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Isolation and characterization of a serine proteinase with thrombin-like activity from the venom of the snake *Bothrops asper*

A.V. Pérez[1,2], A. Rucavado[1], L. Sanz[3], J.J. Calvete[3] and J.M. Gutiérrez[1]

[1]Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica
[2]Facultad de Medicina, Universidad de El Salvador, San Salvador, El Salvador
[3]Laboratorio de Proteinómica Estructural, Instituto de Biomedicina de Valencia, Valencia, España

Correspondence to: J.M. Gutiérrez, Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica
Fax: +506-292-0485. E-mail: jgutierr@icp.ucr.ac.cr

A serine proteinase with thrombin-like activity was isolated from the venom of the Central American pit viper *Bothrops asper*. Isolation was performed by a combination of affinity chromatography on aminobenzamidine-Sepharose and ion-exchange chromatography on DEAE-Sepharose. The enzyme accounts for approximately 0.13% of the venom dry weight and has a molecular mass of 32 kDa as determined by SDS-PAGE, and of 27 kDa as determined by MALDI-TOF mass spectrometry. Its partial amino acid sequence shows high identity with snake venom serine proteinases and a complete identity with a cDNA clone previously sequenced from this species. The N-terminal sequence of the enzyme is VIGGDECNINEHRSLVVLFXSSGFL CAGTLVQDEWVLTAANCDSKNFQ. The enzyme induces clotting of plasma (minimum coagulant dose = 4.1 µg) and fibrinogen (minimum coagulant dose = 4.2 µg) *in vitro*, and promotes defibrin(ogen)ation *in vivo* (minimum defibrin(ogen)ating dose = 1.0 µg). In addition, when injected intravenously in mice at doses of 5 and 10 µg, it induces a series of behavioral changes, i.e., loss of the righting reflex, opisthotonus, and intermittent rotations over the long axis of the body, which closely resemble the 'gyroxin-like' effect induced by other thrombin-like enzymes from snake venoms.

Key words: Snake venom; *Bothrops asper*; Serine proteinase; Thrombin-like serine proteinase; Defibrin(ogen)ation

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Viperid snakes of the genus *Bothrops* sp inflict the vast majority of snakebites in Central and South America (1,2). In Central America and parts of northern South America, the species *Bothrops asper* is responsible for the majority of cases (2). Envenomation by this species is associated with prominent local pathological effects, i.e., myonecrosis, hemorrhage, edema, dermonecrosis and blistering (3), as well as with systemic alterations characterized by coagulopathy, hemorrhage, cardiovascular shock, and acute renal failure (2,4,5). Alterations in hemostasis leading to thrombocytopenia, platelet hypoaggregation, defibrin(ogen)ation and disseminated intravascular coagulation are often observed in victims of *B. asper* bite envenomation (5-7). Such complex hemostatic disturbances potentiate the profuse bleeding initiated by the disruptive action of hemorrhagic metalloproteinases in the microvasculature (7,8).

Viperid venom-induced defibrin(ogen)ation is the consequence of the action of venom procoagulant components on the clotting system *in vivo*, since they cause the formation of microthrombi in the circulation, with the concomitant consumption of fibrinogen and the activation of the fibrinolytic system, which results in the appearance of fibrin(ogen)-degradation products (9). These effects result in alteration of

laboratory clotting test parameters, such as whole blood clotting time, prothrombin time and activated partial thromboplastin time (6,9). Procoagulant components in *Bothrops* sp venoms include zinc-dependent metalloproteinases that activate factor X and prothrombin, as well as serine proteinases that directly convert fibrinogen into fibrin (10,11). In the case of *B. asper*, previous investigations have described a prothrombin activator, which is a class P-III metalloproteinase named basparin A (12), and a thrombin-like serine proteinase, named asperase (13,14); however, the characterization of the latter is limited to molecular mass estimation and amino acid composition, with no sequence information available. On the other hand, this venom is devoid of factor X activators (15). Owing to the pathophysiological relevance of coagulant enzymes in this medically relevant snake venom, in the present paper we describe the isolation of a thrombin-like serine proteinase, the determination of its partial amino acid sequence, and the characterization of its *in vitro* coagulant and *in vivo* defibrin(ogen)ating activities.

Serine proteinase was purified by a two-step chromatographic protocol. Samples of 250 mg *B. asper* venom, obtained from adult specimens collected in the Pacific region of Costa Rica, were dissolved in 5 mL 50 mM Tris-HCl, 0.4 M NaCl, pH 9.0. After centrifugation at 500 *g* for 10 min, the supernatant was applied to a benzamidine-Sepharose 4 Fast Flow affinity chromatography column (3 x 12 cm; Amersham Biosciences, Uppsala, Sweden), previously equilibrated with the same buffer. The sample was passed several times through the column in order to maximize binding. After elution of the unbound fraction, a 0.1 M glycine-HCl, pH 3.0, buffer was applied to the column and the absorbance of the eluting material was monitored at 280 nm. Fractions of 3 mL were collected into tubes containing 0.5 mL 0.5 M Tris buffer, pH 8.8. Throughout fractionation, fibrinogen-clotting activity was assessed in order to identify fractions with thrombin-like activity. To this end, 200 µL of a 4 mg/mL bovine fibrinogen (Sigma-Aldrich, St. Louis, MO, USA) in 0.12 M NaCl, 40 mM phosphate, pH 7.2 (PBS) was incubated with 100 µL dilutions of the fractions. Proteins from the peaks showing fibrinogen-clotting activity were diafiltered and concentrated by ultrafiltration against 50 mM Tris-HCl, 50 mM KCl buffer, pH 9.0. The concentrated material was applied to a 3 x 8 cm DEAE-Sepharose column previously equilibrated with the dialysis buffer. After elution of the unbound material, a linear KCl gradient (50 mM to 0.75 M) in the same Tris buffer was developed in a total volume of 500 mL. Active peaks were diafiltered against distilled water and lyophilized. Homogeneity was assessed by reverse-phase HPLC on a C4 column (Vydac; 250 x 4.6 mm; flow rate 1.0 mL/min) using an Agilent 1100 instrument (Tokyo, Japan) and a linear gradient from 0 to 60% acetonitrile in 0.1% trifluoroacetic acid.

For molecular mass analysis and amino acid sequencing, the fibrinogen-clotting protein purified by DEAE-Sepharose chromatography was submitted to reverse-phase HPLC using an ETTAN™ LC HPLC system (Amersham Biosciences, Piscataway, NJ, USA) and a Lichrosphere RP100 C_{18} column (250 x 4 mm, 5 µm particle size) eluted at 1 mL/min with a linear gradient of 0.1% TFA in water (solution A) and acetonitrile (solution B) employing the following chromatographic conditions: isocratically (5% B) for 10 min, followed by linear gradients of 5-15% B over 20 min, 15-45% B over 120 min, and 45-70% B over 20 min. Protein detection was at 215 nm. Fractions were collected manually and dried in a Speed-Vac instrument (Savant, Ramsey, MN, USA). The molecular mass of the purified protein was determined by SDS-PAGE (16), run under reducing conditions on 12% polyacrylamide gels and by MALDI-TOF mass spectrometry (using a Voyager DE-Pro™ instrument, Applied Biosystem, Foster City, CA, USA, and sinapinic acid as the matrix) and electrospray ionization mass spectrometry using an Applied Biosystem Qtrap™ mass spectrometer (17) operated in Enhanced Multiple Charge mode in a 600-1700-m/z range.

The purified protein was subjected to N-terminal sequence analysis using a Procise instrument (Applied Biosystems) following manufacturer instructions. For internal sequence determination, the protein band of ~30 kDa was excised from a Coomassie brilliant blue-stained SDS-PAGE and subjected to automated reduction with DTT and alkylation with iodoacetamide, and in-gel digestion with sequencing grade bovine pancreas trypsin (Roche, San Cugart del Vallés, Barcelona, Spain) using a ProGest digestor (Genomic Solutions, Cambridgeshire, UK) following manufacturer instructions. A 0.65-µL amount of the tryptic peptide mixtures (total volume of ~20 µL) was spotted onto a MALDI-TOF sample holder, mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile containing 0.1% TFA, dried, and analyzed with a Voyager-DE Pro MALDI-TOF mass spectrometer (Applied Biosystems) operated in delayed extraction and reflector modes. A tryptic peptide mixture of *Cratylia floribunda* seed lectin (SwissProt accession code P81517) prepared and previously characterized in our laboratory was used as mass calibration standard (mass range, 450-3300 Da).

For peptide sequencing, the protein digest mixture was loaded onto a nanospray capillary column and subjected to electrospray ionization tandem mass spectrometric analysis using a QTrap mass spectrometer (Applied Biosystems) (17) equipped with a nanospray source (Protana, Odense, Denmark). Doubly- or triply-charged ions of se-

lected peptides from the MALDI-TOF mass fingerprint spectra were analyzed in Enhanced Resolution MS mode and the monoisotopic ions were fragmented using the Enhanced Product Ion tool with Q_0 trapping. Enhanced Resolution was performed at 250 amu/s across the entire mass range. Settings for MS/MS experiments were as follows: Q1 - unit resolution; Q1 to Q2 collision energy, 30-40 eV; Q3 entry barrier, 8 V; linear ion trap Q3 fill time, 250 ms, and Q3 scan rate, 1000 amu/s. Collision-induced dissociation spectra were interpreted manually or using the on-line form of the MASCOT program at http://www.matrixscience. com.

The coagulant activity of fractions and purified enzyme was assessed on citrated human plasma and on a 4 mg/mL solution of bovine fibrinogen, dissolved in PBS, as described above. Samples of 100 µL fractions or various concentrations of the enzyme were added to 200 µL plasma or fibrinogen, previously incubated at 37°C. Clotting times were recorded, and the minimum coagulant doses (MCD) for plasma or fibrinogen were determined; MCD corresponds to the amount of enzyme that induces clotting in 60 s (18). *In vivo* alterations were assessed by intravenous (*iv*) injection of various amounts of the enzyme dissolved in 100 µL PBS, in a group of four CD-1 mice (18-20 g body weight); controls received the same volume of PBS under otherwise identical conditions. One hour after injection, mice were bled under anesthesia. Blood was placed in dry clean glass tubes and left at room temperature; the formation of clots was regularly assessed by tilting the tubes, and clotting time was recorded (18). In addition, mice injected *iv* were observed for behavioral changes occurring during the first minutes after injection. Experiments involving mice were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUA) of the University of Costa Rica.

Fractionation of *B. asper* venom by affinity chromatography on benzamidine-Sepharose yielded a peak, which bound to the gel, having fibrinogen-clotting activity (Figure 1A). This fraction was separated into five peaks on a DEAE-Sepharose column eluted with a linear KCl gradient (Figure 1B). Only peak 1 showed fibrinogen-clotting activity. When analyzed by reverse-phase HPLC, this peak had one main component (Figure 1C). The yield of this enzyme was low, as only 2 mg was recovered from an initial amount of 1.5 g venom, thus corresponding to approximately 0.13% of *B. asper* venom. The apparent molecular mass of this thrombin-like enzyme was 32 kDa (non-reduced)/24 kDa (reduced), as determined by SDS-PAGE (Figure 1C, inset). MALDI-TOF mass spectrometry evidenced three molecular species of molecular masses 27,067, 27,356, and 27,649 Da (± 27 Da). The mass difference of 290-293 Da may indicate that the purified enzyme is a mixture of glycoforms differing in their degree of sialylation (mass increment due to sialylation = 292 Da).

N-terminal sequence analysis (Figure 2) revealed 48 residues with high identity with previously characterized thrombin-like serine proteinases from viperid snake venoms (19). Moreover, the internal sequences of 14 peptides resulting from tryptic digestion also showed characteristic sequences of venom serine proteinases. All the sequences determined showed 100% identity with a deduced amino acid sequence of a cDNA clone derived from RNA isolated from the venom gland of *B. asper* from Costa Rica (UniProtKB/TrEMBL entry Q072L6; Figure 2). Therefore, we may conclude that this sequence corresponds to the thrombin-like serine proteinase described in this communication. The cDNA-deduced sequence of *B. asper* serine proteinase exhibits a single potential N-glycosylation site at position 20 of the mature protein. The mass difference between the cDNA-deduced sequence (isotope-averaged molecular mass = 25308 Da, assuming that its 12 cysteines are involved in the formation of disulfide bonds) and the MALDI-TOF estimated molecular masses support the hypothesis that each of the three purified thrombin-like enzyme glycoforms bears a single di-, mono-, or non-syalilated, fucosylated complex-type dianntenary glycan chain (calculated mass increments of 2352.1, 2060.1, and 1768.1 Da, respectively). On the other hand, the similarity in molecular mass with asperase, a thrombin-like serine proteinase isolated from this venom by Ortiz and Gubensek (13), suggests that the enzyme described here is likely to correspond to asperase.

The MCD of the serine proteinase was 4.1 µg for human plasma and 4.2 µg for bovine fibrinogen. When the enzyme was incubated with 20 mM PMSF for 30 min at room temperature before addition to fibrinogen, the clotting activity was abrogated. The MCD of crude *B. asper* venom for plasma and fibrinogen are 0.5 µg and 80 µg, respectively (15). Taken together, these results clearly indicate that a) *B. asper* venom contains other more active procoagulant components, which very likely correspond to prothrombin-activating metalloproteinases (12,15), and b) the amount of thrombin-like serine proteinase in *B. asper* venom, and thus its contribution to the clotting activity is low, in agreement with the low yield obtained with our fractionation protocol. Therefore, serine proteinases play a secondary role in the clotting activity of *B. asper* venom. This conclusion is supported by previous experiments in which metalloproteinases were inhibited by the peptidomimetic hydroxamate batimastat. Under these conditions, the MCD of *B. asper* venom increased from 0.5 µg (uninhibited venom) to 50 µg (batimastat-inhibited venom) (15). Moreover, inhibition of venom serine proteinases by PMSF had little effect on the coagulant activity of

the venom (15). Clearly, therefore, metalloproteinases constitute the most important coagulant components of *B. asper* venom.

Upon *iv* injection in mice, the serine proteinase induced defibrin(ogen)ation. Blood collected from control mice injected with PBS clotted within 2 min, whereas blood collected from mice injected with 10, 5, 2.5, and 1.25 µg of the enzyme were unclottable; blood from mice receiving 0.5 µg showed normal clotting. The minimum defibrin(ogen)ating dose, i.e., the minimum dose that induced defibrin(ogen)ation in all mice tested, corresponded to 1.0 µg. Thus, this enzyme has a potent defibrin(ogen)ating activity. The role of serine proteinases in defibrin(ogen)ation induced by *B. asper* venom has been previously assessed in experiments in which venom metalloproteinases were inhibited by batimastat (15). As in the case of *in vitro* coagulant activity, inhibition of metalloproteinases greatly reduced the defibrin(ogen)ating activity of this venom, further supporting the proposed pre-

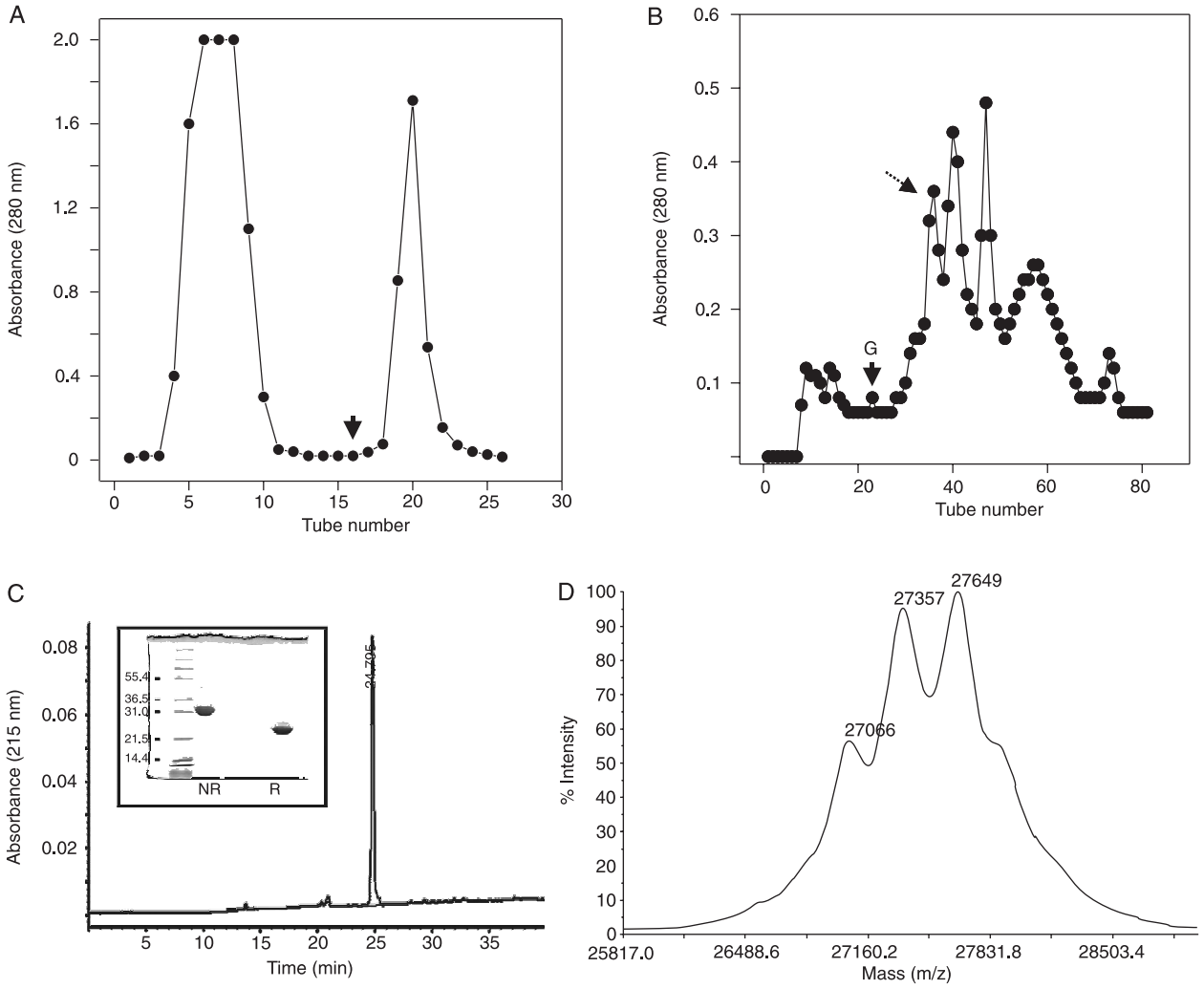

Figure 1. Isolation of a thrombin-like serine proteinase from *Bothrops asper* venom. *A*, Venom (250 mg) was dissolved in 50 mM Tris-HCl, 0.4 M NaCl, pH 9.0, and applied to a benzamidine-Sepharose 4 Fast Flow column. After elution of unbound material, a 0.1 M glycine-HCl buffer, pH 3.0, was applied (arrow) and the eluted material was collected into tubes containing 0.5 M Tris buffer, pH 8.8. *B*, The benzamidine-binding fraction isolated in *A* was further fractionated by ion-exchange chromatography on a DEAE-Sepharose column, equilibrated with 50 mM Tris-HCl, 50 mM KCl buffer, pH 9.0. After elution of unbound material, a linear gradient (G) of KCl from 50 mM to 0.75 M was developed in the same Tris buffer. Only the first peak collected after the onset of the gradient (discontinuous arrow) had thrombin-like activity. *C*, Reverse-phase HPLC analysis of the active peak after DEAE-Sepharose separation, carried out on a C4 column at a flow rate of 1.0 mL/min, using a linear gradient from 0 to 60% acetonitrile in 0.1% trifluoroacetic acid. The inset shows an SDS-PAGE of the purified protein run under reducing (R) and non-reducing (NR) conditions. *D*, MALDI-TOF mass spectrum of the purified thrombin-like enzyme.

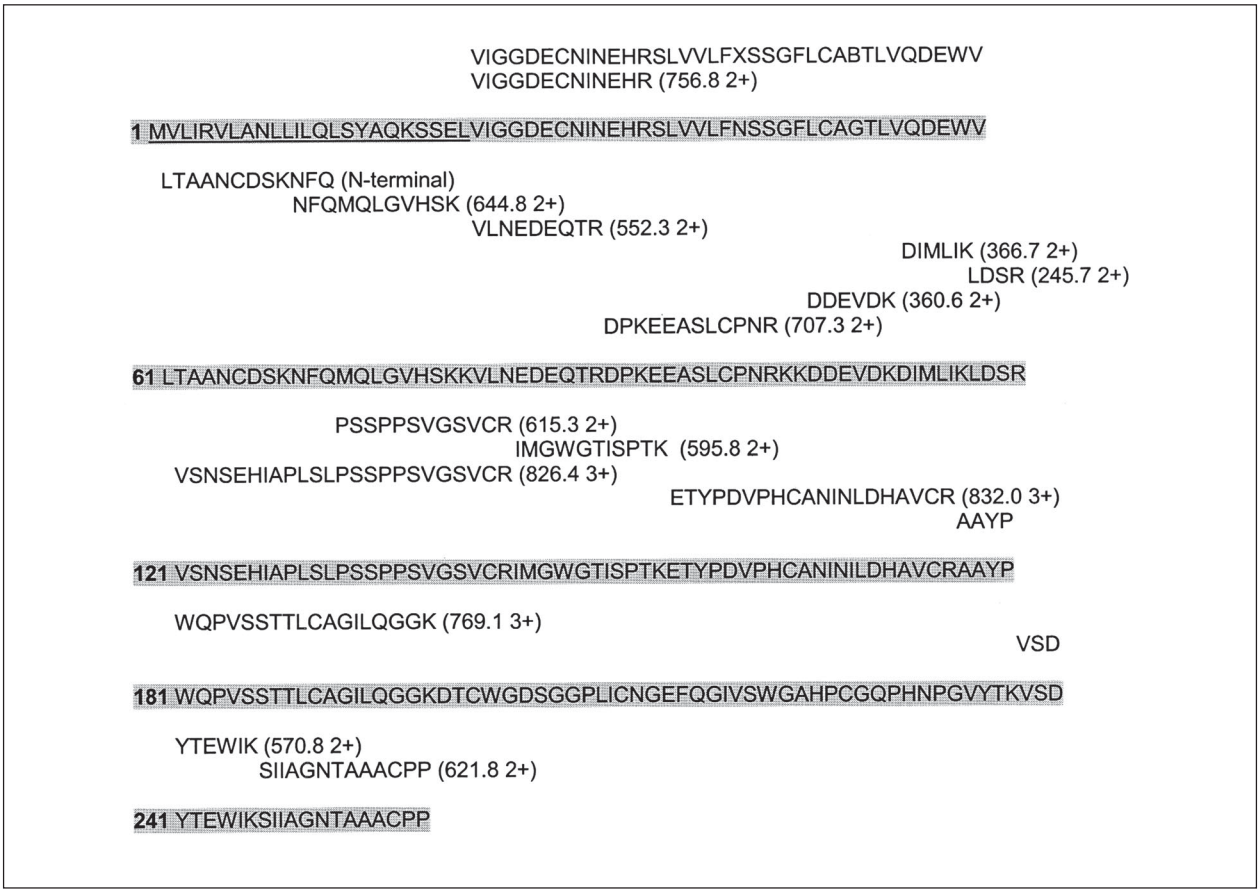

Figure 2. Alignment of the N-terminal sequence of *Bothrops asper* serine proteinase determined by automated Edman degradation (N-terminal), and internal amino acid sequences gathered by collision-induced dissociation tandem mass spectrometry of doubly- and triply-charged tryptic peptides (the m/z value is indicated at the end of each peptide), with the deduced amino acid sequence of a cDNA clone derived from RNA isolated from the venom gland of *B. asper* (highlighted in gray) (UniProtKB/TrEMBL entry Q072L6). The underlined residues correspond to the signal peptide. There is a 100% identity between the sequenced peptides and the deduced sequence.

dominant role of metalloproteinases over serine proteinases in *B. asper*-induced coagulopathy.

Intravenous administration of doses of 10 and 5 µg of the serine proteinase induced a series of behavioral changes in mice within the first 2-3 min after injection. These were characterized by loss of the righting reflex, opisthotonus, and intermittent rotations over the long axis of the body. These effects persisted during approximately 10 min, after which the animals apparently recovered. No such effect was observed with doses below 5 µg. This effect is very similar to that described for gyroxin, a thrombin-like serine proteinase isolated from *Crotalus durissus terrificus* venom (20). Similar observations were made with thrombin-like serine proteinases from other venoms (20). It is likely that this 'gyroxin-like' effect is typical of thrombin-like serine proteinases from viperid snake venoms, although the underlying mechanism remains unknown.

In conclusion, a thrombin-like serine proteinase, present in low concentration, was isolated from the venom of *B. asper* from Costa Rica. It is likely that this enzyme corresponds to asperase, isolated from this venom (13,14), and to a previously described sequence (UniProtKB/TrEMBL entry Q072L6). The enzyme displays fibrinogen-clotting activity *in vitro* and defibrin(ogen)ating activity *in vivo*, and is likely to play a role, albeit not a predominant one, in the coagulopathy characteristic of envenomation inflicted by *B. asper* bites.

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