Biochemical and Biophysical Research Communications **276,** 29–34 (2000)
doi:10.1006/bbrc.2000.3419, available online at http://www.idealibrary.com on IDEAL®

Edema Induction by the Disintegrin-like/Cysteine-Rich Domains from a *Bothrops atrox* Hemorrhagin

Jorge H. Petretski,* Milton M. Kanashiro,* Flávia R. Rodrigues,* Elias W. Alves,† Olga L. T. Machado,† and Thereza L. Kipnis*,[1]

**Laboratório de Biologia do Reconhecer, Universidade Estadual do Norte Fluminense, Av. Alberto Lamego, 2000, Campos dos Goytacazes, Rio de Janiero, Cep 28015-620, Brazil; and †Laboratório de Quimica e Função de Proteínas e Peptídeos, Universidade Estadual do Norte Fluminense, Brazil*

Received August 10, 2000

Viperine and crotaline snake venoms contain one or more hemorrhagic metalloproteases called hemorrhagins. The most potent hemorrhagins belong to the P-III class and have, in addition to the protease domain, disintegrin-like and cysteine-rich domains. Although proteolytic degradation of vascular endothelium basement membrane has been established to be the main factor responsible for hemorrhage, several studies reveal other factors that actually do facilitate this process. Recent evidence has shown that the nonprotease domains of the P-III class hemorrhagins are able to inhibit the platelet aggregation by blocking essential procoagulant integrins on platelets. In this study we report the identification of a hemorrhagin from *Bothrops atrox* venom. This enzyme, a P-III class metalloprotease, undergoes an apparent spontaneous degradation, releasing a proteic fragment containing the disintegrin-like/cysteine-rich domains. This fragment shows the capability to induce an edematogenic process, suggesting the existence of a still unknown nonenzymatic mechanism of vascular permeability increase.

Key Words: **hemorrhagins; snake venom metalloproteases; edema; disintegrin-like.**

Envenoming by *Bothrops* snakes results in local and systemic effects that develop simultaneously. Local lesions include edema, pain, erythema, ecchymosis, bullae, cyanosis, necrosis, and cellulitis (1). Hemorrhage, blood-clotting, phospholipase A_2 and proteolytic activities, long detected in *Bothrops* venoms, have been implicated in the pathogenesis of these lesions and symptoms (2).

The snake venom hemorrhagins are zinc-metalloproteinases with molecular masses ranging from approximately 20 kDa to over 100 kDa and sharing a conserved proteinase domain (3). Based on their published molecular masses these proteins are distributed into four main classes: P-I class, the smaller hemorrhagins, having molecular masses of 20 to 30 kDa and containing only the proteinase domain; P-II class, the medium-size enzymes with molecular masses of 30 to 50 kDa, having proteinase and disintegrin-like domains; P-III class, the most potent hemorrhagic toxins, with molecular masses of 50 to 80 kDa, having besides the proteinase and disintegrin-like domains, a third cysteine-rich domain; and, P-IV class with molecular masses of 80 to 100 kDa, includes the less toxic hemorrhagins characterized by the presence in their molecules of the proteinase, the disintegrin-like, the cysteine-rich, and the lectin domains (4, 5).

Cumulative evidences indicate that escape of blood from vessels into extravascular tissues induced by snake venom hemorrhagins, follows disruption of the basement membrane underlying vascular endothelial cells. Impairment of the blood coagulation system resulting from platelet aggregation inhibition mediated by the disintegrin-like domain and fibrinogenolysis (6) may contribute to increase the lesions. This hypothesis is compatible with data demonstrating that the most potent hemorrhagins belong to the P-III class, containing disintegrin-like and cysteine-rich domains as well as the protease domain in their molecule (3). Although the main reason for hemorrhage is probably due to the proteolytic disruption of the basement membrane, there have been several contradictions between the proteolytic and hemorrhagic potencies (7).

In this study, we report in *B. atrox* venom, the existence of one hemorragin with molecular mass (m.m.) of 55 kDa, probably belonging to PIII class. This 55 kDa enzyme exhibited a rapid degradation, generating several fragments. Two of these fragments, with m.m. around 26 kDa, were isolated and partially sequenced, presenting at their amino-terminal sequence a high

[1] To whom correspondence should be addressed. E-mail: kipnis@cbb.uenf.br.

0006-291X/00 $35.00

homology with the spacer region connecting protease and disintegrin-like domains. The size of the fragments leads us to deduce that they possess also the cysteine-rich domain. During degradation the proteolytic activity of the fraction containing the 55 kDa enzyme is rapidly reduced but not abolished, suggesting that these domains have a function in the modulation of catalytic activity. The 26 kDa fragments were able to induce edema, demonstrating the existence of a non-enzymatic action in vascular permeability enhancement, directly caused by the disintegrin-like and/or cysteine-rich domains. The data suggests that this still unknown mechanism could be involved in the higher hemorrhagic potency observed in P-III class snake venom metalloproteinases.

MATERIALS AND METHODS

Materials. *Bothrops atrox* venom was provided by the Laboratório de Herpetologia, Instituto Butantan, São Paulo, Brazil. This serpent colony was originally initiated with specimens captured in Tucuruí, Pará State, Brazil. Venom was filtered through a 0.45-μm membrane, lyophilized, divided into 10 mg aliquots and stored at −20°C. Sephacryl S-100-HR and HPLC column DEAE − 5PW were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and Bio-Rad Laboratories (CA), respectively. Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Protein purification. Fifty mg of crude venom were solubilized in 6 ml of 50 mM Tris-HCl buffer, pH 8.0, plus 0.15 M NaCl, filtered through a 0.45-μm millipore filter and applied on a Sephacryl S100-HR column (2.5 cm × 67 cm) equilibrated with the same buffer. The chromatography was performed at 4°C and each fraction was assayed for relative protein concentration, proteolytic activity, relative hemorrhagic activity and edematogenic activity. The pooled fractions were dialyzed against water at 4°C and freeze dried. This material was applied on a DEAE-5PW HPLC anionic exchange column equilibrated with 2.5 mM Tris-HCl, pH 8.3. The protein elution was performed at a flow rate of 0.5 ml/min, using a NaCl plus 2.5 mM Tris-HCl, pH 7.5, gradient as indicated in the figures. The peaks of interest were collected, dialyzed against water at 4°C, freeze-dried and stored at −20°C.

Electrophoresis. SDS-PAGE was performed, as previously described (8) using 15% (w/v) minigels (Bio-Rad Labs., CA) and the protein bands were revealed by the Coomassie blue staining method.

Proteolytic activity assay. Unless specifically stated the proteolytic activity was assayed mixing 25 μl of the fraction to be tested with 0.4 ml of a reaction medium containing 2 mg/ml of azocasein, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.15 M NaCl and 0.05 M Tris-HCl, pH 7.5. After incubation at 35°C, the reaction was stopped by adding 0.1 ml of tricloroacetic acid 25%. The reaction solution was centrifuged at 2000*g* for 5 min. 0.3 ml of supernatant was mixed with 0.3 ml of 2 N NaOH and the solution absorbance was measured at 440 nm. One unit of proteolytic activity was arbitrarily defined as the amount of protein needed to increase 1.0 unit of the absorbance at 440 nm in 1 h.

Hemorrhagic activity assay. Hemorrhagic activity was estimated by injecting intracutaneously (i.c.) 50 μL of the test samples in the abdominal skin of BALB/c mice. After 1 h, mice were anesthetized, killed and the abdominal skin removed. A spot of ≥3 × 3 mm was considered a positive hemorrhagic reaction. The spots were arbitrarily scored from 0 (zero hemorrhagy) to 6 (maximum hemorhagy).

Edematogenic activity assay. Fifty μl of the sample tests were subcutaneously (sc) injected into the posterior left paw of CF1 mice. An equal volume of buffer was injected into the right paw. After 1 h the thickness of each paw was measured in millimeters, using a caliper and the edema estimated as the percent difference between the left and the right paw thickness.

N-terminal sequence determination. N-terminal sequences of the Western-blotted proteins were analyzed on a Shimadzu PPSQ-10 Automated Protein Sequencer performing Edman degradation. PTH-amino acids were detected at 269 nm after separation on a reverse phase C18 column (4.6 × 250 mm) under isocratic conditions, according to the manufacturer's instructions.

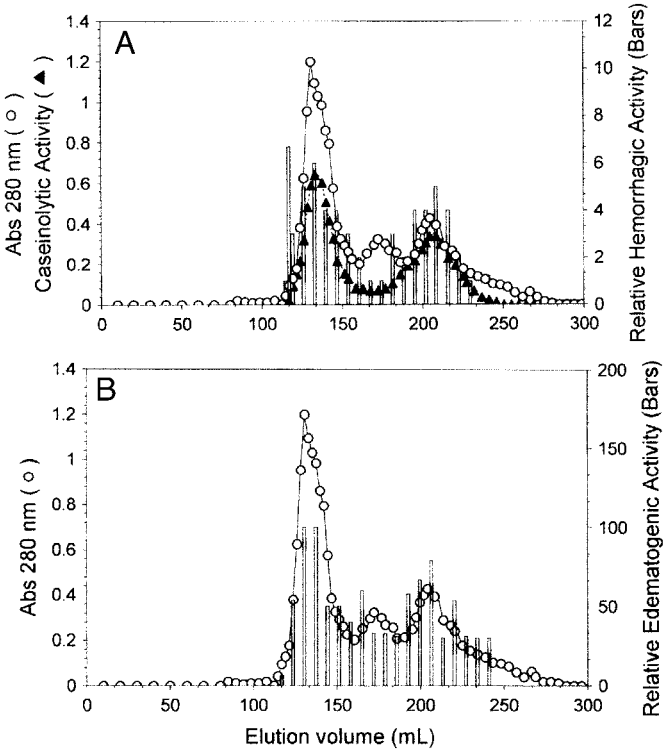

FIG. 1. Size exclusion chromatography of crude *Bothrops atrox* venom. 50 mg of crude venom was solubilized and applied on a Sephacryl S-100 HR column (2.5 × 67.0 cm) as described under Materials and Methods. Relative protein concentration of collected fractions was monitored at 280 nm (open circles, A and B). Proteolytic activity (triangles, A), hemorrhagic activity (bars, 1A) and edematogenic activity (bars, B) were estimated, as described under Materials and Methods.

RESULTS

Crude snake venom from *B. atrox* exhibiting patent caseinolytic, edematogenic and hemorrhagic activities was fractionated by size exclusion chromatography on a Sephacryl S-100 HR column. Proteins were eluted in three major peaks. Proteolytic, hemorrhagic and edematogenic activities were eluted essentially in the first and third protein peaks (Figs. 1A and 1B). The fractions 120 to 150 were pooled and termed HIII pool (hemorrhagins of the PIII-class). After concentration the pool was submitted to DEAE-5PW HPLC ionic exchange chromatography. The pool was resolved into

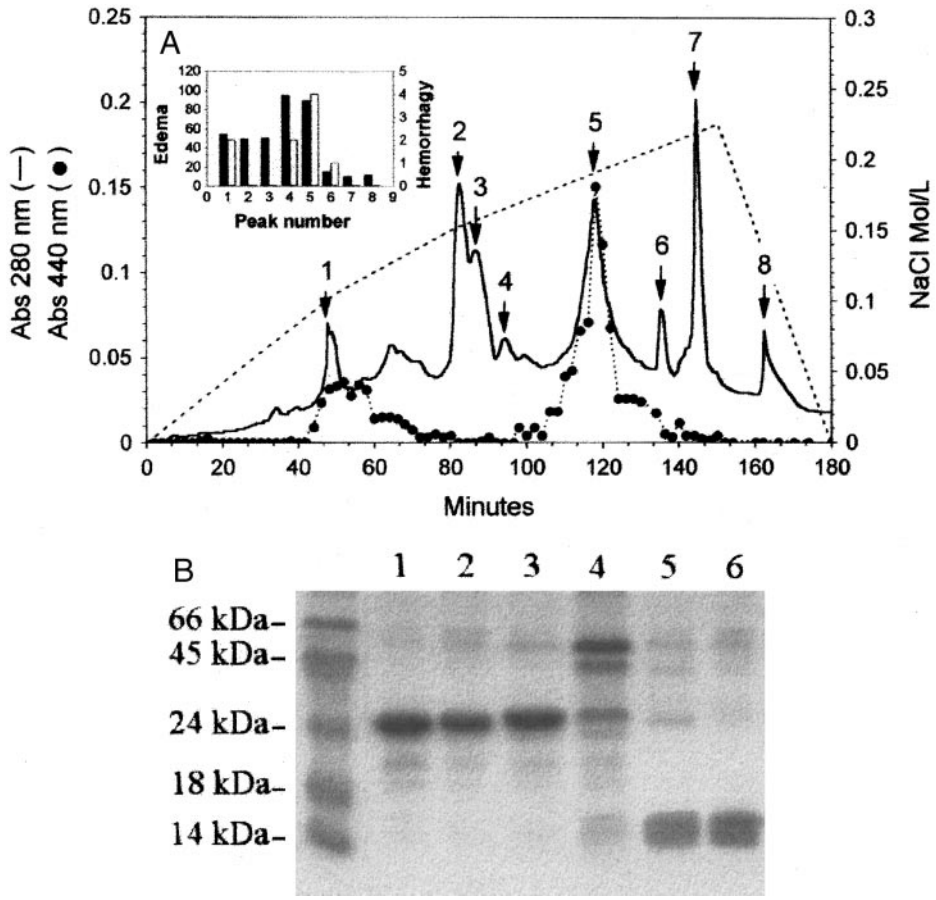

FIG. 2. Ion-exchange chromatography of the HIII pool. (A) The HIII pool was applied on a DEAE-5PW HPLC column, equilibrated with 2.5 mM Tris-Cl, pH 8.3, and eluted with a buffered (2.5 mM Tris-HCl, pH 7.5) NaCl gradient as shown in the figure (dashed line), relative protein (line) concentration was monitored at 280 nm and azocasein proteolysis (circles) were assayed as described under Materials and Methods. Inset: Relative edematogenic activity (black bars) and relative hemorrhagic activity (open bars), were estimated as described under Materials and Methods using 6 μg of each peak. (B) Electrophoretic (15% SDS-PAGE) profile of the peaks 2 to 7. Lane 1, peak 2 (5 μg); lane 2, peak 3 (5 μg); lane 3, peak 4 (5 μg); lane 4, peak 5 (15 μg); lane 5, peak 6 (5 μg); lane 6, peak 7 (5 μg).

eight distinct protein peaks (Fig. 2A and inset). Proteolytic activity was detected in the peaks 1 and 5 and was completely abolished by preincubation in 5 mM of EDTA or EGTA (data not shown), while edematogenic activity spread from peak 1 to peak 5, the hemorrhagic activity was concentrated mostly in the protein peaks 1 and 5 (Fig. 2A). The protein peaks 2, 3, 4, 5, 6 and 7 were collected, concentrated and named HIII-2, HIII-3, HIII-4, HIII-5, HIII-6 and HIII-7 fractions, respectively. A SDS-PAGE (15%) analysis of these protein peaks showed the presence, in addition to a protein band of around 55 kDa, of a protein band with a molecular mass of 26 kDa in peaks 2, 3, 4, and 5. Whilst the former band became more intense from peaks 2 to 5, the latter protein band became progressively less pronounced in the peaks 5, 6 and 7. A concomitant progressive appearance of a 14 kDa protein band also occurred, in addition peak 5 showed also a band of 40 kDa that disappeared in the consecutive peaks (Fig. 2B).

In former preparations, attempting to adjust the ionic gradient, the observation of variations in the area of the chromatogram peaks and in the relative intensity of the bands obtained from HIII-5 (data not shown), suggested that the HIII-5 fraction could be undergoing degradation with concomitant generation of other bands. The protein containing in this fraction was quite stable at low ionic strength but, when dissolved in solutions of NaCl above 0.085 M, it was quickly converted into fragments of 40 and 26 kDa (Fig. 3A). During the degradation of the 55 kDa protein the proteolytic activity was quickly reduced in the first two hours of incubation, remaining constant until 6 h and decaying slowly thereafter. The edematogenic activity also decreased, but in a slightly slower manner (Fig. 3B).

With the aim of identifying these 55 kDa cleavage products, the fractions HIII-2, HIII-4 and HIII-5 were submitted to a 15% SDS-PAGE. The protein bands of around 26 kDa from HIII-2 and HIII-4 and the protein

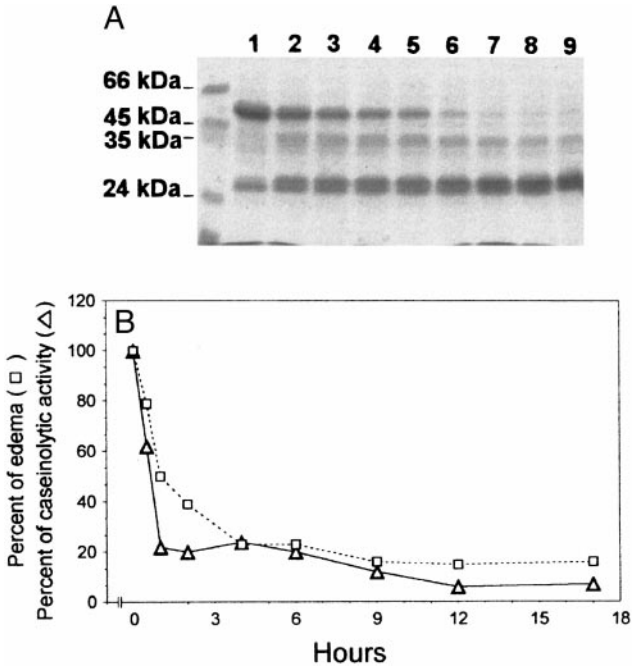

FIG. 3. Degradation of the 55 kDa protein from the HIII-5 fraction. 450 μg of a freshly prepared HIII-5 fraction was incubated in 20 mM Tris-HCl, pH 8.0, plus 150 mM NaCl at 35°C. At indicated times 45 μg were withdrawn and used to analyze the protein profile and estimate the proteolytic and edematogenic activities. (A) Electrophoretic (15% SDS-PAGE) profile of HIII-5 at different incubation times, 7.5 μg of protein were applied on each lane. Lane 1, 0 time, lane 2, 0.5 h; lane 3, 1 h; lane 4, 2 h; lane 5, 4 h; lane 6, 6 h; lane 7, 9 h; lane 8, 12 h; and lane 9, 17 h. (B) The proteolytic (HIII-5, 15 μg) and the edematogenic (HIII-5, 15 μg) activities were estimated as described under Materials and Methods. Both activities were expressed as percentage, considering as 100% the activities levels at 0 time of incubation.

bands with 55 and 40 kDa from HIII-5 were excised. The attempts to sequence the 55 and 40 kDa bands from HIII-5 fraction were unsuccessful, indicating that the amino-terminal region of these proteins is blocked. On other hand the amino-terminal sequence of the 26 kDa fragments from HIII-2 and HIII-4 was determined, showing that both fragments starts at the spacer region between the disintegrin-like and protease domains, found in all the hemorrhagins belonging to the P-II and P-III classes (Fig. 4). Based on the published molecular masses of other snake venom metalloproteases it is reasonable to conclude that these fragments are composed by the disintegrin-like and the cysteine-rich domains.

TABLE 1

Comparison of the Edematogenic and Hemorrhagic Activity of HIII-2 and HIII-5

Preparation used (15 μg)	Preincubated without EDTA		Preincubated with 20 mM EDTA	
	Edema	Hemorrhage	Edema	Hemorrhage
HIII-5	71%	Yes	33%	No
HIII-2	57%	No	45%	No

Note. The indicated amounts of the preparations were incubated at 4°C by 30 min in 25 μL of a medium containing 20 mM Tris-HCl, pH 8.0, and 200 mM NaCl with or without 20 mM EDTA. After the incubation the solution was diluted adding an equal volume of deionizated water. The mice were injected as described under Materials and Methods. The control solution was the incubation medium without protein and properly diluted. The results are representative of three independent experiments.

The capacities of the fractions HIII-5 and HIII-2 to induce hemorrhage and edema were compared by assaying them into mice (Table 1). The results demonstrates that both fractions were able to induce edema, but only the HIII-5 fraction was able to produce hemorrhage when injected i.c. in mice. A preincubation with EDTA 20 mM, in order to abolish the proteolytic activity, highly inhibited the edema induced by HI-5 and nearly do not affected the action of HIII-2. The edema was quickly formed being visually detectable in 10 min, reaching its maximum in 60 min (data not shown). The HIII-2 edema forming effect was completely abolished after heating the protein preparations at 100°C for 20 min (data not shown), suggesting that the edematogenic forming mechanism is conformation-dependent.

DISCUSSION

The snake venom hemorrhagic toxins, often termed hemorrhagins, comprise a large group of metallopro-

```
HIII-2 -          1                   I V S P P V C G N E L L E V G
HIII-4 -          1  L I Q E P L G P X I V S P P V
Jararhagin -    201  I I N E P L G T D I V S P P V C G N E L L E V G
Atrolysin A -   197  I L N E P L Q T D I I S P P V C G N E L L E V G
HRIB -          201  I L N A P S K T D I V S P P V C G N E L L E A G
```

FIG. 4. HIII-2 and HIII-4 sequence homology. Amino acid sequence homology between HIII-2, HIII-4, and the snake venom metalloproteases jararhagin (9), atrolysin A (10) and HRIB (11). The numbers indicate the positions of amino acid residues from N-terminus.

teases largely involved in the pathogenesis of envenoming that results from *Bothrops* bites. Approximately 102 metalloproteinases with molecular masses ranging from 15 to 100 kDa have been purified from 35 snake venom species (5). Many of these studies, though, have yet to determine completely the mechanism of action of these proteins.

Size exclusion chromatography resolved *B. atrox* crude venom samples into three major protein peaks. Hemorrhagic, edematogenic and caseinolytic activities were essentially associated with the first and the third of these peaks. When, however, the first protein peak, termed HIII pool, was chromatographed on an ionic exchange column, edematogenic activity could be detected in protein fractions apparently free of hemorrhagic or proteolytic activity. It may be suggested, therefore, that HIII pool contains at least two edematogenic activities, one weakly retained by the DEAE-5PW anionic exchange column, and the other strongly retained and eluted together with the hemorrhagic activity. The proteolytic activity exhibited by samples from protein peaks containing edematogenic and hemorrhagic activities were, however, abrogated if the caseinolytic assay was performed in the presence of the cation chellating agents, EDTA or EGTA. Further studies are required to characterize the mechanism responsible for any correlation between edematogenic and enzymatic proteolytic activities.

The 55 kDa hemorrhagic-inducing fraction enriched from the *B. atrox* snake venom, besides having potent hemorrhagic and edema-inducing properties when assayed *in vivo* exhibited, as expected for a metalloprotease, a cation-dependent proteolytic activity. This protein is quite stable in water, but in the presence of high ionic strength is rapidly converted into smaller polypeptide fragments which include a 26 kDa protein band. Cleavages of the molecule occurs at the spacer region between the protease and disintegrin-like domains. These fragments are endowed only with edematogenic activity but at a lower level than that of the original one. Results suggest that in the original 55 kDa fragment hemorrhagic and edematogenic are concurrent activities.

Based on the published sequences and sizes of three domains of P-III class snake metalloproteinases, it is reasonable to assume that the 26 kDa fragments are composed of the spacer region connected to the disintegrin-like and cysteine-rich domains. It is already well established that the disintegrin-like domain is a platelet aggregation inhibitor by specifically binding to the platelet surface fibrinogen receptor α_{IIb}/β_3 integrin and $\alpha_2\beta_1$ collagen integrin (12, 13). Recently was reported that the cysteine-rich domain is also involved in the inhibition of the collagen-stimulated platelet aggregation (14). It has been speculated that the disintegrin-like domain could also modulate the metalloprotease activity targeting the hemorrhagin to a particular site of action attaching the enzyme to its substrate (15). The degradation pattern observed at Fig. 3 reinforces this rational. Apparently the detachment of the disintegrin-like domain from the protease domain results in a far less active protease. At this moment we are aiming to sequence to completion all the protein fragments generated by the 55 kDa protein proteolysis in order to clarify the degradation process itself and its correlation with reduction of the proteolytic capability.

However, the most striking results presented here are the significant edematogenic process caused by the 26 kDa fragments HIII-2, HIII-3 and HIII-4 (Fig. 2A, inset), even after a pre-incubation with EDTA to avoid interference of any possible protease contamination (HIII-2, Table 1). These data indicate the existence of an unreported nonenzymatic mechanism of vascular permeability increase, probably mediated by the binding of the disintegrin-like, and/or the cysteine-rich domains, at specific sites on the vascular endothelium. Experiments with the objective to elucidate this mechanism are now in progress.

ACKNOWLEDGMENTS

This work was supported by grants from FENORTE, FINEP, CNPQ, and FAPERJ.

REFERENCES

1. Barraviera, B., and Pereira, P. C. M. (1994) Acidentes por serpentes do gênero *Bothrops*. in Venenos Animais: Uma Visão Integrada. (Barraviera, B., Ed.), pp. 261–280. Publicações Científicas Ltda, Rio de Janeiro.
2. Assakura, M. T., Reichl, A. P., Asperti, M. C. A., and Mandelbaum, F. R. (1985) Isolation of the major proteolytic enzyme from the venom of the snake *Bothrops moojeni* (caissara). *Toxicon* **23,** 681–706.
3. Hite, L. A., Jia, J. B., Bjarnason, J. B., and Fox, J. M. (1994) cDNA sequences for four snake venom metalloproteinases: Structure, classification, and their relationship to mammalian reproductive proteins. *Arch. Biochem. Biophys.* **308,** 182–191.
4. Bjarnason, J. B., and Fox, J. W. (1995) Snake venom metalloendopeptidases: Reprolysins. *Methods Enzymol.* **248,** 345–368.
5. Bjarnason, J. B., and Fox, J. W. (1994) Hemorrhagic metalloproteinases from snake venoms. *Pharmacol. Ther.* **62,** 321–372.
6. Kamiguti, A. S., Hay, C. R. M., Theakston, R. D. G., and Zuzel, M. (1996) Insights into the mechanism of hemorrhage caused by snake venom metalloproteinases. *Toxicon* **34,** 627–642.
7. Hati, R., Mitra, P., Sarker, S., and Bhattacharyya, K. K. (1999) Snake venom hemorragins. *Crit. Rev. Toxicol.* **29,** 1–19.
8. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature* **227,** 680–685.
9. Paine, M. J. I., Desmond, H. P., Theakston, R. D. G., and Crampton, J. M. (1992) Purification, cloning, and molecular characterization of a high molecular weight hemorrhagic metalloprotease, jararhagin, from *Bothrops jararaca* venom. *J. Biol. Chem.* **267,** 22869–22876.
10. Fox, J. W., and Bjarnason, J. B. (1995) Atrolysins: Metalloproteinases from *Crotalux atrox* venom. *Methods Enzymol.* **248,** 368–387.
11. Takeya, H., Oda, K., Miyata, T., Omori-Satoh, T., and Iwanaga,

S. (1990) The complete amino acid sequence of the high molecular mass hemorrhagic protein HRIB isolated from the venom of *Trimeresurus flavoviridis*. *J. Biol. Chem.* **265,** 16068–16073.

12. Jia, L. J., Wang, X. M., Shannon, J. D., Bjarnason, J. B., and Fox, J. W. (1997) Function of disintegrin-like/cysteine-rich domains of atrolysin A. *J. Biol. Chem.* **272,** 13094–13102.
13. De Luca, M., Ward, C. M., Ohmori, K., Amdrews, R. K., and Berndt, M. C. (1995) Jaraahagin and jaracetin: Novel snake venom inhibitors of the integrin collagen receptor, $\alpha_2\beta_1$. *Biochem. Biophys. Res. Commun.* **206,** 570–576.
14. Jia, L. J., Wang, X. M., Shannon, J. D., Bjarnason, J. B., and Fox, J. W. (1999) Inhibition of platelet aggregation by the recombinant cysteine-rich domain of the hemorrhagic snake venom metalloproteinase, atrolysin A. *Arch. Biochem. Biophys.* **373,** 281–286.
15. Zhou, Q., Smith, J. B., and Grossmam, M. (1995) Molecular cloning and expression of catrocollastatin, a snake venom protein from *Crotalus atrox* (western diamondback rattlesnake) which inhibits platelet adhesion to collagen. *Biochem. J.* **307,** 411–417.