vig          TSPQETYPDV PHCANINLLD YEVCRAAYAG ..LPATSRLT CAGIL..EGG
contortrixobin  TPIEVTFPDV PHCAYINLLD NAACQPGYPE V.LPEY.RTL CAGIL..EGG
pa-bj           SSTKETYPDV PHCAKINILD HAVCRAAYT. W.WPATSTTL CAGIL..QGG
tsv-pa          IPTKEIYPDV PHCANINILD HAVCRTAYS. WRQVAN.TTL CAGIL..QGG
acc-c           TSPNATLPDV PHCANINILD YAVCQAATK. .GLAA..TTL CAGIL..EGG
rvv-v           STTEDTYPDV PHCTNIFIVK HKWCEPLYP. .WVPADSRTL CAGIL..KGG
trypsin         TS....YPDV LKCLKAPILS DSSCKSAYP. .G.QITSNMF CAGYL..EGG
                                      *                  ***     *

                187         197     204A  208     215    221A   227   233
thrombin        RGDACEGDSG GPFVMKSPFN NRWYQMGIVS WG.EGCDRDG KYGFYTHVFR
crotalase       .IDTCNRDSG GPLIC..... NGQFQ.GIVF WGPDPCAQPD KPGLYTKVFD
calobin         .IDTCRGDSG GPLIC..... NGQFQ.GIAS WGDDPCAQPH KPAAYTKVFD
batroxobin      .IDTCGGDSG GPLIC..... NGQFQ.GILS WGSDPCAEPR KPAFYTKVFD
bothrombin      .IDTCGGDSG GPLIC..... NGQFQ.GILS WGSDPCAEPR KPAFYTKVFD
ancrod          R.DSCNSDSG GPLIC..... NEELH.GIVA RGPNPCAQPN KPALYTSIYD
flavoxobin      .IDTCGFDSG TPLIC..... NGQFQ.GIVS YGGHPCGQSR KPGIYTKVFD
mutobin         .IDTCNRDSG GPLIC..... NGQFQ.GIVF WGPDPCAQPD KPGVYTKVFD
kn-bj2          K.DTCVGDSG GPLIC..... NGQFQ.GIVS WGSDVCGYVL EPALYTKVSD
bilineobin      IKDTCKRDSG .PLIC..... NGQFQ.GIVS WGPKRCAQPR KPALYSKVFD
halystase       K.DTCRGDSG GPLIC..... NGQFQ.GIAS WGDDPCAQPH KPAAYTKVFD
pallabin        K.DTCVGDSG GPLIC..... NGQFQ.GIAS WGDDPCAQPH KPAAYTKVFD
tm-vig          K.DSCVGDSG GPLIC..... NGQFQ.GIVS WGGDPCAGPR EPGVCTNVFD
contortrixobin  K.DTCNYDSG GPLIC..... NGQFQ.GIVS YGAHPCGQSL KPGIYTKVFD
pa-bj           K.DTCEGDSG GPLIC..... NG.LQ.GIVS GGGNPCGQPR KPALYTKVFD
tsv-pa          R.DTCHFDSG GPLIC..... NGIFQ.GIVS WGGHPCGQPG EPGVYTKVFD
acc-c           K.DTCKGDSG GPLIC..... NGQFQ.GILS VGGNPCAQPR KPGIYTKVFD
rvv-v           R.DTCHGDSG GPLIC..... NGQIQ.GIVA GGSEPCGQHL KPAVYTKVFD
trypsin         K.DSCQGDSG GPVVC..... SGKLQ.GIVS WGSG.CAQKN KPGVYTKVCN
                  * *  ***  *               **    *   *

                234         244     252
thrombin        LKKWIQKVID QFGE.....
crotalase       HLDWIQSIIA GEKTVNCP.
calobin         HLDWIQSIIA GNTDASCPP
batroxobin      YLPWIQSIIA GNKTATCP.
bothrombin      YLPWIQSIIA GNKTATCPP
ancrod          YRDWVNNVIA GN..ATCSP
flavoxobin      YNAWIQSIIA GNTAATCLP
mutobin         YLDWIQSVIA GN..TTCS
kn-bj2          YTEWINSIIA GNTTATCPP
bilineobin      HLDWIQSIIA GNKTVNCP.
halystase       HLDWIKSIIA GNTDASCPP
pallabin        HLDWIENIIA GNTDASCPP
tm-vig          HLDWIKGIIA GNTDVTCPL
contortrixobin  YNDWIQSIIA GNTAATCPP
pa-bj           YLPWIESIIA GTTTATCP
tsv-pa          YLDWIKSIIA GNKDATCPP
acc-c           YTDWIQSIIS GNTDATCPP
rvv-v           YNNWIQNIIA GNRTVTCPP
trypsin         YVSWIKQTIA SN.......
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FIGURE 8: Comparison of amino acid sequences of snake venom proteases with human α-thrombin and bovine *β*-trypsin. Points represent gaps introduced to optimize the alignment, according to topological equivalencies; residues identical to those of contortrixobin are starred. All residue positions correspond to the first digit of the residue number [chymotrypsinogen numbering (*39*)]. Relevant references are as follows: α-thrombin B-chain (*39*); crotalase (*54*); calobin (*55*); batroxobin (*56*); bothrombin (*51*); ancrod (*57*); flavoxobin (*53*); mutobin (*58*); kn-bj2 (*59*); bilineobin (*60*); halystase (*50*); pallabin (*61*); tm-vig (*62*); pa-bj (*63*); tsv-pa (*64*); acc-c (*13*); rvv-v (*48*); *β*-trypsin (*65*).

Table 4: Calculated Similarities of Contortrixobin Relative to Most Serine Proteases of Known Sequence Isolated from Snake Venom[a]

enzyme	similarity (%)	no. of residues	biological activity
human α-thrombin	27.6	259	FPA and FPB release
crotalase	56.7	237	FPA release
calobin	64.1	238	FPA release
batroxobin	57.4	231	FPA release
bothrombin	57.8	232	FPA release
ancrod	45.1	234	FPA release
flavoxobin	88.8	236	FPA release
mutobin	62.4	228	FPA release
kn-bj2[b]	64.5	233	FPA release
bilineobin	55.2	235	FPB release
halystase	72.6	238	FPB release[c]
pallabin	71.9	236	FPB release
tm-vig[b]	45.2	233	FPB release
pa-bj[b]	77.7	232	platelet aggregation
tsv-pa[b]	71.5	234	plasminogen activation
acc-c[b]	72.4	231	protein C activation
rvv-V[b]	55.6	236	Factor V activation
bovine β-trypsin	35.0	224	−

[a] The respective chain lengths and biological activities are also reported. The fibrinogenase activity of venombins is preferential and not absolute for FPA or FPB release. [b] kn-bj2, one of the two forms of kinin-releasing and fibrinogen-clotting serine protease from the venom of *Bothrops jararaca*; tm-vig, protease isoform from the venom *Trimeresurus mucrosquamatus* characterized by Val-Ile-Gly in the N-terminus; pa-bj, platelet-aggregating serine protease from the venom of *Bothrops jararaca*; tsv-pa, plasminogen activator from the venom of *Trimeresurus stejnegeri*; acc-c, protein C activator from the venom of *Agkistrodon contortrix contortrix*; rvv-V, Factor V-activating enzyme from Russel viper venom. [c] FPB released from halystase is 28 residues longer than other FPBs listed in the table.

with trypsinogen, when the noncomplementary binding site of the zymogen is forced into a fitting trypsin-like structure upon BPTI complexation (*70*). Accordingly, although the energetic cost exacted by the two mechanisms (active site opening in thrombin and possible rearrangement of binding pocket in contortrixobin) is alike, the structural bases for the formation of the two protease−inhibitor complexes appear to be very different.

Moreover, it has been hypothesized (*71*) that residues 189−220 in the C-terminal sequence of the serine protease family exert an overwhelmingly disproportionate influence, compared with other sequence segments, on the recognition of substrate. This domain, that includes the major portion of the contact area between contortrixobin (or thrombin) and BPTI, accounts for most of the surface around the specificity sites S1, S2, and S3. Consequently, according to this model, the P1−P3 residues (of the substrate) are expected (*72*) to dictate substrate binding in a dominant manner, with primed residues (nomenclature according to *40*) adding to the specificity of recognition to a lesser extent. This model, however, does not seem to fit snake venom proteases. In fact, since (i) the sequence homology with contortrixobin in the 189−220 segment (see Figure 8) is very high but independent of snake proteases' biological activity (76.2% with venombins A; 75.9% with venombins B; 77.6% with proteases acting on substrates different from fibrinogen) and (ii) no evident trend in amino acid substitution as a function of biological activity can be observed (e.g., venombin A vs venombin B), additional binding sites (e.g., the primed ones and/or exosites) in venombins A and B are expected to play a role for fibrinogen biorecognition to an extent not smaller than that of S1, S2, and S3 subsites.

Fibrinogen, the most important substrate of thrombin, is converted into the insoluble fibrin by a precise sequence of thrombin-catalyzed events (*72*): in the reaction pathway, FPA is cleaved in vitro 6−30-fold faster than FPB, depending on the reaction conditions. In contrast, contortrixobin releases FPB preferentially from human fibrinogen, at least under the experimental conditions used in this work (4 times quicker than FPA). Most venombins release FPA from fibrinogen, whereas only a few preferentially attack its β-chains, among these, venombins B purified from *Agkistrodon contortrix contortrix* (*73*), *Agkistrodon halys pallas* (*74*), *Trimeresurus okinavensis* (*75*); *Crotalus atrox* (*76*), *Vipera lebetina* (*77*), *Agkistrodon bilineatus* (*78*), and *Agkistrodon halys blomhoffii* (*50*), in addition to contortrixobin reported in this work. The cleavage of the fibrinogen β-chains produced by these venombins does not occur always at the site typically recognized by thrombin and contortrixobin (i.e., ArgBβ14), but in some cases at another site (i.e., ArgBβ42) as described for halystase (*50*) and protease III from *Crotalus atrox* (*79*). No molecular explanation can be offered for such a difference in behavior among these venombins. On the other hand, some considerations may be proposed to explain the low rate of FPB release observed for contortrixobin with respect to thrombin. Site-directed mutagenesis studies on thrombin demonstrated that deletion of the entire Glu146−Lys149E loop (not present in contortrixobin) reduced the FPB release by 500-fold (*80, 81*). Therefore, the absence of this structural element, directly involved in thrombin's fibrinogen binding, can help us in the understanding the low value of k_c/K_m for FPB release observed for contortrixobin.

As far as the biological significance is concerned, that of contortrixobin as well as that of other venom proteases still need to be clarified. The contortrixobin cleaving activity toward human fibrinogen may gradually affect the coagulation mechanisms, inducing clotting. In fact, the concentration of fibrinogen in human plasma is approximately 18 μM, indicating that contortrixobin reaching the human blood with the snake bite would be half-saturated with the fibrinogen substrate, particularly considering that contortixobin is fully active when injected and that there are no known contortrixobin inhibitors in human plasma. There is, however, a possibility that contortrixobin activity can be neutralized in vivo by the inhibitory potency of other animals' plasma (e.g., by plasma of animals that are snakes' common preys). In fact, a rapid and irreversible neutralization of another venombin B from *Agkistrodon contortrix contortrix* by serpins in rat plasma (but not in human plasma) has been reported (*82*).

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