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Isolation of bothrasperin, a disintegrin with potent platelet aggregation inhibitory activity, from the venom of the snake *Bothrops asper*

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Abstract: The venom of *Bothrops asper* induces severe coagulation disturbances in accidentally envenomed humans. However, only few studies have been conducted to identify components that interact with the hemostatic system in this venom. In the present work, we fractionated *B. asper* venom in order to investigate the possible presence of inhibitors of platelet aggregation. Using a combination of gel filtration, anion-exchange chromatography, and reverse-phase high performance liquid chromatography, we isolated an acidic protein which shows a single chain composition, with a molecular mass of ~8 kDa, estimated by SDS-polyacrylamide gel electrophoresis. Its N-terminal sequence has high similarity to disintegrins isolated from different snake venoms, which are known to bind to cellular integrins such as the GPIIb/IIIa fibrinogen receptor on platelets. The purified protein exerted potent aggregation inhibitory activity on ADP-stimulated human platelets *in vitro*, with an estimated IC_{50} of 50 nM. This biological activity, together with the biochemical characteristics observed, demonstrate that the protein isolated from *B. asper* venom is a disintegrin, hereby named "bothrasperin". This is the first disintegrin isolated from Central American viperid snake species.

Keywords: Snake venom, disintegrin, *Bothrops*, platelet aggregation.

Snake venoms constitute rich sources of proteins that interact with the hemostatic system of vertebrates, either by promoting or by inhibiting particular steps of the coagulation cascade (Markland 1998, Kamiguti *et al*. 1998, Braud *et al*. 2000, Matsui *et al*. 2000). Such proteins may be of central relevance during the clinical course of accidental human envenomations by various snake species, particularly from the families Viperidae and Crotalidae, by inducing striking alterations in the coagulation system that might lead to fatal hemorrhages. In addition, the remarkable specificity of such venom proteins towards defined components of the hemostatic system, in many instances, has opened the possibility to exploit them as molecular tools for the study of coagulation reactions, for the development of *in vitro* diagnostic assays, and for potential therapeutic applications (Niewiarowski *et al*. 1994, Markland 1998, Braud *et al*. 2000).

In Central America, the majority of human envenomations due to snakebites are inflicted by *Bothrops asper*, commonly known as "terciopelo" or "barba amarilla" (Gutiérrez 1995, 2002). As typical of many viperid/crotalid snakes, the venom of *B. asper* induces severe coagulation disturbances (Barrantes *et al*. 1985). Only few studies have been conducted to identify toxins affecting hemostasis in *B. asper* venom, despite their potential relevance for its lethal action. A thrombin-like enzyme has been described (Aragón and Gubensek 1978), and more recently, an inducer of platelet

aggregation (aspercetin) from this venom was reported (Rucavado *et al*. 2001). In the present study, the possible presence of inhibitors of platelet aggregation in the venom of *B. asper* was investigated, resulting in the purification and characterization of a disintegrin, hereby named "bothrasperin".

MATERIALS AND METHODS

Venom. The venom of *Bothrops asper* was a pool obtained from more than 30 specimens collected in the Atlantic region of Costa Rica, and kept at the serpentarium of the Instituto Clodomiro Picado, Universidad de Costa Rica. Immediately after extraction, the venom was centrifuged to remove insoluble debris, lyophilized, and stored at -20°C.

Gel filtration chromatography. Venom samples of 250 mg were dissolved in 0.5 M acetic acid, pH 1.8, and applied to a column of Sephadex G-50 (93 x 2 cm; Pharmacia, Sweden) equilibrated with the same solvent. Elution was carried out at 0.3 ml/min, monitoring proteins with an absorbance detector at 280 nm (Bio-Rad, USA). Fractions of 4 ml were collected, dried in a vacuum centrifuge (Savant SpeedVac System, AES 1010), and stored at -20°C.

Anion-exchange fast protein liquid chromatography (FPLC). Aliquots of 2 mg of fraction III from the gel filtration step were dissolved in 0.05 M ammonium acetate, pH 7.5, and applied to a Mono-Q anion-exchange column using an FPLC instrument (Pharmacia, Sweden). A linear gradient from 0.05 M to 0.24 M ammonium acetate, pH 7.5, was developed in 30 min. Fractions were collected manually, vacuum dried, and finally dissolved in 100 µl of 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2 (PBS) , in order to screen for platelet aggregation inhibitory activity.

High performance liquid chromatography (HPLC). Subfraction III-3 from the anion-exchange chromatography step was applied to a C18 reverse-phase column (15 x 4.6 mm; Vydac, USA) using an HPLC instrument (Waters model 600E, USA). Elution was carried out with a linear gradient from 0 to 60% acetonitrile in 0.1% trifluoroacetic acid, at 1 ml/min during 60 min.

Gel electrophoresis. A triphasic discontinuous polyacrylamide gel electrophoresis system in the presence of sodium dodecylsulphate (SDS-PAGE; Hames 1981) was utilized to monitor the protein composition of venom fractions during purification, as well as to estimate the molecular mass of the isolated disintegrin. Final monomer concentrations of the gel layers were 4% (stacking gel), 10% (upper resolving gel), and 15% (lower resolving gel). A set of very low molecular weight markers (VLMW; Pharmacia, Sweden) was run in parallel to samples, at a constant voltage of 150 V, and proteins were finally visualized by R-250 Coomassie blue staining.

Assay for inhibition of platelet aggregation. Fresh platelet-rich human plasma was prepared by centrifugation of blood from healthy volunteers, at 100 G during 15 min. Aliquots of 450 µl of this preparation were incubated with 10-20 µl of venom fractions for 5 min at 37°C. Then, platelet aggregation was initiated by the addition of 50 µl of 0.1 mM ADP, and monitored by the increase in light transmittance signal using a model 530-VS aggregometer (Chrono-Log Corporation, 530-VS) interfaced to a chart recorder, during 5-8 min. Platelet-poor plasma (450 µl) alone was utilized as a blank, whereas platelet-rich plasma (450 µl) incubated only with ADP (50 µl) was utilized as a positive control for aggregation. All samples were analyzed in duplicate assays. Protein concentration of the samples was estimated by the Bradford colorimetric microassay (Bradford 1976).

N-terminal sequence. The native purified protein was subjected to direct N-terminal sequencing by automated Edman degradation, using a model LF 3000 Beckman sequencer (Beckman, USA).

RESULTS

The separation of *B. asper* venom components by gel filtration on Sephadex G-50 is shown in Fig. 1. All four major peaks obtained (I-IV) caused a direct coagulation of human plasma in the assay for platelet aggregation inhibitory activity, and therefore could not be screened for the presence of aggregation inhibitors. SDS-PAGE analysis revealed that peak I contained the high molecular weight venom components, whereas peak IV had very low amounts of protein, its absorbance being probably due to non-proteinaceous small compounds. Peaks II and III, on the basis of the molecular weight of its components, were selected for further fractionation by anion-exchange FPLC on Mono-Q.

The anion-exchange step resolved 10 subfractions from peak II (not shown), and 11 subfractions from peak III (Fig. 2). Most of these subfractions had direct procoagulant activity on plasma, and were therefore discarded. However, subfraction III-3 (Fig. 2) caused a complete inhibition of platelet aggregation in screening assays. Several III-3 subfractions from different FPLC runs were pooled, and their purity was evaluated by SDS-PAGE, revealing a major band of approximately 8 kDa, and a minor band of 13-14 kDa that became evident only in overloaded gels (Fig. 3). Since the molecular weight of phospholipases A_2 corresponds to 13-15 kDA, an indirect hemolysis assay in agarose gel (Gutiérrez *et al.* 1988) was performed, confirming that this enzymatic activity was contaminating the 8 kDa preparation. Therefore, a third chromatographic step was performed, using RP-HPLC, resulting in the separation of a major sharp peak from few minor contaminants (Fig. 4). This final protein preparation was devoid of phospholipase A_2 activity, and efficiently inhibited platelet aggregation (Fig. 5), thus showing the characteristics of a disintegrin.

By testing a series of different concentrations of the purified disintegrin, an inhibitory concentration 50% (IC_{50}) of 50 nM was calculated as its platelet aggregation inhibitory

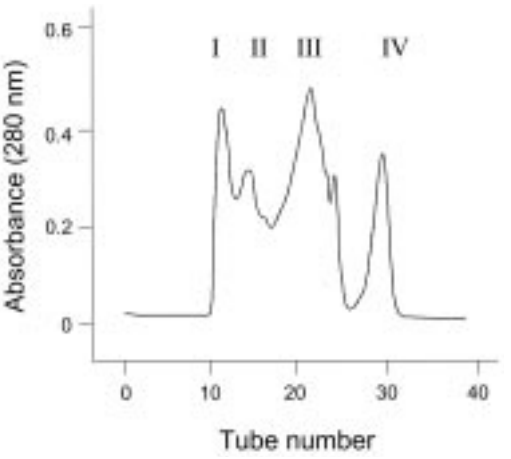

Fig. 1. Gel filtration chromatography of *Bothrops asper* venom on Sephadex G-50. Venom (250 mg) was applied to a G-50 column (93 x 2 cm) equilibrated with 0.5 M acetic acid, pH 1.8. Elution was carried out at 0.3 ml/min, collecting fractions of 4 ml. Proteins were monitored by their absorbance at 280 nm. Main peaks were numbered I to IV, and subjected to further separation.

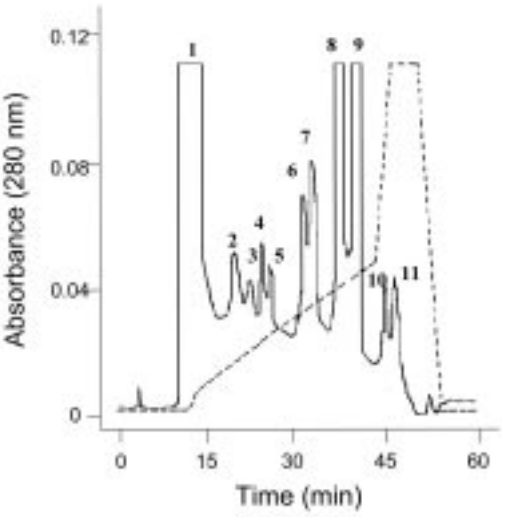

Fig. 2. Anion-exchange chromatography of peak III on Mono-Q-FPLC. Peak III from the gel filtration step (Figure 1) was applied to a Mono-Q-FPLC column, equilibrated with 0.05 M ammonium acetate, pH 7.5, and eluted with a linear gradient (dotted line;) from 0.05 M to 0.24 M ammonium acetate, pH 7.5, in 30 min. Main peaks were numbered 1 to 11. Disintegrin activity was detected in subfraction III-3.

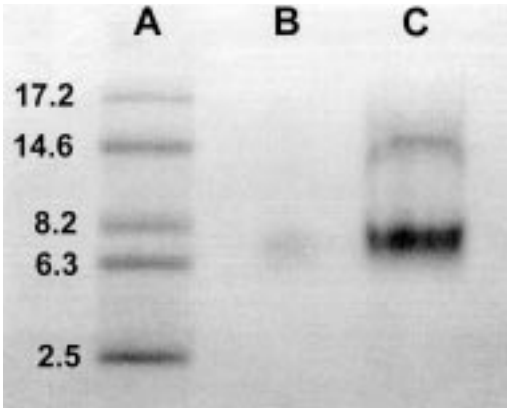

Fig. 3. Triphasic SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of subfraction III-3. Venom subfraction III-3 was analyzed to SDS-PAGE (lane B, 3 µg; lane C: 20 µg) as described in Materials and Methods. A set of very low molecular weight markers (values indicated in kDa) were run in parallel (lane A). The gel was stained with R-250 Coomassie blue.

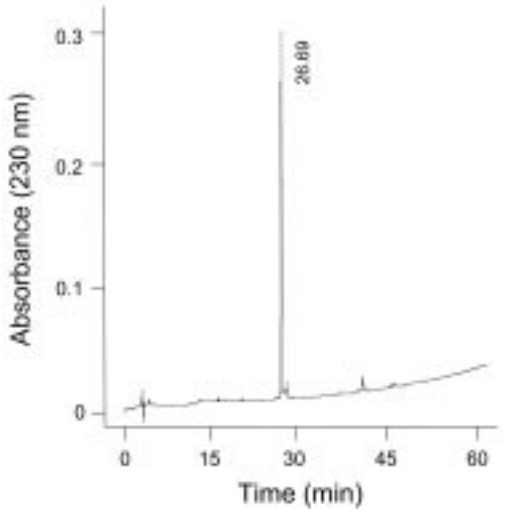

Fig. 4. Reverse-phase high-performance liquid chromatography (RP-HPLC) of subfraction III-3. Subfraction III-3 was applied to a C18 reverse-phase column (15 x 4.6 mm) and eluted with a linear gradient from 0 to 60% acetonitrile in 0.1% trifluoroacetic acid, at 1 ml/min during 60 min. Absorbance was monitored at 230 nm. The retention time of the main peak obtained (26.69 min), corresponding to bothrasperin, is indicated.

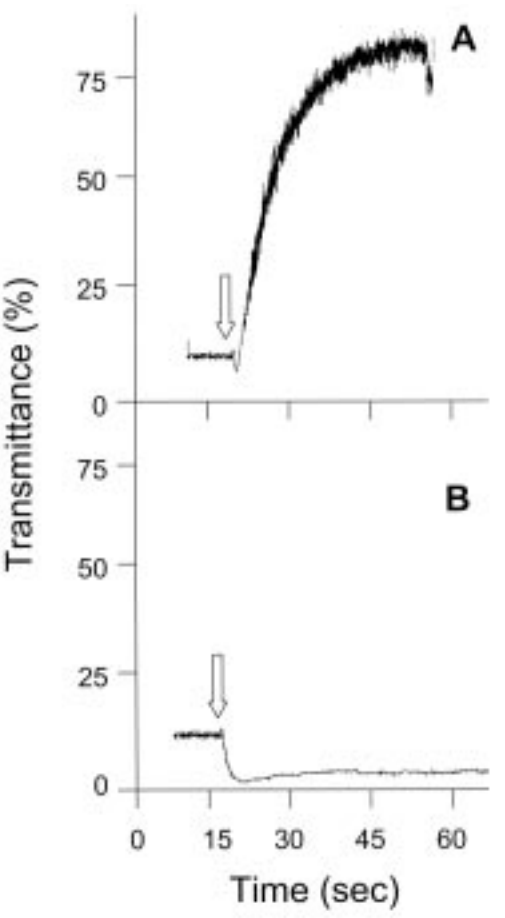

Fig. 5. Inhibition of human platelet aggregation by bothrasperin. (A) ADP was added to platelet-rich human plasma, in order to induce platelet aggregation. (B) plasma was incubated with 1.4 µg of bothrasperin during 5 min at 37°C, before the addition of ADP. Aggregation was monitored by measuring the increase in light transmittance during 5-8 min, as described in Materials and Methods.

potency. The final protein preparation was subjected to N-terminal sequence analysis, in which the first 11 amino acid residues were identified as NH_2-EAGEEXDXGTE (where X is a possible cysteine residue). This partial sequence is compared to that of other disintegrins in Table 1.

TABLE 1
Comparison of the N-terminal amino acid sequence of bothrasperin with other disintegrins isolated from snake venoms

Sequence	Disintegrin	Snake species
EAGEEXDXGTE *	bothrasperin **	*Bothrops asper*
EAGEECDCGTP	jararacin	*Bothrops jararaca*
EAGEECDCGTP	cerastin	*Crotalus cerastes cerastes*
EAGEECDCGAP	lachesin	*Lachesis muta*
EAGEECDCGSP	lutosin	*Crotalus viridis lutosus*
AGEECDCGSP	crotatroxin	*Crotalus atrox*
EAGIECDCGSL	molossin	*Crotalus molossus molossus*
AGEECDCGSP	durissin	*Crotalus durissus durissus*
AGEECDCGSP	viridin	*Crotalus viridis viridis*
EAGEECDCGSP	cereberin	*Crotalus viridis cerebelus*
AGEECDCGSP	basilicin	*Crotalus basilicus*

* "X" indicates an undetermined residue, possibly Cys.
** Present study. Sequence data of other disintegrins is from Scarborough *et al.* (1993).

DISCUSSION

Snake venoms may contain a variety of components with the ability to inhibit platelet aggregation, which, to date, correspond to one of the following types: disintegrins, α-fibrinogenases, 5'-nucleotidases, and phospholipases A_2 (Markland 1998). This study demonstrates that the venom of *B. asper*, the most relevant venomous snake in Central America from the medical point of view, contains at least one component with potent anti-aggregating activity upon human platelets. This protein, here named bothrasperin, was isolated by a combination of gel filtration, ion-exchange, and RP-HPLC chromatographies. It was identified as a disintegrin on the basis of its molecular weight (8 kDa), acidic nature, N-terminal amino acid sequence, and biological activity. Its potency to inhibit human platelet aggregation *in vitro* (IC_{50}) was estimated at approximately 50 nM.

Disintegrins from snake venoms have attracted interest in recent years, due to their clinical potential as platelet aggregation inhibitors in the thrombotic events that are frequently involved in cardiovascular and cerebrovascular disease (Markland 1998). Another relevant area of interest focuses on their anti-angiogenic and anti-metastasic activities in animal cancer models, due to their ability to interact with cellular integrins that participate in the mechanisms of neovascularization and tumor cell invasion (Danen *et al.* 1998, Yeh *et al.* 1998). On the other hand, the precise role of disintegrins in the toxic actions of snake venoms has not been established yet. Some venom hemorrhagic metalloproteinases contain a disintegrin-like domain, which might be released after partial proteolysis (Bjarnason and Fox 1994). Some of these disintegrin-like domains have been shown to interact with integrins (Kamiguti *et al.* 1996, Jia *et al.* 1997). It has been hypothesized that the disintegrin-like domain, by targeting particular cell surface integrins, may have a role in directing the large, multidomain hemorrhagic toxins towards specific locations, therefore enhancing their toxic efficiency (Matsui *et al.* 2000). In addition, if inhibition of platelet aggregation by disintegrins occurs also *in vivo*, hemorrhage induced by venom metalloproteinases might be potentiated, a possibility that awaits to be experimentally evaluated.

The disintegrins usually contain a recognition motif composed by the sequence Arg-Gly-Asp (RGD motif), which confers to them an affinity towards cellular integrins such as the GPIIb/IIIa fibrinogen receptor on platelets

(Zhou *et al.* 1996, Markland 1998), explaining their anti-aggregating activity. In the case of bothrasperin, its N-terminal amino acid sequence showed a high similarity with jararacin, a disintegrin of 73 amino acids (7,739 Da) isolated from the venom of the South American species *Bothrops jararaca* (Scarborough *et al.* 1993). Further characterization of the structural and functional properties of bothrasperin will be of importance to determine its specificity and its role in the pathophysiological alterations occurring in snakebite envenomations by *B. asper*, as well as to explore potentially useful activities related to its ability to inhibit platelet aggregation.

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RESUMEN

El veneno de la serpiente *Bothrops asper* induce graves alteraciones de la coagulación en los humanos accidentalmente envenenados. Sin embargo, se han realizado pocos estudios para identificar los componentes del veneno que interactúan con el sistema hemostático. En el presente trabajo, fraccionamos el veneno de *B. asper* para investigar la posible presencia de inhibidores de la agregación plaquetaria. Empleando una combinación de técnicas cromatográficas (filtración en gel, intercambio aniónico y cromatografía líquida de alto desempeño en fase reversa), aislamos una proteína acídica de cadena simple, con una masa molecular de ~8 kDa, estimada mediante electroforesis en gel de poliacrilamida con SDS. Su secuencia de aminoácidos N-terminal muestra una alta similitud con la de disintegrinas aisladas de diferentes venenos de serpiente, las cuales se unen a integrinas celulares como el receptor de fibrinógeno GPIIb/IIIa de las plaquetas. La proteína purificada ejerce una potente acción inhibitoria sobre la agregación *in vitro* de plaquetas humanas estimuladas con ADP, con una IC_{50} estimada en 50 nM. Esta actividad biológica, sumada a las características bioquímicas observadas, demuestran que la proteína aislada del veneno de *B. asper* es una disintegrina, a la cual denominamos "bothrasperina". Esta es la primera disintegrina aislada de una especie de vipéridos de Centroamérica.

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