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Purification and characterization of patagonfibrase, a metalloproteinase showing α-fibrinogenolytic and hemorrhagic activities, from *Philodryas patagoniensis* snake venom

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Abstract

Venoms of Colubridae snakes are a rich source of novel compounds, which may have applications in medicine and biochemistry. In the present study, we describe the purification and characterization of a metalloproteinase (patagonfibrase), the first protein to be isolated from *Philodryas patagoniensis* (Colubridae) snake venom. Patagonfibrase is a single-chain protein, showing a molecular mass of 53,224 Da and an acidic isoelectric point (5.8). It hydrolyzed selectively the Aα-chain of fibrinogen and when incubated with fibrinogen or plasma, the thrombin clotting time was prolonged. Prominent hemorrhage developed in mouse skin after intradermal injection of patagonfibrase. When administered into mouse gastrocnemius muscle, it induced local hemorrhage and necrosis, and systemic bleeding in lungs. Patagonfibrase showed proteolytic activity toward azocasein, which was enhanced by Ca^{2+} and inhibited by Zn^{2+}, cysteine, dithiothreitol and Na_2EDTA. Patagonfibrase impaired platelet aggregation induced by collagen and ADP. Thus, patagonfibrase may play a key role in the pathogenesis of disturbances that occur in *P. patagoniensis* envenomation, and may be used as a biological tool to explore many facets of hemostasis.

Keywords: Colubrid snake venom; Fibrinogen; Hemorrhage; Metalloproteinase; Coagulation; Platelets

1. Introduction

While many investigations have dealt with purification and characterization of venom components from front-fanged snakes (e.g. Viperidae and Elapidae), little is known about the composition and biological activities of venoms from the polyphyletic family Colubridae, the world's largest snake family. This is probably because these species are mainly rear- or mid-fanged, which means that not only is it more difficult for them to bite physically a human being, but also because it is much harder to extract venom for research purposes [1–3]. Dangerously venomous colubrids are less commonly encountered, and serious bites typically require longer contact time, and thus they pose a minor problem to humans compared with the two major families of medically important land snakes, the Viperidae and the Elapidae [2].

Venoms of Colubridae snakes are a rich source of novel compounds, which may have applications in medicine and biochemistry. Currently, few colubrid venom proteins have been isolated and characterized, primarily because of lack of sufficient starting material [3,4].

Philodryas is a genus of rear-fanged colubrid snakes which is found in South America, from Amazonas to Patagonia [5,6]. The properties of crude venoms from *Philodryas olfersii* and *Philodryas patagoniensis* have been studied extensively [5,7–10].

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However, isolation and characterization of components have been only carried out from *P. olfersii* venom [6,11–14].

Philodryas patagoniensis, an opisthoglyphous snake with a well-developed Duvernoy's gland connected to a grooved tooth, is found in Argentina, Bolivia, Brazil, Paraguay and Uruguay [15,16]. Human envenomation by this colubrid snake, although rare and not yet reported to be lethal, is characterized by pain, swelling, heat and ecchymotic lesions on the bitten limb, bearing a striking resemblance to local signs and symptoms of *Bothrops* sp. envenomation [17,18]. Similarly, *P. patagoniensis* venom (=Duvernoy's gland secretion) induces edema in the mouse foot pad, as well as hemorrhage and necrosis in mouse skin and gastrocnemius muscle [7,9]. Furthermore, it shows high proteolytic and fibrin(ogen)olytic activities [7,10], but it is devoid of thrombin-like, procoagulant and phospholipase A_2 activities [9,10]. The present study is the first report on the purification and characterization of an α-fibrinogenolytic and hemorrhagic metalloproteinase, named patagonfibrase, isolated from the venom of *P. patagoniensis*.

2. Materials and methods

2.1. Materials

A pool of *P. patagoniensis* venom was obtained from specimens captured in northeastern Argentina and then maintained at the serpentarium of the local Zoo, Corrientes, Argentina. Specimens were milked by introducing an 100-μl micropipette under each fang, according to the procedure described by Ferlan and co-workers [19]. Venom was lyophilized and thereafter kept frozen at − 20 °C. When required, venom was dissolved in 50 mM Tris–HCl buffer (pH 7.4) and filtered through a 0.22 μm Millipore filter to remove insoluble material. Bicinchoninic acid (BCA) protein assay kit, bovine serum albumin (BSA), phenylmethanesulfonyl fluoride (PMSF), dithiothreitol (DTT), L-cysteine, collagen, bovine thrombin, adenosine 5′-diphosphate (ADP), ristocetin and azocasein were obtained from Sigma Chemical (St. Louis, MO, USA). Molecular mass standards were obtained from Bio-Rad (USA). All other chemicals were of analytical reagent grade.

2.2. Animals

To determine the minimal hemorrhagic dose (MHD) of patagonfibrase, outbred male Swiss mice, weighing from 18 to 22 g, supplied by the Animal House of Butantan Institute, were used. To determine local and systemic damage induced by patagonfibrase, male CF-1 mice, weighing from 18 to 20 g, supplied by the Animal House, College of Veterinarian Sciences, University of Northeastern Argentina, were used. All experiments followed the ethical standards for animal experiments in toxinological research recommended by the International Society of Toxinology [20].

2.3. Purification of patagonfibrase

Venom was dissolved in buffer A (50 mM Tris–HCl, pH 7.4) and applied to a Mono-Q 5/50 GL column (1 ml) (GE Healthcare, Sweden), previously equilibrated in the same buffer, using a fast protein liquid chromatography (FPLC) system. The column was washed with 10 volumes of buffer A and elution was carried out with an increasing linear gradient of 0–0.3 M NaCl in buffer A, at a flow rate of 1 ml/min. Protein concentration was monitored by measuring the absorbance at 280 nm. Fractions of 1 ml were collected and assayed for proteolytic activity, using azocasein and fibrinogen as substrates (see below), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on selected fractions. Fractions containing proteolytic activity were pooled and dialyzed against buffer A prior to being applied to an HiTrap Blue HP column (1 ml) (GE Healthcare, Sweden) equilibrated with buffer A, using an ÄKTA design system. After washing the column with 20 volumes of buffer A (flow rate of 0.5 ml/min), bound proteins were eluted with a linear gradient of 0–1.5 M NaCl in buffer A, at a flow rate of 1 ml/min. Protein concentration was monitored by measuring the absorbance at 280 nm. One-ml fractions were collected, screened for caseinolytic and fibrinogenolytic activities, and pooled.

To evaluate the purity of the isolated metalloproteinase, reversed-phase high performance liquid chromatography (HPLC) was run on a Sephasil Peptide C 8 column, previously equilibrated with solvent A [0.1% trifluoroacetic acid (TFA) in water], using an ÄKTA design system. The enzyme was eluted with 0–100% increasing linear gradient of solvent B (0.1% TFA/90% acetonitrile) at a flow rate of 1 ml/min for 60 min. The elution profile was monitored at 215 and 280 nm.

2.4. Protein quantification

Protein concentration of samples was determined by BCA protein assay, using BSA as standard [21].

2.5. SDS-PAGE

Protein samples were electrophoresed on 12% polyacrylamide slab gels following the method of Laemmli [22] and then silver stained [23].

2.6. Mass spectrometry

Approximately 0.5 μg protein in 50% acetonitrile was spotted onto sinapinic acid matrix and allowed to dry. Mass spectrum was obtained using an Applied Biosystems Voyager DE Pro MALDI-TOF mass spectrometer, operating in delayed extraction and linear mode [24].

2.7. Isoelectric focusing (IEF)

The isoelectric point (pI) of patagonfibrase (216 ng) was determined by performing isoelectric focusing in a PhastSystem Apparatus (GE Healthcare, Sweden) with precast polyacrylamide gels (PhastGel IEF 3–10). Calibration standards (GE Healthcare, Sweden) were between pH 3.5 and 9.3. Gels were silver stained [23].

2.8. Fibrinogenolytic activity

Specific cleavage of fibrinogen by crude venom, fractions and active enzyme was determined by SDS-PAGE using 12% polyacrylamide gels. Two hundred microliters of 2 mg/ml bovine fibrinogen [25] dissolved in buffer A were incubated at 37 °C with 10 μl of sample. At various time intervals, aliquots were withdrawn from the digestion mixture, and then denatured and reduced by boiling for 7 min with denaturing solution (4% SDS, 20% glycerol and 20% 2-mercaptoethanol) for SDS-PAGE. For inhibition studies, patagonfibrase (21 μg/ml, final concentration) was incubated with the following inhibitors: ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), PMSF or DTT (2 mM in all cases, final concentration) for 1 h at 37 °C before incubation with bovine fibrinogen.

2.9. Thrombin clotting time

Fibrinogenolytic activity was also estimated by incubating 100 μl of 2 mg/ml bovine fibrinogen [25] or human citrated plasma with 10 μl of 1.7 mg/ml patagonfibrase at 37 °C for 10 min. Then, 50 μl of 2 U/ml bovine thrombin were added and the clotting time recorded.

2.10. Caseinolytic activity

An azocasein assay [26] was used to determine specific proteolytic activity of patagonfibrase. Two-fold serial dilutions of patagonfibrase were tested in order to construct a dose–response curve. One unit was defined as the amount of enzyme yielding an increase in absorbance of 1.0 per min at 450 nm. Specific activity was expressed as units/mg protein.

2.11. Effects of pH, temperature, divalent cations and inhibitors on patagonfibrase activity

The effect of pH, temperature, divalent cations and inhibitors on patagonfibrase activity was tested using the caseinolytic assay as previously described [26]. The optimum pH of patagonfibrase (21 μg/ml) was determined over a pH range of 5.0–11.0, using acetate (pH 5.0), HEPES (pH 6.0–7.0), and Tris–HCl (7.5–11.0) buffers (all 50 mM and containing 100 mM NaCl). The temperature in which patagonfibrase (21 μg/ml) showed its maximum activity was examined by incubating the reaction mixture at the optimum pH and at 25, 37 or 55 °C for 90 min. The effect of $MgCl_2$, $CaCl_2$, $ZnCl_2$, Na_2EDTA, DTT, L-cysteine (5 mM in all cases, final concentration) and PMSF (2 mM, final concentration) on patagonfibrase activity was tested by incubating the enzyme (21 μg/ml) with these compounds.

2.12. Inhibition of patagonfibrase by human serum

Equal volumes of patagonfibrase solution (21 μg/ml, final concentration) and human serum (two-fold serial dilutions) were incubated for 30 min at 37 °C. The residual proteolytic activity of patagonfibrase was tested using the caseinolytic assay as described above.

2.13. Hemorrhagic activity

Hemorrhagic activity was evaluated using a modification of the method described by Gonçalves and Mariano [27]. Different doses of the purified protein (0.125–1.000 μg) were injected i.d. in mice, using 3–5 animals per dose. Two hours after injection, animals were sacrificed and the skin was removed. Hemorrhagic haloes were immediately cut, fragmented, and added to tubes containing 4 ml of Drabkin's reagent. The tissue was homogenized and the reaction mixtures were incubated in absence of light at room temperature for 24 h. Thereafter, tubes were centrifuged at 1900×*g* for 10 min. The absorbance of the supernatant at 540 nm was determined in a spectrophotometer (Micronal, Brazil), and then hemoglobin concentration was calculated [27]. The MHD was defined as the minimal concentration of protein able to induce an increase of three times in hemoglobin concentration in relation to that of a control tissue. To test inhibition of hemorrhagic activity, patagonfibrase (8 μg/ml) was incubated with the following inhibitors: Na_2EDTA or PMSF (2 mM in both cases, final concentration) for 1 h at 37 °C prior to injections.

2.14. Local and systemic damage induced by patagonfibrase in mice

Groups of four mice were injected in the right gastrocnemius muscle with 8 μg of *P. patagoniensis* venom or 8 μg of patagonfibrase dissolved in 200 μl of 50 mM Tris–HCl buffer (pH 7.4). Control mice were injected with 200 μl of 50 mM Tris–HCl buffer (pH 7.4) under identical conditions. Twelve hours after injection, mice were anesthetized with chloral hydrate i.p. (300 mg/kg) and blood samples were collected from the abdominal portion of the aorta. Serum was obtained for the enzymatic determination of creatine kinase (CK) levels using an Ultraviolet Kinetic Kit (Sigma, USA). For histological analysis, mice were sacrificed by an overdose of anesthetic 12 h after injection, and small samples of muscle, kidney, heart, liver, and lung were collected and fixed in Bouin's solution for 24–48 h. Thereafter, the tissue samples were dehydrated in a graded alcohol series and embedded in paraffin. Five μm sections were cut and stained with hematoxylin and eosin to be examined under a light microscope. The control samples were processed as described above.

2.15. Platelet aggregation assay

The effect of patagonfibrase on the aggregation of washed human platelets or platelet-rich plasma (PRP) was tested as described previously [28]. Platelet count was ascertained to 300×10^9/l and platelet aggregation was performed in a Chrono-log lumiaggregometer (model 560VS) by the method of Born [29]. One μg/ml collagen, 0.2 U/ml bovine thrombin or 10 μM ADP (final concentrations) were used as agonists for platelet aggregation in washed platelet suspensions; to test platelet aggregation in PRP, 10 μM ADP or 1.5 mg/ml ristocetin (final concentrations) were used as agonists. Bovine fibrinogen was added (150 μg/ml, final concentration) before testing ADP-induced platelet aggregation in washed platelet suspensions.

2.16. Statistical analysis

Where appropriate, the results were expressed as mean±standard deviation (SD). Differences between groups were compared using one-way analysis of variance (ANOVA) followed by Dunnett's test. A value of $p<0.05$ indicated statistical significance.

3. Results

3.1. Purification of patagonfibrase

A fibrinogenolytic and hemorrhagic metalloproteinase from *P. patagoniensis* venom (patagonfibrase) was purified in two chromatographic steps. Venom fractionation by anion exchange chromatography on a Mono-Q column yielded three major protein peaks (Fig. 1A). Caseinolytic and fibrinogenolytic activities were only found in peaks 1 (basic) and 2 (acidic). Active fractions in peak 2 were pooled, desalted, concentrated and chromatographed on a HiTrap Blue HP column (Fig. 1B). Caseinolytic and fibrinogenolytic activities were found in peaks 2 and 3 of Blue HP column. Peak 3 showed several bands on SDS-PAGE, while peak 2 was homogenous by SDS-PAGE (data not shown), and the enzyme present in peak 2 was investigated further. The homogeneity of this purified protein was also verified by C8 reversed-phase HPLC (Fig. 1C). A summary of the purification procedure for patagonfibrase is shown in Table 1. The protein yield was 2.8% with a 15.6% recovery of enzyme activity. The specific activity of the purified protein was 4.84 units/mg, corresponding to a purification factor of 6.2.

The relative molecular mass of patagonfibrase determined by SDS-PAGE under non-reducing and reducing conditions was 57.8 and 57.3 kDa, respectively (Fig. 1D), evidencing that it exists as a single polypeptide chain protein. Mass spectral analyses of the intact protein yielded a molecular mass value of 53,224 Da (Fig. 1E), and the broad peak with multiple apparent isoforms suggested that the protein was glycosylated. Patagonfibrase also showed a single band following isoelectric focusing, with a pI of 5.8 (data not shown).

3.2. Fibrinogenolytic activity

Patagonfibrase degraded the Aα-chain of fibrinogen molecule, whereas the Bβ-chain was essentially unaffected. The γ-chain was resistant, even when the incubation time was prolonged to 24 h (Fig. 2A). Based on these characteristics, patagonfibrase was classified as an α-fibrinogenase. Digestion of Aα-chain by patagonfibrase resulted in the appearance of high relative molecular mass degradation products, particularly polypeptides of 44, 43 and 42 kDa. When patagonfibrase was incubated with fibrinogen or plasma, no clot was produced. Moreover, when thrombin was added to the incubation mixtures, patagonfibrase caused a prolongation of clotting time of human citrated plasma (>120 s; control: 27 s) and bovine fibrinogen (98 s; control: 9 s).

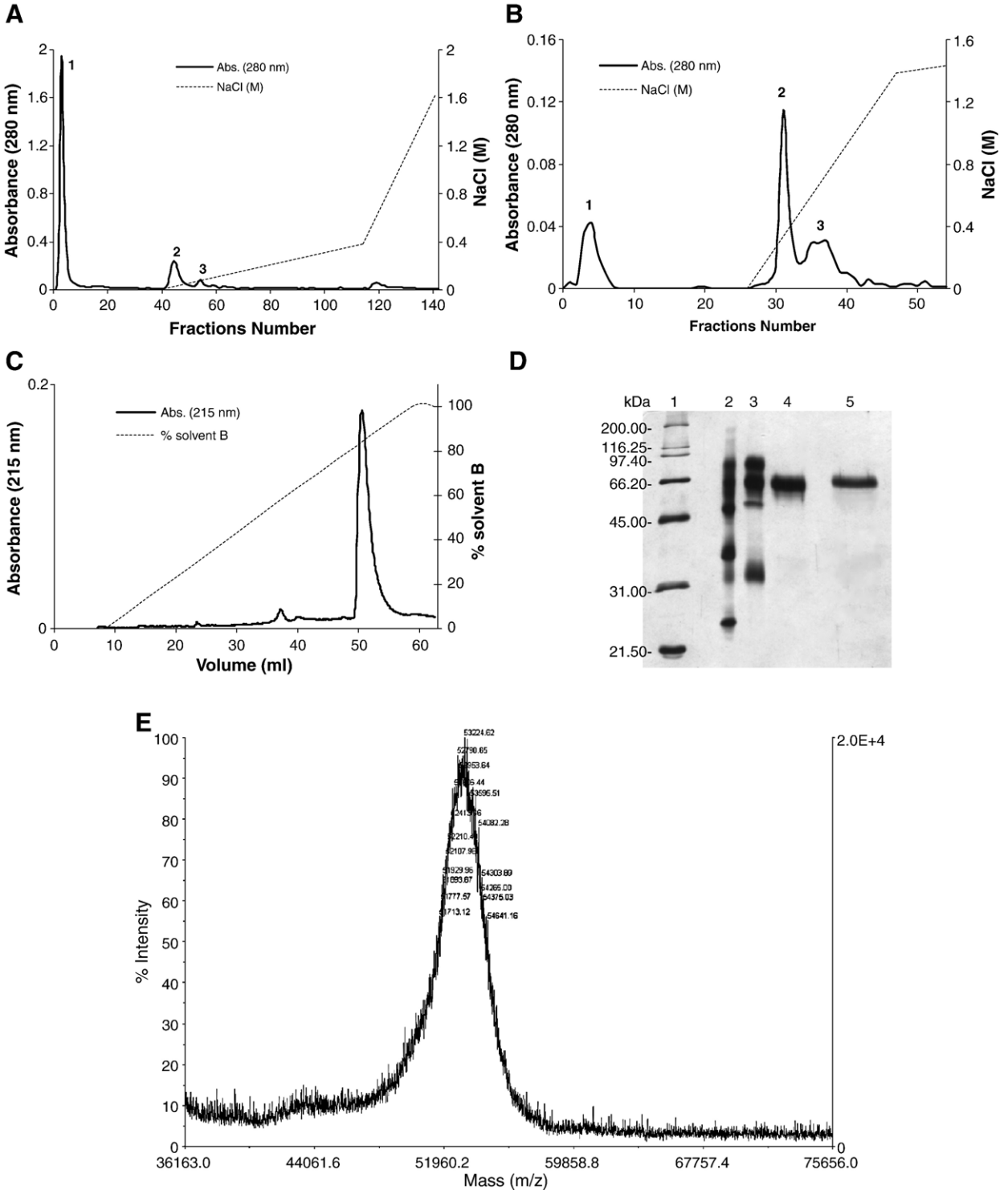

Fig. 1. Purification of patagonfibrase from *P. patagoniensis* venom. (A) Anion-exchange chromatography of *P. patagoniensis* venom on a Mono-Q column. Fractions were monitored at 280 nm and assayed for caseinolytic and fibrinogenolytic activities. Peaks 1 and 2 displayed caseinolytic and fibrinogenolytic activities. (B) Affinity chromatography on a HiTrap Blue HP column of the active acidic pool (peak 2) from the previous step. Fractions were monitored at 280 nm and assayed for caseinolytic and fibrinogenolytic activities. Peak 2 degraded azocasein and bovine fibrinogen and showed a single band on SDS-PAGE. (C) Reversed-phase HPLC of peak 2 from the previous step on a C8 column equilibrated with solvent A. The protein was eluted with a 0–100% increasing linear gradient of solvent B and monitored at 215 nm. (D) SDS-PAGE in 12% gel of *P. patagoniensis* venom, active acidic pool from Mono-Q column and patagonfibrase. Lane 1—molecular mass standards: myosin (200,000 Da), β-galactosidase (116,250 Da), phosphorylase b (97,400 Da), BSA (66,200 Da), egg albumin (45,000 Da), carbonic anhydrase (31,000 Da), and trypsin inhibitor (21,500 Da). Lane 2—crude venom (1.26 μg) under non-reducing conditions. Lane 3—active acidic pool from Mono-Q column (1.26 μg) under non-reducing conditions. Lane 4—patagonfibrase (1.26 μg) under non-reducing conditions. Lane 5—patagonfibrase (1.26 μg) under reducing conditions. Gel was silver stained. (E) Mass spectrometry of patagonfibrase. Approximately 0.5 μg protein was spotted onto sinapinic acid matrix. The broad peak and multiple apparent isoforms suggest that the protein is glycosylated.

Table 1
Purification of patagonfibrase from the venom of *Philodryas patagoniensis*

Step	Protein			Enzymatic activity		
	mg	%	Total (units)	Yield %	Specific activity (units/mg) [a]	Purification factor (fold)
Crude venom	18.2	100	14.1	100	0.78±0.06	1.0
Mono-Q	4.3	23.6	4.4	31.2	1.02±0.07	1.3
HiTrap Blue HP	0.5	2.8	2.2	15.6	4.84±0.27	6.2

[a] These values represent the mean±SD (n=4).

Inhibition studies showed that DTT and Na_2EDTA were able to abolish completely the fibrinogenolytic activity of patagonfibrase (Fig. 2B). However, PMSF had no effect on this activity.

3.3. Caseinolytic activity

Hydrolysis of azocasein was observed after incubation of patagonfibrase and *P. patagoniensis* crude venom, with specific activities of 4.84 and 0.78 units/mg of protein, respectively. The effect of pH on caseinolytic activity was also assayed, and the optimum pH for patagonfibrase was noticed at pH 7.5; incubation of patagonfibrase at pH values below 6.0 or above 9.5 resulted in an abrupt decrease in activity (data not shown). The optimum temperature for the caseinolytic activity of patagonfibrase was observed at 37 °C. Patagonfibrase was thermolabile since its activity was reduced to about 20% of its original value after incubation at 55 °C for 90 min (data not shown). Furthermore, patagonfibrase was affected by divalent metal ions (Fig. 3). Ca^{2+} caused an increase in about 40% of its activity, but Mg^{2+} did not affect it; Zn^{2+} inhibited almost all caseinolytic activity (about 90%). Patagonfibrase was completely inhibited by DTT, while Na_2EDTA and L-cysteine inhibited it in about 95% and 90%, respectively (Fig. 3). On the other hand, PMSF, an inhibitor of serine proteinases, had no effect on it. Pre-incubation of patagonfibrase with human serum, which contains endogenous proteinase inhibitors, did not eliminate its caseinolytic activity (data not shown).

3.4. Hemorrhagic activity

Patagonfibrase induced hemorrhage after intradermal injection in mice, with a MHD of 0.27 μg. Hemorrhagic activity of patagonfibrase was totally abolished after incubation with Na_2EDTA. However, PMSF had no effect on this activity (Fig. 4).

3.5. Local and systemic damage induced by patagonfibrase in mice

When *P. patagoniensis* crude venom or purified patagonfibrase was injected i.m. in the gastrocnemius, evident hemorrhage, edema and myonecrosis were observed concomitant with an inflammatory reaction characterized by the presence of a polymorphonuclear leukocyte infiltrate (Fig. 5). In agreement with these observations, significant increments in serum CK activity were noticed after i.m. injection of either venom or patagonfibrase (Fig. 6).

Systemic alterations were also investigated after i.m. injection of patagonfibrase. Both venom and patagonfibrase

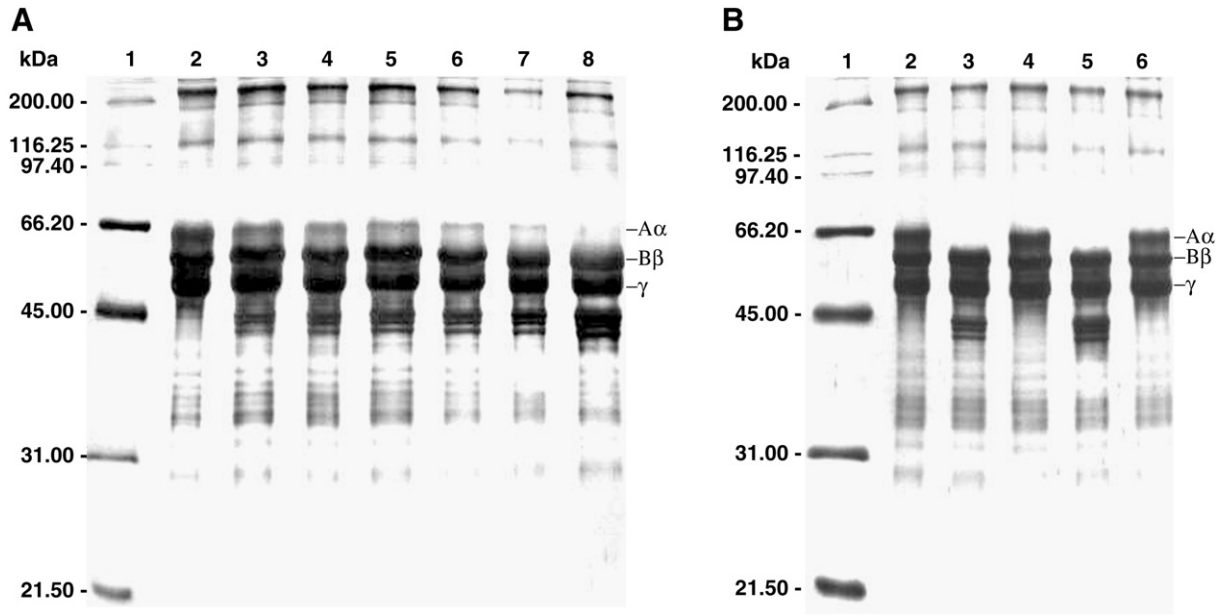

Fig. 2. Fibrinogen degradation by patagonfibrase. (A) SDS-PAGE analysis of bovine fibrinogen after digestion by patagonfibrase in 12% gel under reducing conditions; note the rapid loss of the Aα subunit. Lane 1—molecular mass standards (same as Fig. 1D). Lane 2—control of bovine fibrinogen sample incubated at 37 °C for 24 h with no patagonfibrase. Lanes 3–8—bovine fibrinogen samples after incubation at 37 °C with patagonfibrase (0.42 μg) for 5, 15, 30, 60, 120 min and 24 h, respectively. Gel was silver stained. (B) Effect of inhibitors on the digestion of fibrinogen by patagonfibrase, analyzed by SDS-PAGE (12% gel), under reducing conditions. Patagonfibrase (0.21 μg) was incubated with inhibitors for 1 h at 37 °C before the incubation with bovine fibrinogen at 37 °C for 2 h. Lane 1—molecular mass standards (same as Fig. 1D). Lane 2—no patagonfibrase. Lane 3—patagonfibrase and no inhibitor. Lanes 4–6—patagonfibrase and 2 mM inhibitors (Na_2EDTA, PMSF or DTT, respectively). Gel was silver stained.

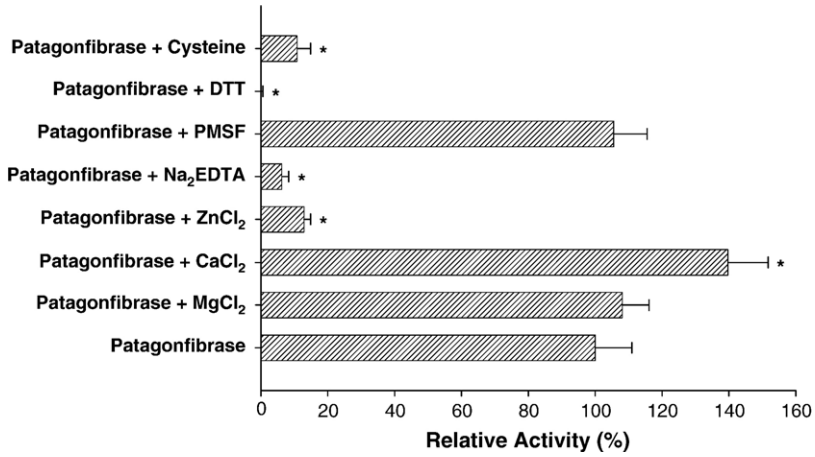

Fig. 3. Enzymatic properties of patagonfibrase. Effect of divalent metals and inhibitors (5 mM, final concentration, except for PMSF, 2 mM) on caseinolytic activity of patagonfibrase. Bars represent the mean±SD of four individual experiments. Asterisks indicate statistically significant differences between treatments and patagonfibrase ($p<0.01$).

induced multifocal hemorrhage in lungs, evidenced by the presence of erythrocytes in alveolar spaces. Mixed inflammatory infiltrate of polymorphonuclear and mononuclear cells that dilated the alveolar septa, and intense diffuse congestion of pulmonary parenchyma, were also observed (Fig. 5). In contrast, no histological change was induced by patagonfibrase in heart, kidney and liver tissues (data not shown).

3.6. Platelet aggregation

When added to washed human platelet suspensions, patagonfibrase at concentrations up to 174 nM (final concentration) induced no platelet aggregation per se. However, it inhibited collagen-induced platelet aggregation, and 50% inhibition was obtained with 129 nM patagonfibrase. Inactivation of the enzyme with 121 μM Na_2EDTA (final concentration) produced no change in this inhibition (data not shown). When 1 μg/ml collagen and 5 μg/ml patagonfibrase (final concentrations) were pre-incubated at 37 °C for 5 min, collagen could trigger platelet aggregation in the same way as adding the agonist after 2 min incubation of washed human platelet suspensions with patagonfibrase. In addition, patagonfibrase exhibited 64% inhibition of ADP-induced aggregation at a final concentration of 174 nM. This inhibition was enhanced to 93% by pre-incubating the enzyme with fibrinogen at 37 °C for 2 min. Thrombin- and ristocetin-induced platelet aggregation were not inhibited by patagonfibrase.

4. Discussion

In the present study, we have purified to homogeneity the first protein derived from *P. patagoniensis* venom. Apparently, *P. patagoniensis* venom contains several fibrinogenolytic enzymes [10], and herein we purified one of them in two steps, using ion exchange and affinity chromatography. It was named "patagonfibrase" (*P.* ***patagon****iensis* **fibr**inogen**ase**), according to the nomenclature system proposed previously [30].

Patagonfibrase degraded fibrinogen and azocasein, and these activities were strongly inhibited by Na_2EDTA, DTT and cysteine, suggesting that it is a metalloproteinase, and that intramolecular S–S bond and free-SH groups are essential for its catalytic activity. Patagonfibrase activity was enhanced by calcium ions, implying that this divalent cation is important for structural stabilization [31]. In contrast, patagonfibrase was inhibited by addition of Zn^{2+}, similar to effects of zinc ions on a hemorrhagin isolated from *Deinagkistrodon acutus* venom [32] and the major metalloproteinase isolated from *Crotalus viridis oreganus* venom [33]. Such modifications are caused by the

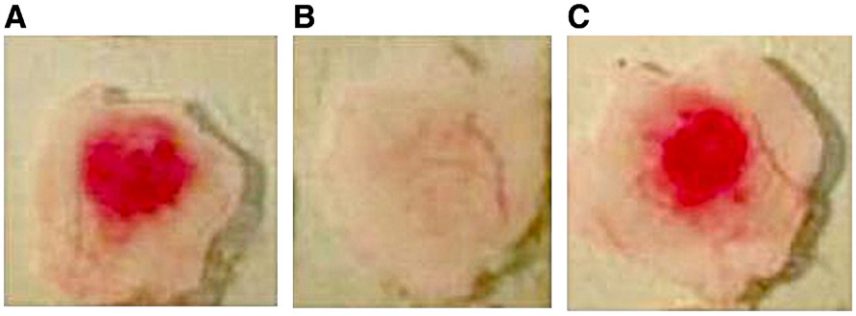

Fig. 4. Inhibition of the hemorrhagic activity of patagonfibrase. (A) Patagonfibrase (0.40 μg) alone. (B) Patagonfibrase incubated with 2 mM Na_2EDTA. (C) Patagonfibrase incubated with 2 mM PMSF.

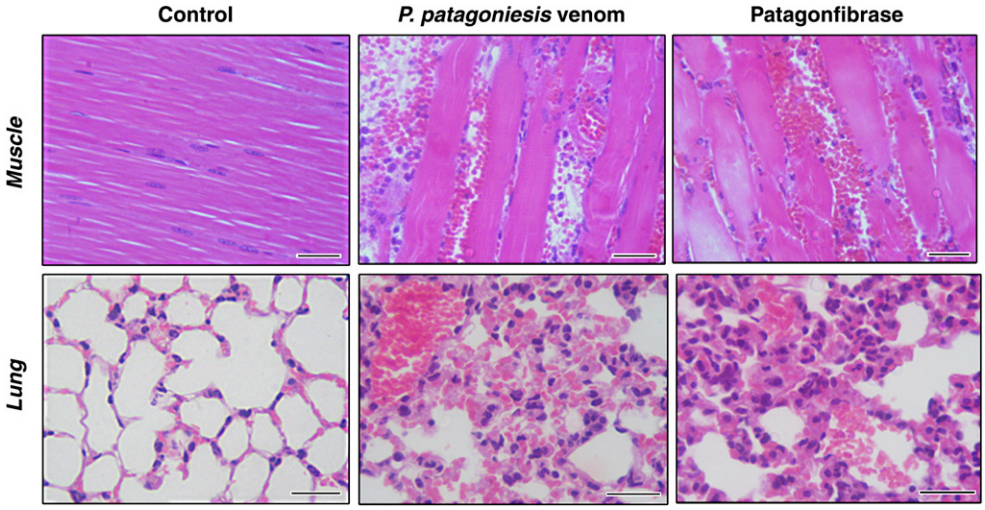

Fig. 5. Light micrographs showing the histopathological changes induced by crude *P. patagoniensis* venom or patagonfibrase in mouse gastrocnemius muscle and lung. Muscle: note cell necrosis, inflammatory infiltrate of polymorphonuclear leukocytes, and extravasation of erythrocytes induced by both crude *P. patagoniensis* venom and patagonfibrase. Lung: note extravasation of erythrocytes in some alveolar spaces, mixed inflammatory infiltrate of polymorphonuclear and mononuclear cells in the alveolar septa, and congestion of blood vessels induced by both crude *P. patagoniensis* venom and patagonfibrase. Sections were stained with hematoxylin and eosin. Scale bar, 30 μm.

binding of zinc to non-catalytic ion-binding sites, resulting in a conformation change that leads to the loss of proteolytic activity [32].

Patagonfibrase degraded only the Aα-chain of fibrinogen throughout the incubation period examined, suggesting that it was an α-fibrinogenase, according to Markland [34]. The Aα-chain specificity is typical of the majority of fibrinogenolytic snake venom metalloproteinases (SVMPs) [35]. While some of them degrade preferentially, although not exclusively, the Aα-chain of fibrinogen – such as BjussuMP-I from *Bothrops jararacussu* venom, which degrades the Aα-chain within 15 min and the Bβ-chain within 6 h [36] –, others degrade exclusively the Aα-chain of fibrinogen, such as jararhagin from *Bothrops jararaca* venom [37] and the enzyme isolated in this work. Additionally, the latter showed a high fibrinogenolytic activity since Aα-chain degradation could be noticed after a short period of incubation. This is an important difference regarding other α-fibrinogenases, which require a longer period of incubation to demonstrate their fibrinogenolytic activity, e.g. berytractivase from *Bothrops erythromelas* venom [38]. Patagonfibrase treatment did not result in clotting of fibrinogen per se, despite the fact it could degrade fibrinogen; therefore, it is unlikely that this enzyme produces fibrinopeptides A and/or B, which are required for the initiation of fibrin polymerization. In addition, the proteolytic activity on fibrinogen resulted in impairment of fibrin clot formation by thrombin. As incubation of patagonfibrase with plasma also prolonged thrombin clotting time, it is likely that proteinase inhibitors present in plasma cannot inhibit the fibrinogenolytic activity of this enzyme. Additionally, patagonfibrase could not clot plasma by itself, which suggests that this enzyme is unable to activate prothrombin into thrombin. In this regard, patagonfibrase is different from other α-fibrinogenase, such as EoVMP2 from *Echis ocellatus* venom, which cleaves not only fibrinogen but also prothrombin [39].

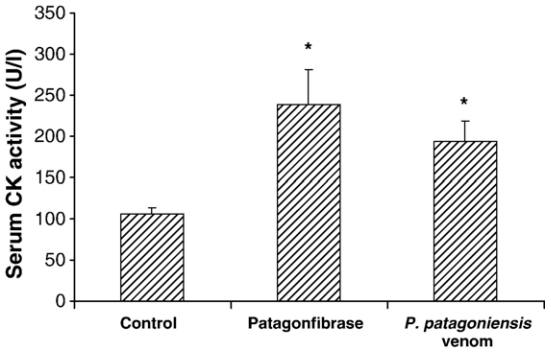

Fig. 6. Changes in mouse serum CK levels 12 h after i.m. injection of *P. patagoniensis* venom or patagonfibrase. Control mice received 50 mM Tris–HCl buffer (pH 7.4) alone. Serum CK activity was expressed in units/l. Bars represent the mean±SD of four individual experiments. Asterisks indicate statistically significant differences between treatments and control ($p<0.01$).

Purified patagonfibrase presented only one band in SDS-PAGE, under reducing and non-reducing conditions, demonstrating that it is a single polypeptide chain protein, with a relative molecular mass around 57.5 kDa. This is similar to 58-kDa α-fibrinogenolytic metalloproteinase isolated from *P. olfersii* venom, named PofibH. However, mass spectrometry showed a mass of 53.2 kDa, displaying a broad spectrum typical of glycosylated proteins. Fry and collaborators have already demonstrated molecular masses consistent with patagonfibrase (~51–52 kDa) when analyzing *P. patagoniensis* venom by mass spectrometry [40]. Both patagonfibrase and PofibH are acidic proteins with similar pIs, 5.8 and 4.6, respectively [6].

Patagonfibrase induced hemorrhage in mouse skin and gastrocnemius muscle, similar to crude *P. patagoniensis* venom [7]. The hemorrhagic activity of patagonfibrase is most likely correlated with its proteolytic activity, i.e., with its ability to degrade various components of basement membrane, disturbing thereby the interaction between the basement membrane and

endothelial cells, and resulting in hemorrhage [41–43]. The inhibition of hemorrhage by Na_2EDTA suggests that proteolytic activity is essential for its capacity to induce hemorrhage. Therefore, patagonfibrase is a proteolytic enzyme that actively acts not only on fibrinogen but also on several other protease substrates, such as basement membrane components. Although its fibrinogenolytic activity does not appear to be essential for inducing hemorrhage, it may play an important role by interfering with blood coagulation and the formation of hemostatic plug.

Several fibrinogenolytic enzymes also possess hemorrhagic activity [34,44], especially α-fibrinogenases. Conversely, other fibrinogenolytic enzymes have been reported to show no hemorrhagic activity [34,35]. Assakura and co-workers [6] have isolated and characterized five fibrin(ogen)olytic enzymes from the venom of *P. olfersii:* four of them are metalloproteinases (PofibC$_1$, C$_2$, C$_3$ and H) and one is a serine proteinase (PofibS). PofibC$_1$, C$_2$ and C$_3$ showed proteolytic activity on casein, but they were devoid of hemorrhagic activity. PofibH is an α-fibrin (ogen)ase with high hemorrhagic (MHD = 0.2 μg) and low caseinolytic (1.73 units/mg) activity [6]. Patagonfibrase is similar to PofibH in several respects: (1) high relative molecular mass, (2) acidic isoelectric point, (3) dependence of enzymatic activity on divalent cations and disulfide bridges, (4) proteolytic activity toward fibrinogen and casein, and (5) potent hemorrhagic activity. On account of the last activity, they may be deemed one of the most important hemorrhagic principles present in both *Philodryas* venoms. Hemorrhagic activity of patagonfibrase is nearly 4 times higher than alternagin (MHD = 1 μg) [45], a hemorrhagic factor isolated from the venom of *Bothrops alternatus*, which is the main species responsible for snakebites in northeastern Argentina, and it is comparable to the hemorrhagic levels of BaH1 (MHD = 0.2 μg) [46], the main hemorrhagin of *Bothrops asper* venom. This potent hemorrhagic activity of patagonfibrase, along with its increased bleeding tendency by degrading fibrinogen, may have important clinical implications in *P. patagoniensis* bites.

In addition to hemorrhage, patagonfibrase induces myonecrosis after i.m. injection. A variety of hemorrhagic metalloproteinases have been reported to induce muscle cell degeneration [47–49]. As shown above, patagonfibrase is a metalloproteinase with high hemorrhagic activity, and therefore we speculate that muscle necrosis induced by patagonfibrase develops secondarily to the ischemia provoked in skeletal muscle as a consequence of microvasculature injury. This is the mechanism suggested for most snake venom hemorrhagic metalloproteinases that cause muscle damage [42].

Our observations also indicate that i.m. injection of patagonfibrase can induce systemic bleeding in lungs. Similar damage was observed when 2 mg jararhagin – the best studied hemorrhagic component of *Bothrops jararaca* venom – was injected s.c. in rats [50]. It is important to note that in the present work the amount of injected patagonfibrase was 8 μg, an amount much less than that of jararhagin. The capacity of patagonfibrase to induce systemic hemorrhage likely depends on its ability to hydrolyze peptide bonds of basal lamina components that are critical for the stability of extracellular matrix scaffold [41], as well as its resistance to the inhibitory action of plasma proteinase inhibitors, mainly α-macroglobulins [51]. In addition, patagonfibrase degrades fibrinogen and inhibits collagen- and ADP-dependent platelet aggregation, and thereby is similar to acurhagin from *Deinagkistrodon acutus* venom [26]. Both actions could result in the impairment of thrombus formation and therefore may contribute to systemic hemorrhage.

The finding that both native and enzymatically-inactive patagonfibrase possess a similar ability to interfere with the platelet-collagen interaction suggests that its metalloproteinase activity does not play a crucial role for inhibiting platelet aggregation. Based on the results that both collagen pre-incubated with the enzyme and collagen added after incubation of platelet suspensions with patagonfibrase could induce similar values of platelet aggregation, it is likely that the inhibitory activity on collagen-induced aggregation could result from the binding of the enzyme to platelet collagen receptors without affecting collagen itself, as described for many SVMPs affecting platelet function [52]. Regarding ADP-induced platelet aggregation, patagonfibrase likely inhibits aggregation by both destroying intact fibrinogen and consequently by generating fibrinogen degradation products which act as competitive inhibitors of platelet–fibrinogen bridging formation, as it was suggested for L4 from *Agkistrodon halys brevicaudatus* venom [53].

Based on its molecular mass, intense hemorrhagic activity, ability to induce not only local but also systemic bleeding, and inhibitory activity on collagen- and ADP-induced platelet aggregation, patagonfibrase may be hypothesized to belong to the P-III class of SVMPs, which are composed of metalloproteinase, disintegrin-like and cysteine-rich domains [41]. Transcriptomic data of *P. olfersii* venom gland, complemented by proteomic analysis of its secretion, have revealed that P-III class SVMPs are the major components of this venom and likely the main cause of most toxic effects observed during *Philodryas* sp. envenomation [14]. However, the correct classification of patagonfibrase will only be accomplished when the nucleotide sequence is known. Attempts to elucidate the amino acid sequence of patagonfibrase, which is N-terminally blocked, are currently in progress. Amino acid sequence analysis of this SVMP from a novel source (family Colubridae) will illuminate similarities and divergences between Colubridae and Viperidae snake venoms which have occurred during their separate evolutionary histories.

In conclusion, we have purified a metalloproteinase from *P. patagoniensis* venom with caseinolytic, α-fibrinogenolytic, hemorrhagic, myotoxic and platelet aggregation inhibitory activities, and as a prominent venom component, it likely plays a key role in the pathogenesis of disturbances that occur in human envenomations by *P. patagoniensis*. Due to its multiple effects on hemostasis, patagonfibrase may be of considerable importance because of its use as a biological tool to explore many facets of hemostasis. The presence of this potent proteinase in a Colubridae snake venom further demonstrates that mechanisms to produce tissue damage (related to trophic roles of venom) are common to all families of venomous snakes.

Moreover, patagonfibrase will be important for studies of the structure–function and evolutionary relationships of SVMPs.

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References

[1] R.E. Hill, S.P. Mackessy, Characterization of venom (Duvernoy's secretion) from twelve species of colubrid snakes and partial sequence of four venom proteins, Toxicon 38 (2000) 1663–1687.
[2] A.S. Kamiguti, R.D.G. Theakston, N. Sherman, J.W. Fox, Mass spectrophotometric evidence for P-III/P-IV metalloproteinases in the venom of the Boomslang (*Dispholidus typus*), Toxicon 38 (2000) 1613–1620.
[3] S.P. Mackessy, Biochemistry and pharmacology of colubrid snake venoms, J. Toxicol., Toxin Rev. 21 (2002) 43–83.
[4] B.G. Fry, N.G. Lumsden, W. Wuster, J.C. Wickramaratna, W.C. Hodgson, R.M. Kini, Isolation of a neurotoxin (α-colubritoxin) from a nonvenomous colubrid: evidence for early origin of venom in snakes, J. Mol. Evol. 57 (2003) 446–452.
[5] M.T. Assakura, M.G. Salomão, G. Puorto, F.R. Mandelbaum, Hemorrhagic, fibrinogenolytic and edema forming activities of the venom of the colubrid snake *Philodryas olfersii* (green snake), Toxicon 30 (1992) 427–438.
[6] M.T. Assakura, A.P. Reichl, F.R. Mandelbaum, Isolation and characterization of five fibrin(ogen)olytic enzymes from the venom of *Philodryas olfersii* (green snake), Toxicon 32 (1994) 819–831.
[7] O. Acosta, L.C. Leiva, M.E. Peichoto, S. Maruñak, P. Teibler, L. Rey, Hemorrhagic activity of Duvernoy's gland secretion of the xenodontine colubrid *Philodryas patagoniensis* from the north-east region of Argentina, Toxicon 41 (2003) 1007–1012.
[8] O. Acosta de Pérez, L.C. Leiva de Vila, M.E. Peichoto, S. Maruñak, R. Ruíz, P. Teibler, C. Gay, L. Rey, Edematogenic and myotoxic activities of the Duvernoy's gland secretion of *Philodryas olfersii* from the north-east region of Argentina, Biocell 27 (2003) 363–370.
[9] M.E. Peichoto, O. Acosta, L. Leiva, P. Teibler, S. Maruñak, R. Ruíz, Muscle and skin necrotizing and edema-forming activities of Duvernoy's gland secretion of the xenodontine colubrid *Philodryas patagoniensis* from the north-east of Argentina, Toxicon 44 (2004) 589–596.
[10] M.E. Peichoto, L.C. Leiva, L.E. Guaimás Moya, L. Rey, O. Acosta, Duvernoy's gland secretion of *Philodryas patagoniensis* from the northeast of Argentina: its effects on blood coagulation, Toxicon 45 (2005) 527–534.
[11] J. Prado-Franceschi, S. Hyslop, J.C. Cogo, A.L. Andrade, M. Assakura, M.A. Cruz-Höfling, L. Rodrigues-Simioni, The effects of Duvernoy's gland secretion from xenodontine colubrid *Philodryas olfersii* on striated muscle and the neuromuscular junction: partial characterization of a neuromuscular fraction, Toxicon 34 (1996) 459–466.
[12] J. Prado-Franceschi, S. Hyslop, J.C. Cogo, A.L. Andrade, M. Assakura, A.P. Reichl, M.A. Cruz-Höfling, L. Rodrigues-Simioni, Characterization of a myotoxin from the Duvernoy's gland secretion of the xenodontine colubrid *Philodryas olfersii* (green snake): effects on muscle and the neuromuscular junction, Toxicon 36 (1998) 1407–1421.
[13] B.G. Fry, N. Vidal, J.A. Norman, F.J. Vonk, H. Scheib, S.F. Ramjan, S. Kuruppu, K. Fung, S.B. Hedges, M.K. Richardson, W.C. Hodgson, V. Ignjatovic, R. Summerhayes, E. Kochva, Early evolution of the venom system in lizards and snakes, Nature 439 (2006) 584–588.
[14] A.T. Ching, M.M. Rocha, A.F. Paes Leme, D.C. Pimenta, M.F. Furtado, S.M. Serrano, P.L. Ho, I.L. Junqueira-de-Azevedo, Some aspects of the venom proteome of the Colubridae snake *Philodryas olfersii* revealed from a Duvernoy's (venom) gland transcriptome, FEBS Lett. 580 (2006) 4417–4422.
[15] J.A. Peters, B. Orejas-Miranda, Catalogue of the neotropical squamata. Part I. Snakes, Bull. U.S. Nat. Mus. 297 (1970) i–viii + 1–347.
[16] M.B. Rocha, F.B. Molina, Considerações Preliminares Sobre a Biologia e Manejo de *Philodryas Patagoniensis*, Parelheira, em Cativerio (Colubridae, Ophidia), Resumos do XIV Congres. Brasil. de Zoología, 1987, p. 134.
[17] M.E. Araújo, A.C. dos Santos, Cases of human envenoming caused by *Philodryas olfersii* and *Philodryas patagoniensis* (Serpentes: Colubridae), Rev. Soc. Bras. Med. Trop. 30 (1997) 517–519.
[18] S.A. Nishioka, P.V.P. Silveira, *Philodryas patagoniensis* bite and local envenoming, Rev. Inst. Med. Trop. São Paulo 36 (1994) 279–281.
[19] I. Ferlan, A. Ferlan, T. King, F.E. Russel, Preliminary studies on the venom of the colubrid snake *Rhabdophis subminiatus* (red-necked keelback), Toxicon 21 (1983) 570–574.
[20] J. Meier, B. Banks, E.E. Creppy, G. Habermehi, F. Kornalik, C.Y. Lee, D. Mebs, P. Rosenberg, R.D.G. Theakston, Ethical standards for animal experiments in toxinological research, Toxicon 31 (1993) 9–12.
[21] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[22] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[23] H. Blum, H. Beier, H.J. Gross, Improved silver staining of plant proteins. RNA and DNA in polyacrylamide gels, Electrophoresis 8 (1987) 93–99.
[24] S.P. Mackessy, N.M. Sixberry, W.H. Heyborne, T. Fritts, Venom of the Brown Treesnake, *Boiga irregularis*: ontogenetic shifts and taxa-specific toxicity, Toxicon 47 (2006) 537–548.
[25] R. Biggs, The preparation of general reagents and coagulation factors, in: R. Biggs (Ed.), Human Blood Coagulation, Haemostasis and Thrombosis, Blackwell Scientific Publications, Oxford, 1976, pp. 657–669.
[26] W.J. Wang, T.F. Huang, Purification and characterization of a novel metalloproteinase, acurhagin, from *Agkistrodon acutus* venom, Thromb. Haemost. 87 (2002) 641–650.
[27] L.R.C. Gonçalves, M. Mariano, Local haemorrhage induced by *Bothrops jararaca* venom: relationship to neurogenic inflammation, Mediat. Inflamm. 9 (2000) 101–107.
[28] M.L. Santoro, M.C.C. Sousa-e-Silva, L.R.C. Gonçalves, S.M. Almeida Santos, D.F. Cardoso, I.L. Laporta-Ferreira, M. Saiki, C.A. Peres, I.S. Sano-Martins, Comparison of the biological activities in venoms from three subspecies of the South American rattlesnake (*Crotalus durissus terrificus, C. durissus cascavella* and *C. durissus collilineatus*), Comp. Biochem. Physiol. 122C (1999) 61–73.
[29] G.V.R. Born, Aggregation of blood platelets by adenosine diphosphate and its reversal, Nature 194 (1962) 927–929.
[30] H. Pirkle, N. Marsh, Nomenclature of exogenous hemostatic factors, Thromb. Haemost. 66 (1991) 264.
[31] J.B. Bjarnason, J.W. Fox, Hemorrhagic toxins from snake venoms, J. Toxicol., Toxin Rev. 7 (1988–1989) 121–209.
[32] Z. Zhu, W. Gong, X. Zhu, M. Teng, L. Niu, Purification, characterization and conformational analysis of a haemorrhagin from the venom of Agkistrodon acutus, Toxicon 35 (1997) 283–292.
[33] S.P. Mackessy, Characterization of the major metalloprotease isolated from the venom of the northern pacific rattlesnake, *Crotalus viridis oreganus*, Toxicon 34 (1996) 1277–1285.
[34] F.S. Markl Jr., Inventory of α- and β-fibrinogenases from snake venoms, Thromb. Haemost. 65 (1991) 438–443.
[35] F.S. Markland, Snake venoms and the hemostatic system, Toxicon 36 (1998) 1749–1800.
[36] M.V. Mazzi, S. Marcussi, G.B. Carlos, R.G. Stabeli, J.J. Franco, F.K. Ticli, A.C. Cintra, S.C. Franca, A.M. Soares, S.V. Sampaio, A new hemorrhagic

metalloprotease from *Bothrops jararacussu* snake venom: isolation and biochemical characterization, Toxicon 44 (2004) 215–223.
[37] A.S. Kamiguti, J.R. Slupsky, M. Zuzel, C.R. Hay, Properties of fibrinogen cleaved by Jararhagin, a metalloproteinase from the venom of *Bothrops jararaca*, Thromb. Haemost. 72 (1994) 244–249.
[38] M.B. Silva, M. Schattner, C.R. Ramos, I.L. Junqueira-de-Azevedo, M.C. Guarnieri, M.A. Lazzari, C.A. Sampaio, R.G. Pozner, J.S. Ventura, P.L. Ho, A.M. Chudzinski-Tavassi, A prothrombin activator from *Bothrops erythromelas* (sjararaca-da-seca) snake venom: characterization and molecular cloning, Biochem. J. 369 (2003) 129–139.
[39] J.M. Howes, A.S. Kamiguti, R.D. Theakston, M.C. Wilkinson, G.D. Laing, Effects of three novel metalloproteinases from the venom of the West African saw-scaled viper, *Echis ocellatus* on blood coagulation and platelets, Biochim. Biophys. Acta 1724 (2005) 194–202.
[40] B.G. Fry, W. Wuster, S.F. Ryan Ramjan, T. Jackson, P. Martelli, R.M. Kini, Analysis of Colubroidea snake venoms by liquid chromatography with mass spectrometry: evolutionary and toxinological implications, Rapid Commun. Mass Spectrom. 17 (2003) 2047–2062.
[41] J.B. Bjarnason, J.W. Fox, Hemorrhagic metalloproteinases from snake venoms, Pharmacol. Ther. 62 (1994) 325–372.
[42] J.M. Gutiérrez, A. Rucavado, Snake venom metalloproteinases: their role in the pathogenesis of local tissue damage, Biochimie 82 (2000) 841–850.
[43] J.M. Gutiérrez, A. Rucavado, T. Escalante, C. Díaz, Hemorrhage induced by snake venom metalloproteinases: biochemical and biophysical mechanism involved in microvessel damage, Toxicon 45 (2005) 997–1011.
[44] N. Marsh, Inventory of haemorrhagic factors from snake venoms, Thromb. Haemost. 71 (1994) 793.
[45] D.H. Souza, M.R. Iemma, L.L. Ferreira, J.P. Faria, M.L. Oliva, R.B. Zingali, S. Niewiarowski, H.S. Selistre-de-Araujo, The disintegrin-like domain of the snake venom metalloprotease alternagin inhibits $\alpha_2\beta_1$ integrin-mediated cell adhesion, Arch. Biochem. Biophys. 384 (2000) 341–350.
[46] G. Borkow, J.M. Gutiérrez, O. Ovadia, Isolation and characterization of synergistic hemorrhagins from the venom of the snake *Bothrops asper*, Toxicon 31 (1993) 1137–1150.
[47] A. Franceschi, A. Rucavado, N. Mora, J.M. Gutiérrez, Purification and characterization of BaH4, a hemorrhagic metalloproteinase from the venom of the snake *Bothrops asper*, Toxicon 38 (2000) 63–77.
[48] J.M. Gutiérrez, M. Romero, J. Núñez, F. Chaves, G. Borkow, M. Ovadia, Skeletal muscle necrosis and regeneration after injection of BaH1, a hemorrhagic metalloproteinase isolated from the venom of the snake *Bothrops asper* (Terciopelo), Exp. Mol. Pathol. 62 (1995) 28–41.
[49] A. Rucavado, B. Lomonte, M. Ovadia, J.M. Gutiérrez, Local tissue damage induced by BaP1, a metalloproteinase isolated from *Bothrops asper* (Terciopelo) snake venom, Exp. Mol. Pathol. 63 (1995) 186–199.
[50] A.S. Kamiguti, R.D. Theakston, H. Desmond, R.A. Hutton, Systemic haemorrhage in rats induced by a haemorrhagic fraction from *Bothrops jararaca* venom, Toxicon 29 (1991) 1097–1105.
[51] K. Anai, M. Sugiki, E. Yoshida, M. Maruyama, Inhibition of a snake venom hemorrhagic metalloproteinase by human and rat α-macroglobulins, Toxicon 36 (1998) 1127–1139.
[52] A.S. Kamiguti, Platelets as targets of snake venom metalloproteinases, Toxicon 45 (2005) 1041–1049.
[53] O.H. Jeon, D.S. Kim, Molecular cloning and functional characterization of a snake venom metalloprotease, Eur. J. Biochem. 263 (1999) 526–533.