ELSEVIER

Toxicon 45 (2005) 527–534

TOXICON

www.elsevier.com/locate/toxicon

Duvernoy's gland secretion of *Philodryas patagoniensis* from the northeast of Argentina: its effects on blood coagulation

M.E. Peichoto[a], L.C. Leiva[a], L.E. Guaimás Moya[b], L. Rey[c], O. Acosta[d,*]

[a]*Cátedra de Química Biológica I, Facultad de Ciencias Exactas y Naturales y Agrimensura, Universidad Nacional del Nordeste (UNNE), Av. Libertad 5470, Corrientes 3400, Argentina*

[b]*Cátedra de Patología Quirúrgica, Facultad de Ciencias Veterinarias, Universidad Nacional del Nordeste (UNNE), Sargento Cabral 2139, Corrientes 3400, Argentina*

[c]*Serpentario del Zoológico de la ciudad de Corrientes, Av. Costanera Gral., San Martín 99, Corrientes 3400, Argentina*

[d]*Cátedra de Farmacología, Facultad de Ciencias Veterinarias, Universidad Nacional del Nordeste (UNNE), Sargento Cabral 2139, Corrientes 3400, Argentina*

Received 5 July 2004; accepted 10 December 2004

Abstract

Duvernoy's gland secretion of *Philodryas patagoniensis* exhibits high hemorrhagic activity, containing enzymes that are able to degrade the vascular wall. In this work we aim to determine if the secretion can also affect the hemostatic system by causing changes in blood coagulation. Procoagulant and coagulant activities were evaluated on plasma and fibrinogen, respectively. The delay in the thrombin clotting time of fibrinogen previously incubated with the secretion was also determined. Specific hydrolysis of fibrinogen and fibrin incubated with the secretion at different time intervals was shown by electrophoresis on polyacrylamide gel. To determine the structural characteristics of the enzymes degrading fibrinogen and fibrin, secretion were incubated in the presence of 45 mM Na_2EDTA, 40 mM Benzamidine, and/or 2 mM PMSF before the incubation with fibrinogen or fibrin, respectively. The effect in vivo was investigated in adult male rats injected with different dose of secretion, aliquots of blood were withdrawn at different time intervals, and the fibrinogen concentration was determined. Duvernoy's gland secretion of *P. patagoniensis* did not clot plasma or fibrinogen. It exhibited a potent fibrinogenolytic activity degrading the Aα-chain faster than the Bβ-chain, whereas γ-chain was resistant. This latter corresponded with a strong delay in the thrombin clotting time of fibrinogen (4 mg/ml) pre-incubated with the secretion, being 9.53 μg the amount of protein from Duvernoy's gland secretion that increased the thrombin clotting time from 20 to 60 s. In vivo, the loss of rat plasma fibrinogen was proportional to the amount of secretion injected. The secretion also hydrolyzed fibrin degrading the α-monomer. Inhibition studies with Na_2EDTA, Benzamidine, and/or PMSF showed that metalloproteinases and serinoproteinases are the main enzymes responsible for the hydrolyzing activity on fibrinogen and fibrin. All these results demonstrate that Duvernoy's gland secretion of *P. patagoniensis* possesses enzymes able to hydrolyze plasma components playing a relevant role in the blood coagulation. These hydrolyzing activities and those acting on the wall of blood vessels let the secretion exhibit a high hemorrhagic activity, which may result in permanent sequelae or even cause the death of the victims bitten by this colubrid snake.

Keywords: *Philodryas patagoniensis*; Blood-clotting activity; Fibrinolytic activity; Fibrinogenolytic activity

* Corresponding author. Tel./fax: +54 378 342 5753.
E-mail address: patmed@vet.unne.edu.ar (O. Acosta).

0041-0101/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2004.12.016

1. Introduction

Reports on toxic saliva in colubrid snakes are few and the chances of being bitten by colubrid snakes are rare, because of the anatomical disadvantage of having fangs situated behind its other teeth. Nevertheless, some species have caused severe reactions in humans. Paralysis, respiratory failure, hemorrhage and death have been observed (Assakura et al., 1992, 1994; Silva and Buononato, 1983/84; Kamiguti et al., 2000). The toxic effects associated with bites from certain species of colubrids are the result of toxins secreted from the Duvernoy's gland (DG) (Weistein and Kardong, 1994). However, not all colubrid snakes apparently possess DG and not all secretions from DG are toxic (Weistein and Kardong, 1994). In those colubrids that produce toxic secretions the result of envenoming may be very mild to fatal depending on the snake, the victim and the amount of secretion injected into the victim (Kamiguti et al., 2000). While many reports are found on venom constituents of snakes belonging to the families *Elapidae*, *Hydrophidae*, *Crotalidae* and *Viperidae*, because of the small quantity of secretion of the DG, very little has been done to elucidate the composition and biological activities of colubrid venoms (Assakura et al., 1992, 1994; Kamiguti et al., 2000). It is worthwhile to point out that non-clotting blood and hemorrhage, usually associated with viperid envenomations, are the most striking clinical manifestations of colubrid envenomations (Vest, 1978; Visser and Chapman, 1978; Silva and Buononato, 1983/84). Among the snakes belonging to the *Colubridae* family, the genus *Philodryas* are widespread all over South America and are considered as not venomous (Jorge and Ribeiro, 1990). However, they have a DG, whose secretion possesses such toxicity as to cause serious lesions (Assakura et al., 1992, 1994; Acosta et al., 2003; Acosta de Pérez et al., 2003; Peichoto et al., 2004).

Only a few cases of human envenoming by *Philodryas* have been reported in the literature (Fowler and Salomão, 1994; Kuch and Jesberger, 1993; Nickerson and Henderson, 1976; Silva and Buononato, 1983/84; Ribeiro et al., 1999; Nishioka and Silveira, 1994; Araújo and dos Santos, 1997; Kuch, 1999). Those cases are mainly restricted to *Philodryas olfersii* (green snake), which is the *Philodryas* species whose secretion has been studied more extensively so far (Assakura et al., 1992, 1994; Prado-Franceschi et al., 1996, 1998; Acosta de Pérez et al., 2003).

Philodryas patagoniensis, opistoglyphous snake with a well-developed Duvernoy's gland connected with a grooved tooth, is found in South America: Argentina, Bolivia, Brazil, Paraguay and Uruguay (Peters and Orejas-Miranda, 1970; Rocha and Molina, 1987). Regarding to Duvernoy's gland secretion, very little is known, since although there are records of envenoming cases by this colubrid snake, they are rare (Nishioka and Silveira, 1994; Araújo and dos Santos, 1997). The local signs and symptoms caused by *P. patagoniensis* can be confused with those found in bothropic accidents, which is necessary to be avoided, in order to prevent people from receiving the specific antivenom, which could have harmful effects on the patient's health (Nishioka and Silveira, 1994). In previous studies (Acosta et al., 2003; Peichoto et al., 2004), we have demonstrated that Duvernoy's gland secretion of *P. patagoniensis* exhibits dermonecrotic, myonecrotic and edema-forming activities, and lethality comparable to many bothropic venoms. We have also demonstrated that it possesses high hemorrhagic activity, containing enzymes (hemorrhagic metalloproteinases) that damage vascular endothelium by perforating the basement membrane of vessel walls. The aim of this investigation was to attempt to confirm that DG secretion of *P. patagoniensis*, which inhabits the northeast region of Argentina, can also affect the hemostatic system by causing changes in blood coagulation.

2. Material and methods

2.1. Duvernoy's gland (DG) secretion of P. patagoniensis

DG secretion was obtained from eight adult specimens measuring 90–120 cm, being wild adult snakes captured in the northeast region of Argentina and kept at the herpetarium of 'Facultad de Ciencias Exactas y Naturales y Agrimensura, Universidad Nacional del Nordeste' (Corrientes, Argentina). The snakes were milked by introducing a 100 μl micropipet over each fang following the procedure of Ferlan et al. (1983). The secretion was lyophilized; after that, it was kept frozen at −20 °C. When required, the secretion was dissolved with phosphate buffered saline solution, pH 7.4. The small amount of insoluble material was centrifuged and the clear supernatant was applied for studies.

The protein content was estimated assuming that an absorbance of 1.183 at 280 nm corresponded to 1 mg of protein/ml.

2.2. Animals

Sprague-Dawley male rats (220–270 g body weight) were supplied by 'Bioterio de la Facultad de Ciencias Veterinarias, Universidad Nacional del Nordeste' (Corrientes, Argentina). Food (chow rat diet) was withdrawn 12–14 h before the experiment, but the animals had free access to water. Temperature in the animal room was 23±2 °C and the relative humidity was between 35 and 65%. Lights in the animal room were on from 6 AM to 6 PM. Four animals per group were used, as stated in each experimental design.

2.3. Blood-clotting activity

Two hundred microliters of standard citrated solution of human plasma or 2 mg/ml fibrinogen (Human fibrinogen type I, Sigma) were mixed with 100 μl of DG secretion

sample (1 mg/ml) and the clotting time was recorded at 37 °C (Theakston and Reid, 1983).

2.4. Delay of the thrombin clotting time

To 200 μl of a solution of 4 mg/ml fibrinogen (Human fibrinogen type I, Sigma) were added aliquots of 50 μl of solutions containing the following quantities of DG secretion: 4.65; 9.3 and 18.6 μg. The mixtures were pre-incubated at 37 °C for 10 min, then 50 μl of thrombin from human plasma (Sigma) 10 NIH units/ml were added and the clotting times recorded (Assakura et al., 1994).

2.5. Specific hydrolysis of fibrinogen

Specific cleavage of fibrinogen by DG secretion was shown by electrophoresis on 12% polyacrylamide gels. One hundred microliters of fibrinogen (Human fibrinogen type I, Sigma) at a concentration of 5 mg/ml in 0.1 M Tris–HCl buffer (pH 8.0) containing 0.5 mM $CaCl_2$ and 0.02% NaN_3 were incubated at 37 °C with 100 μl of DG secretion at a concentration of 250 μg/ml in the buffer mentioned above, corresponding to a ratio of 20:1 (w/w). At various time intervals, aliquots of 30 μl were withdrawn from the digestion mixture. The samples were denatured and reduced by boiling for 5 min with 30 μl of denaturing solution (5% urea, 4% SDS and 1% 2-mercaptoethanol in 35 mM phosphate buffer, pH 7.0) before the run on SDS-polyacrylamide gel. For inhibition studies, DG secretion was incubated in the presence of 45 mM Na_2EDTA, 40 mM Benzamidine, and/or 2 mM PMSF, for 1 h at room temperature before the incubation with human fibrinogen (Assakura et al., 1994).

2.6. Fibrinolytic activity

A modified plaque assay (Rojas et al., 1987) was used. Citrated human plasma was diluted 1:2 with a buffer containing 50 mM Tris, 70 mM $(NH_4)_2SO_4$, 90 mM NaCl, 0.70 mM $MgCl_2$, pH 7.5. Twenty-two milliliters were then transferred to plastic dishes (13×8 cm), and 3.0 ml of 0.25 M $CaCl_2$ and 132 μl of human thrombin 10 NIH units/ml were added to produce a clot. After 60 min of incubation at 37 °C, wells of 2 mm diameter were made in the clot, and 10 μl of solutions containing different amounts of DG secretion (from 1.00 to 50.00 μg) were applied to each well. Incubations were carried out at 37 °C for 18 h, after which diameters of the fibrinolytic halos were measured. The minimum fibrinolytic concentration (MFC) was defined as the concentration of DG secretion (final, mg/ml) that induced a fibrinolytic halo of 12 mm diameter.

2.7. Specific hydrolysis of fibrin

Specific cleavage of fibrin by DG secretion was shown by electrophoresis on 12% polyacrylamide gels. Fibrin clots were prepared following the procedure of Willis and Tu (1988). Thrombin, 0.1 ml containing 10 NIH unit/ml, was added to 0.1 ml of a 1% fibrinogen solution in 0.1 M Tris–HCl buffer (pH 8.0) containing 0.5 mM $CaCl_2$ and 0.02% NaN_3. The fibrin clot was allowed to form for 1 h at room temperature. After 1 h, DG secretion at a concentration of 250 μg/ml in 0.1 M Tris–HCl buffer (pH 8.0) containing 0.5 mM $CaCl_2$ and 0.02% NaN_3 was added to the clots [corresponding to a ratio of 1:20 (w/w)] and incubated at 37 °C for various time intervals. Aliquots of 30 μl were withdrawn from the digestion mixture and the reaction was stopped by boiling for 5 min with 30 μl of denaturing solution (5% urea, 4% SDS and 1% 2-mercaptoethanol in 35 mM phosphate buffer, pH 7.0) before the run on SDS-polyacrylamide gel. For inhibition studies, DG secretion was incubated in the presence of 45 mM Na_2EDTA, 40 mM Benzamidine, and/or 2 mM PMSF, for 1 h at room temperature before the incubation with fibrin clots (Assakura et al., 1994).

2.8. Sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was performed on 12% or 12–18% gradient polyacrylamide slab gels following the method of Laemmli (1970). Bromophenol blue was used as a tracking dye. Gels were stained with Coomassie Brilliant Blue R250 or with silver by the procedure of Blum et al. (1987).

2.9. Effect of P. patagoniensis DG secretion on the rat plasma fibrinogen

The method described by Assakura et al. (1994) was used. Adult male rats were previously anesthetized with i.p. injection of cloral hydrate (300 mg/kg) and heparinized with 1000 IU/kg, i.v. Four rats were used for each dose of 0.23, 0.45 and 0.9 mg in 0.3 ml of DG secretion in phosphate buffered saline solution, pH 7.4. Control rats were injected with 0.3 ml of phosphate buffered saline solution, pH 7.4. Aliquots (1 ml) of blood were withdrawn 15, 30 and 60 min after injection. Blood control (1 ml) was removed 15 min before injection. Administration of DG secretion and removal of blood samples were made through a polyethylene catheter introduced into the iliac vein. In order to maintain a constant blood volume, each blood sample collected was replaced by injecting an equivalent volume of 0.9% NaCl. Twenty microliters of rat plasma were added to 180 μl of 0.9% NaCl and clotted with 50 μl of 25 NIH units/ml of thrombin. The clotting times were measured at 37 °C. The fibrinogen concentration was determined from a calibration curve prepared from a fibrinogen reference (Clauss, 1957).

2.10. Statistical analysis

All experiments were repeated at least four times. The amount of protein that caused a shift from 20 to 60 s on

the thrombin clotting time, and the MFC were estimated by linear regression, adjusted to the minimum square method. The results of the loss of rat plasma fibrinogen after intravenous injection of *P. patagoniensis* DG secretion were expressed as the mean ± SD. The significance of differences between means was assessed by ANOVA followed by Dunnet's test for multiple comparisons among groups. *P* values less than 0.05 were considered to be statistically significant.

3. Results

The protein composition of the DG secretion of *P. patagoniensis*, as analyzed by electrophoresis on SDS-polyacrylamide gel, showed a wide distribution of proteins of varying molecular weights (Fig. 1).

DG secretion of *P. patagoniensis* did not clot citrated human plasma or purified human fibrinogen, even after 15 min of incubation at 37 °C. These results indicate absence of thrombin-like and procoagulant enzymes in DG secretion. On the other hand, the clotting time of human fibrinogen by thrombin was delayed in the presence of DG secretion of *P. patagoniensis*. This delay was proportional to the amount of DG secretion in the incubation mixture ($r = 0.996$). The amount of protein that caused a shift from 20 to 60 s on the thrombin clotting time was of 9.53 μg. To confirm that the DG secretion, without clotting, is hydrolyzing fibrinogen in vitro, it was incubated with this substrate and analyzed by SDS-gel electrophoresis (Fig. 2A). In 2 min, the Aα-chain was completely hydrolyzed (data not shown), whereas the Bβ-chain almost completely disappeared after 30 min of incubation. The γ-chain was resistant, being stable after 2 h of hydrolysis. Within the first 15 min of hydrolysis, degradation products of 23, 36, 38 and 45 kDa were observed. After 60 min, degradation products of 23 and 36 kDa began to be hydrolyzed, whereas a new band with molecular mass less than 14 kDa appeared. Fibrinogenolytic activity was not completely inhibited by Na_2EDTA alone (metalloproteinase inhibitor), Benzamidine alone (serine-proteinase competitive inhibitor), or PMSF alone (serine-proteinase covalent irreversible inhibitor). However, the fibrinogenolytic activity was completely abolished when the DG secretion was simultaneously incubated with Na_2EDTA y Benzamidine (Fig. 2B), or Na_2EDTA y PMSF (Fig. 2C). These results show that some of the fibrinogenases present in the studied DG secretion are metalloproteinases capable to degrade Aα- and Bβ-chains, and others are serineproteinases exhibiting ability to degrade only Aα-chain throughout the incubation period examined.

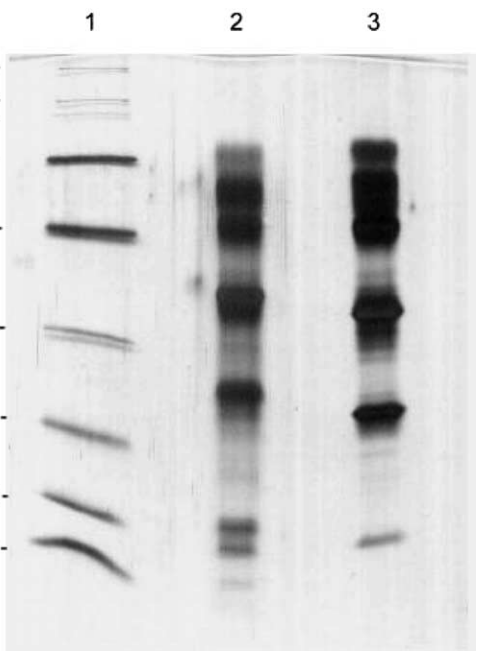

Fig. 1. Protein bands of *Philodryas patagoniensis* DG secretion on a 12–18% gradient gel under reducing (2) and non-reducing (3) conditions. Lane 1, molecular-weight markers: myosin (200.00 kDa), β-galactosidase (116.25 kDa), phosphorilase b (97.40 kDa), serum albumin (66.20 kDa), egg albumin (45.00 kDa), carbonic anhydrase (31.00 kDa), trypsin inhibitor (21.50 kDa), lysozyme (14.40 kDa), and aprotinin (6.50 kDa). Lane 2, crude DG secretion (8 μg) after denaturation with SDS and reduction with 2-mercaptoethanol. Lane 3, crude DG secretion (8 μg) after only denaturation with SDS. The gel was silver stained.

Duvernoy's gland secretion of *P. patagoniensis* exhibited intense fibrinolytic activity when tested by a modified plaque assay. Diameters of the fibrinolytic halos were proportional to the amount of DG secretion injected. The obtained linear relationship ($r = 0.992$) let us determine the MFC, finding a low value (1.50 mg/ml). When the DG secretion was tested for hydrolyzing fibrin, the α-monomer was completely digested in 30 min; while γ–γ dimer, β-monomer and γ-monomer were resistant, remaining stable after 2 h of hydrolysis (Fig. 3A). The major degradation fragment was a polypeptide with a molecular weight of 40 kDa. Fibrinolytic activity was not completely inhibited by Na_2EDTA alone (metalloproteinase inhibitor), or Benzamidine alone (serineproteinase competitive inhibitor). However, the fibrinolytic activity was completely abolished when the DG secretion was simultaneously incubated with Na_2EDTA y Benzamidine (Fig. 3B). When the DG secretion was incubated with PMSF (serineproteinase covalent irreversible inhibitor), the results were the same that with Benzamidine (data not shown). These results show that metalloproteinases and serineproteinases, with different intensities of degradation on the α-monomer of fibrin, are present in the studied DG secretion.

The effect of *P. patagoniensis* DG secretion in vivo was investigated in adult male rats. The experiments showed that the rat plasma fibrinogen decreases rapidly in the 15 min after injection, and this loss is proportional to the amount of DG secretion injected (Fig. 4). A tendency to the physiological restatement of plasma fibrinogen is

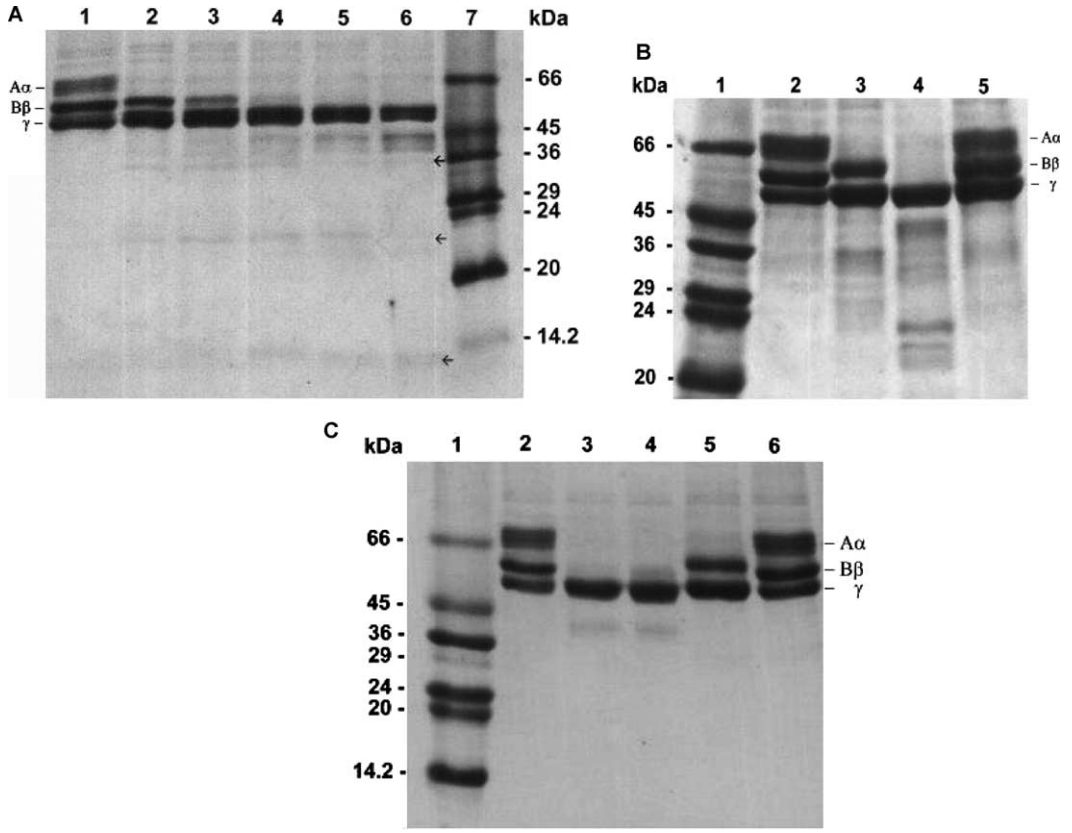

Fig. 2. (A) SDS-PAGE analysis of reduced human fibrinogen after digestion by DG secretion of *P. patagoniensis* in a 12% gel under reducing conditions. Lane 1, human fibrinogen control incubated at 37 °C for 10 min without DG secretion. The Aα- (63 kDa), Bβ- (56 kDa), and γ- (47 kDa) chains of fibrinogen are indicated on the left. Lane 2–6, human fibrinogen samples after incubation at 37 °C with DG secretion at a ratio 20:1 (w/w) for 5, 15, 30, 60 and 120 min, respectively. Lane 7, molecular-weight markers: bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14.20 kDa). The gel was stained with Coomassie Brilliant Blue R250. Arrows indicate faintly visible protein bands. (B) Effect of inhibitors on the digestion of fibrinogen by DG secretion of *P. patagoniensis* in a 12% gel under reducing conditions. Lane 1, molecular-weight markers: bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), and trypsin inhibitor (20 kDa). Lane 2, human fibrinogen control incubated at 37 °C for 10 min without DG secretion. Lane 3, human fibrinogen incubated at 37 °C for 120 min with DG secretion + Na_2EDTA. Lane 4, human fibrinogen incubated at 37 °C for 120 min with DG secretion + Benzamidine. Lane 5, human fibrinogen incubated at 37 °C for 120 min with DG secretion + Na_2EDTA + Benzamidine. The gel was stained with Coomassie Brilliant Blue R250. (C) Effect of inhibitors on the digestion of fibrinogen by DG secretion of *P. patagoniensis* in a 12% gel under reducing conditions. Lane 1, molecular-weight markers: bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14.20 kDa). Lane 2, human fibrinogen control incubated at 37 °C for 10 min without DG secretion. Lane 3, human fibrinogen incubated at 37 °C for 120 min with DG secretion. Lane 4, human fibrinogen incubated at 37 °C for 120 min with DG secretion + PMSF. Lane 5, human fibrinogen incubated at 37 °C for 120 min with DG secretion + Na_2EDTA. Lane 6, human fibrinogen incubated at 37 °C for 120 min with DG secretion + Na_2EDTA + PMSF. The gel was stained with Coomassie Brilliant Blue R250.

evidenced from 60 min of injection of the lower assayed dose. The circulant plasma fibrinogen after DG secretion treatment, different from the plasma control, produced a loose clot with thrombin. It is important to clarify that there was a drop in the level of rat plasma fibrinogen in the control rats over time because each blood sample collected was replaced by injecting an equivalent volume of 0.9% NaCl which caused a progressive dilution of the blood samples.

4. Discussion

Minton and Weinstein (1987) showed that the Duvernoy's secretions of colubrid snakes possess as complex activities as venoms of many proteroglyphous snakes. The many activities exhibited by the DG secretion of *P. patagoniensis* (Acosta et al., 2003; Peichoto et al., 2004; this work) and the molecular complexity demonstrated in SDS-PAGE corroborate the findings of these authors.

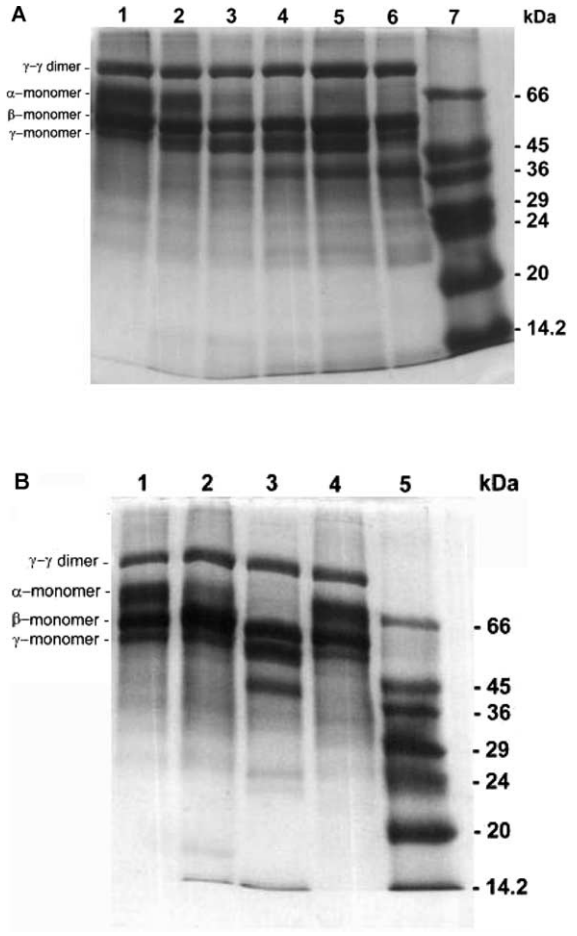

Fig. 3. (A) SDS-PAGE analysis of reduced human fibrin after digestion by DG secretion of *P. patagoniensis* in a 12% gel under reducing conditions. Lane 1, human fibrin control incubated at 37 °C for 10 min without DG secretion. γ–γ dimer, α-monomer, β-monomer, and γ-monomer are indicated on the left. Lane 2–6, human fibrin samples after incubation at 37 °C with DG secretion at a ratio 20:1 (w/w) for 5, 15, 30, 60 and 120 min, respectively. Lane 7, molecular-weight markers: bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14.20 kDa). The gel was stained with Coomassie Brilliant Blue R250. (B) Effect of inhibitors on the digestion of fibrin by DG secretion of *P. patagoniensis* in a 12% gel under reducing conditions. Lane 1, human fibrin control incubated at 37 °C for 10 min without DG secretion. Lane 2, human fibrin incubated at 37 °C for 120 min with DG secretion + Na_2EDTA. Lane 3, human fibrin incubated at 37 °C for 120 min with DG secretion + Benzamidine. Lane 4, human fibrin incubated at 37 °C for 120 min with DG secretion + Na_2EDTA + Benzamidine. Lane 5, molecular-weight markers: bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14.20 kDa). The gel was stained with Coomassie Brilliant Blue R250.

It is common to find consumption of clotting factors and blood incoagulability accompanied by hemorrhage in victims of snakebite. Various reviews on this subject have been published (Seegers and Ouyang, 1979; Ouyang et al., 1992; Hutton and Warrell, 1993; Kamiguti and Sano-Martins, 1995; Kamiguti et al., 1998; Markland, 1998).

In a previous study (Acosta et al., 2003) we have studied the hemorrhage associated with *P. patagoniensis* envenomation. We have showed that DG secretion of *P. patagoniensis* exhibits high proteolytic activity on bovine casein (143 U/mg), being much more proteolytic than the venom of *Bothrops alternatus* (yarará grande) (20 U/mg) (Ruiz de Torrent et al., 2001), *Bothrops* species mostly responsible for the ophidic accidents in northeast region of Argentina. The main enzymes responsible for this activity are metalloproteinases, such as Assakura et al. (1992) described for *Philodryas olfersii* venom. We have also demonstrated that the strong hemorrhagic activity is caused by DG secretion metalloproteinases that degrade proteins of the basement membrane in the blood vessel wall, leading this action to the loss of capillary integrity with resultant hemorrhage at the local site.

Coagulation disturbance is the other striking activity in the colubrid venoms. DG secretion of *P. patagoniensis* has neither thrombin-like activity that converts fibrinogen to fibrin nor procoagulant enzymes that produce thrombin; DG secretion of *Philodryas olfersii* does not have them either (Assakura et al., 1992, 1994). However, coagulant and procoagulant enzymes are very commonly found in venoms of *Bothrops* species (Gené et al., 1989; Acosta de Pérez et al., 1996).

We have demonstrated (Peichoto et al., 2004) that DG secretion of *P. patagoniensis* has not phospholipase A_2 activity. Therefore, the anticoagulant activity of this secretion is not due to inhibition of the prothrombinase complex. This latter is the mechanism by which some snake venom phospholipases exert their anticoagulant action by degrading phospholipids involved in the prothrombinase complex (Markland, 1998).

As in the venom of *P. olfersii* (Assakura et al., 1992), fibrin(ogen)olytic enzymes are present in DG secretion of *P. patagoniensis*. Some of them are metalloproteinases with specificity directed preferentially towards the Aα-chain and with somewhat lower activity towards the Bβ-chain, since there is substantial degradation of the β-chain with increasing time. Other of them are serine proteinases with fibrin(ogen)olytic activity directed exclusively towards the Aα-chain. Both types of proteinases are fibrinogenolytic and fibrinolytic, but none of these enzymes exhibits activity with the γ-chain of fibrin or fibrinogen.

These enzymes differ substantially from plasmin which cleaves peptide bonds at the carboxy-terminal side of lysine residues in the α-, β- and γ-chains of fibrin(ogen) (Markland, 1998), sites different than those cleaved by the enzymes present in the secretion studied, since in this case the γ-chain is not affected. Therefore, these enzymes do not activate plasminogen by an enzymatic action, leading to plasmin generation.

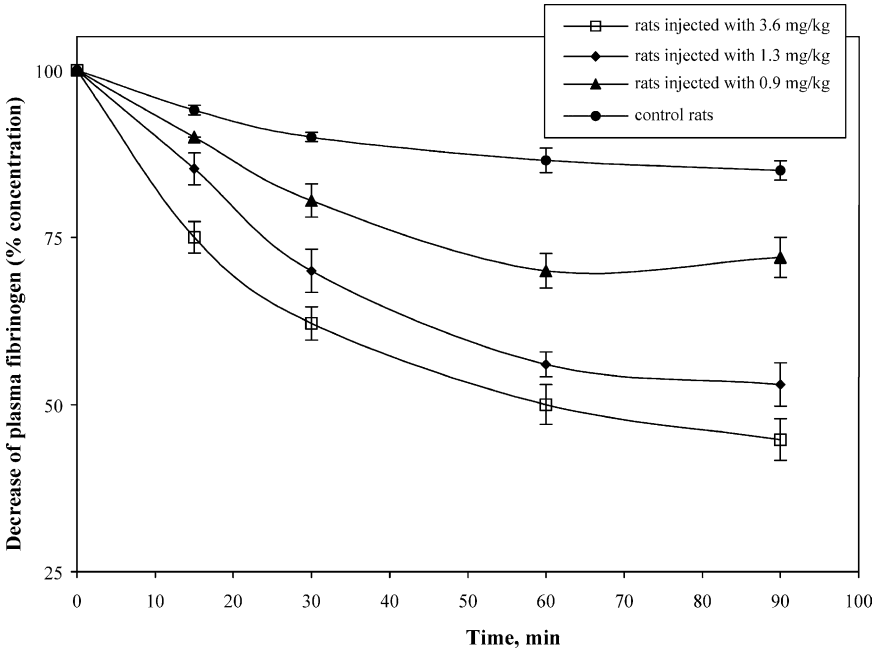

Fig. 4. Loss of rat plasma fibrinogen after intravenous injection of *P. patagoniensis* DG secretion. Three different dose of DG secretion were used. Results are presented as means ± SD of four isolated experiments. All rats injected with DG secretion show significantly lower plasma fibrinogen decreases than the control rats ($P < 0.01$). Tests were performed as described in Section 2.

The peptides of human fibrin(ogen) released by *P. patagoniensis* DG secretion are similar from those of *P. olfersii* venom (Assakura et al., 1992).

DG secretion of *P. patagoniensis* produces in vivo a decrease of the rat's plasma fibrinogen probably due to the fibrinogenolytic enzymes. This was already observed with *P. olfersii* venom (Assakura et al., 1994).

We can conclude that the extensive hemorrhage caused by DG secretion of *P. patagoniensis*, which may result in permanent sequelae or even cause the death of the victims bitten by this colubrid snake, is the result of the synergistic action of metalloproteinases that degrade the extracellular matrix surrounding blood vessels, and fibrin(ogen)olytic enzymes that interfere with hemostasis. Moreover, it is possible that the metalloproteinases that possess hemorrhagic activity are, at least in part, the very same enzymes that are responsible for the cleavage of fibrinogen and fibrin. This latter will be deciphered when purification and characterization of components of DG secretion of *P. patagoniensis* are carried out. The data presented in this paper give insight into future directions for research into DG secretion of *P. patagoniensis* and potentially more effective treatments of envenoming by this colubrid snake.

Acknowledgements

The authors would like to thank Professor Beatriz Avanza for supplying *Philodryas patagoniensis* specimens and Roberto Aguirre for his technical assistance in extraction of Duvernoy's gland secretion (Facultad de Ciencias Exactas y Naturales y Agrimensura, Universidad Nacional del Nordeste). This work was financially supported by 'Secretaría General de Ciencia y Técnica, Universidad Nacional del Nordeste' (Project No. PI 610). M.E. Peichoto is the recipient of a fellowship from 'Consejo Nacional de Investigaciones Científicas y Tecnológicas—CONICET', Argentina. This work was carried out in partial fulfillment of the requirements for the PhD degree for M.E. Peichoto at the University of Buenos Aires, Argentina.

References

Acosta, O., Leiva, L.C., Peichoto, M.E., Maruñak, S., Teibler, P., Rey, L., 2003. Hemorrhagic activity of Duvernoy's gland secretion of the xenodontine colubrid *Philodryas patagoniensis* from the north-east region of Argentina. Toxicon 41, 1007–1012.

Acosta de Pérez, O.C., Koscinczuk, P., Gutiérrez, J.M., 1996. Actividades hemorrágica y coagulante del veneno de *Bothrops alternatus* de Argentina. Acta Bioquímica Clínica Latinoamericana XXX, 401–405.

Acosta de Pérez, O., Leiva de Vila, L.C., Peichoto, M.E., Maruñak, S., Ruíz, R., Teibler, P., Gay, C., y Rey, L., 2003. Edematogenic and myotoxic activities of the Duvernoy's gland secretion of *Philodryas olfersii* from the north-east region of Argentina. Biocell 27, 363–370.

Araújo, M.E., dos Santos, A.C., 1997. Cases of human envenoming caused by *Philodryas olfersii* and *Philodryas patagoniensis* (Serpentes: Colubridae). Rev. Soc. Bras. Med. Trop. 30, 517–519.

Assakura, M.T., Salamão, M.G., Puorto, G., Mandelbaum, F.R., 1992. Hemorrhagic, fibrinogenolytic and edema forming activities of the venom of the colubrid snake *Philodryas olfersii* (green snake). Toxicon 30, 427–438.

Assakura, M.T., Reichl, A.P., Mandelbaum, F.R., 1994. Isolation and characterization of five fibrin(ogen)olytic enzymes from the venom of *Philodryas olfersii* (green snake). Toxicon 32, 819–831.

Blum, H., Beier, H., Gross, H.J., 1987. Improved silver staining of plant proteins. RNA and DNA in polyacrylamide gels. Electrophoresis 8, 93–99.

Clauss, V.A., 1957. Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. Acta Haematol. 17, 237–246.

Ferlan, I., Ferlan, A., King, T., Russel, F.E., 1983. Preliminary studies on the venom of the colubrid snake *Rhabdophis subminiatus* (red-necked keelback). Toxicon 21, 570–574.

Fowler, I.R., Salomão, M.da G., 1994. Activity patterns in the colubrid snake genus *Philodryas* and their relationship to reproduction and snakebite. Bull. Chicago Herp. Soc. 29, 229–232.

Gené, J.A., Roy, A., Rojas, G., Gutiérrez, J.M., Cerdas, L., 1989. Comparative study on coagulant, defibrinating, fibrinolytic and fibrinogenolytic activities of Costa Rican crotaline snake venoms and their neutralization by a polyvalent antivenom. Toxicon 27, 841–848.

Hutton, R.A., Warrell, D.A., 1993. Action of snake venom components on the haemostatic system. Blood Rev. 7, 176–189.

Jorge, M.T., Ribeiro, L.A., 1990. Acidentes por serpentes peçonhentas do Brasil. Revista da Associação Médica Brasilera 36, 66–77.

Kamiguti, A.S., Sano-Martins, I.S., 1995. South American snake venoms affecting haemostasis. J. Toxicol. Toxin Rev. 14, 359–374.

Kamiguti, A.S., Zuzel, M., Theakston, R.D.G., 1998. Snake venom metalloproteinases and disintegrins: interactions with cells. Braz. J. Med. Biol. Res. 31, 853–862.

Kamiguti, A.S., Theakston, R.D.G., Sherman, N., Fox, J.W., 2000. Mass spectrophotometric evidence for P-III/P-IV metalloproteinases in the venom of the Boomslang (*Dispholidus typus*). Toxicon 38, 1613–1620.

Kuch, U., 1999. Notes on two cases of human envenomation by the South American colubrid snakes *Philodryas olfersii latirostris* (COPE, 1862) and *Philodryas chamissonis* (WIEGMANN, 1834). Herpetozoa 12, 11–16.

Kuch, U., Jesberger, U., 1993. Human envenomation from the bite of the South American colubrid snake species *Philodryas baroni* Berg, 1895. Snake 25, 63–65.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Markland, F.S., 1998. Snake venoms and the hemostatic system. Toxicon 36, 1749–1800.

Minton, S.A., Weinstein, S.A., 1987. Colubrid snake venoms: immunologic relationships, electrophoretic patterns. Copeia 1987, 993–1000.

Nickerson, M.A., Henderson, R.W., 1976. A case of envenomation by the South American colubrid, *Philodryas olfersii*. Herpetologica 32, 197–198.

Nishioka, S.de.A., Silveira, P.V.P., 1994. *Philodryas* patagoniensis bite and local envenoming. Rev. Inst. Med. Trop. São Paulo 36, 279–281.

Ouyang, C., Teng, C.-M., Huang, T.-F., 1992. Characterization of snake venom components acting on blood coagulation and platelet function. Toxicon 30, 945–966.

Peichoto, M.E., Acosta, O., Leiva, L., Teibler, P., Maruñak, S., Ruíz, R., 2004. 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, 589–596.

Peters, J.A., Orejas-Miranda, B., 1970. Catalogue of the Neotropical Squamata. Part I. Snakes. Bull. US Nat. Mus. 297 (i–viii), 1–347.

Prado-Franceschi, J., Hyslop, S., Cogo, J.C., Andrade, A.L., Assakura, M., Cruz-Höfling, M.A., Rodrigues-Simioni, L., 1996. 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, 459–466.

Prado-Franceschi, J., Hyslop, S., Cogo, J.C., Andrade, A.L., Assakura, M., Reichl, A.P., Cruz-Höfling, M.A., Rodrigues-Simioni, L., 1998. 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, 1407–1421.

Ribeiro, L.A., Puorto, G., Jorge, M.T., 1999. Bites by the colubrid snake *Philodryas olfersii*: a clinical and epidemiological study of 43 cases. Toxicon 37, 943–948.

Rocha, M.B., Molina, F.B. 1987. Considerações preliminares sobre a biología e manejo de *Philodryas patagoniensis*, parelheira, em cativerio (Colubridae, Ophidia). In: Resumes do XIV Congres. Brasil. de Zoología, 134.

Rojas, G., Gutiérrez, J.M., Gené, J.A., Gómez, M., Cerdas, L., 1987. Neutralización de las actividades tóxicas y enzimáticas de cuatro venenos de serpientes de Guatemala y Honduras por el antiveneno polivalente producido en Costa Rica. Rev. Biol. Trop. 35 (1), 59–67.

Ruiz de Torrent, R., Leiva, L.C.A., y Acosta de Pérez, O., 2001. Efecto proteolítico de venenos de serpientes del género *Bothrops* (yarará) de la Argentina y su neutralización por un antiveneno bivalente. Acta Toxicol. Argent. 9 (1), 9–12.

Seegers, W.H., Ouyang, C., 1979. Snake venoms and blood coagulation. Handbook Exp. Pharmacol. 52, 684–750.

Silva, M.V., Buononato, M.A., 1983/84. Relato clínico de envenenamiento humano por *Philodryas olfersii*. Mem. Inst. Butantan 47/48, 121–126.

Theakston, R.D.G., Reid, H.A., 1983. Development of simple standard assay procedures for the characterization of snake venoms. Bull. WHO 61, 949–956.

Vest, D.K., 1978. Some effects and properties of Duvernoy's gland secretion from Hypsiglena torquata texana (Texas night snake). Toxicon 26, 417–419.

Visser, J., Chapman, D.S., 1978. Snakes and snake bites, in: Purnell, Mc.D. (Ed.), Venomous Snakes and Management of Snake Bite in Southern Africa, pp. 61–131.

Weistein, S.A., Kardong, K.V., 1994. Properties of Duvernoy's secretions from opisthoglyphous and aglyphous colubrid snakes. Toxicon 32, 1161–1185.

Willis, T.W., Tu, A.T., 1988. Purification and biochemical characterization of atroxase, a nonhemorrhagic fibrinlolytic protease from western diamondback rattlesnake venom. Biochemistry 27, 4769–4777.