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Isolation and biological characterization of a basic phospholipase A_2 from *Bothrops jararacussu* snake venom

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ABSTRACT: A phospholipase A_2 has been isolated from *Bothrops jararacussu* venom from snakes that inhabit the northeast region of Argentina. The present study describes *in vivo* and *in vitro* biological activities of phospholipase A_2 from *B. jararacussu* as well as isolation details of its. Venom was obtained by milking of adult snakes which were housing in wood reptile cages of varying dimensions in heated (20–30ºC) rooms. Snakes received a weekly diet of mice and water was available ad libitum for drinking and soaking. The enzyme was purified by gel filtration on a Sephadex G-75 column followed by ion exchange chromatography on a SP-Sephadex C25 column. The major peak belonging to proteins was retained in the cation exchanger and then eluted using a concentration gradient of KCl that exhibited phospholipase activity.

This basic PLA_2 consists of a single polypeptide chain with a molecular mass of 15.6 kDa. It had a high indirect hemolytic activity and produced a significant paw edema reaction in mice. The enzyme showed a low lethality (LD_{50} 148.6 µg) when was administered i.p. but exhibited elevated myotoxic effects *in vivo* by increasing plasma CK activity of injected mice, corroborated results by the histological observations of samples of gastrocnemius muscle. Myonecrosis is the result of intense destruction of muscular fibers that involves local infiltration of inflammatory cells and leads to the highest peak of CK level just after 1 hour mice injection. Moreover, the isolated enzyme showed anticoagulant activity, evaluated on sheep platelet-poor plasma which recalcification time was prolonged after incubation with the isolated phospholipase A_2. These findings showed that this phospholipase, isolated by only two simple chromatographic steps, possesses high edematogenic and myotoxic activities. However, despite the low lethal activity, this enzyme would contribute markedly to the pathophysiology of the bothropic envenomation.

Introduction

The *B. jararacussu*, one of the most dreaded snakes of Brazil, southern Bolivia, Paraguay and northeastern Argentina, is a heavily-built pit viper which may grow to a length of 2.2 m (Milani *et al*., 1997). Its venom has a lethal potency much higher than venoms of most of other Bothrops species found in this region of Argentina (*B. alternatus, B.neuwiedii, B.jararaca, B.moojeni)* approaching that of the neotropical rattlesnake *Crotalus durissus terrificus* (Schöttler, 1951; Sanchez *et al*., 1992). Local envenoming is characterized by pain, swelling, bleeding, and occasionally blisters, abscess formation and necrosis. Signs of systemic envenoming include gingival haemorrhage, ecchymosis and consumption coagulopathy (Milani *et al*., 1997; Cardoso *et al*., 1993).

Venom from *B. jararacussu* specimens that inhabit tropical forests of Brazil is widely studied and characterizated. It showed to be a proteolytic, coagulant, necrotizing and haemorrhagic venom (Sanchez *et al*., 1992), due to the presence of proteases, phospholipases and trombin-like enzymes. Besides, it exhibited

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high myotoxic and edematogenic activities (dos-Santos *et al*., 1992). Among its components, Phospholipases A_2 are mainly responsible of these injuries. Thus, several PLA_2 have been isolated from *B. jararacussu* venom. In that sense Andrião-Escarso and coworkers (2000) have isolated two myotoxic phospholipases named Bothroptoxin I and II (BthTX-I and BthTX-II), another basic PLA_2 with high enzymatic activity able to form multimeric complex was isolated by Bonfim *et al*. (2001). More recently, Ketelhut *et al*. (2003) isolated and characterized four myotoxic acidic PLA_2s.

Despite *B. jararacussu* venom is a full characterized secretion, there is scarce information regarding to the venom of this *Bothrops* specie that inhabits in the north-east region of Argentina. Considering the variability in venom composition associated with geographical location, (Chippaux and Goyffon, 1991; Rodrigues *et al*., 1998; Francischetti *et al*., 2000) it is necessary further studies about our local snake due to the high incidence of fatal cases of *B. jararacussu* poisoning. For this reason it is needed the isolation of those components principally responsible of venom intoxication.

In this article we report either the isolation or the biochemical and biological characterization of basic phospholipase A_2 from *Bothrops jararacussu* snake venom from Argentina. Results are compared with those obtained from the whole venom. In addition, a comparative analysis among snake venom phospholipases A_2 belonging to *Bothrops jararacussu,* from other areas of Latin America, is presented.

Material and Methods

Chemicals and reagents

Bothrops jararacussu adult snakes proceeding from the North-East of Argentina were kept in the Serpentarium of Misiones, Argentina. Venom was collected by milking of venom glands in sterile glass recipient, then desiccated and stored at -20ºC. Molecular weight markers, Sephadex G-75, SP Sephadex C25 were purchased from Sigma. All other reagents were laboratory grade.

Phospholipase A_2 Isolation (PLA_2)

Desiccated crude venom from *Bothrops jararacussu* (20 μg) was extracted with 250 μl of 10 mM sodium phosphate buffer, pH 7.2. It was then cleared by centrifugation for 5 min at 480 ∞ *g.* The supernatant was loaded on a Sephadex G-75 column (1.0 ∞ 50 cm) equilibrated with the same buffer. Fractions of 0.75 ml/tube were collected at a flow rate of 7.5 ml/h. Absorbance was monitored at 280 nm using a Cam Spec M330 spectrophotometer.The fractions were tested for phospholipase activity (see *Hemolitic activity, described below)*. In order to check the presence of others venom enzymatic components (thrombin-like and metalloproteases) coagulant and hemorrhagic activities (see ***Thrombin-like activity and Hemorrhagic activity assay respectively,*** *described below)* were also tested on the collected fractions. Those fractions containing phospholipase activity and no hemorrhagic and coagulant activities were pooled. Therefore, fractions belong to peak SIII from the gel filtration step were dissolved in 0.5 ml of 50 mM Tris, 100mM KCl, pH 7.0. Subsequently, this fraction was applied on a 1.0 ∞ 5.0 cm column of SP Sephadex C-25, and eluted with the same buffer. After elution of the non- absorbed proteins a linear gradient of KCl from 0.1 to 0.5 M in the same buffer, pH 7, was applied. Fractions of 1 ml were collected at 18 ml/h.

All steps of the purification procedure were carried out at room temperature (25ºC).

The fractions displaying phospholipase activity (measured through radial indirect hemolysis technique) were pooled and submitted to characterization.

Estimation of proteins

The protein determination of venom fractions was determined by measuring the absorbance at 280 nm in a UV-visible CamSpec M330 spectrophotometer with an optical path of 1 cm, based upon the assumption that the absorbance of the 1 mg/ml of protein of venom fraction was 1.138, the same as that of crude venom (de Haas *et al*., 1968).

Sodium dodecyl sulfate (SDS)- polyacrylamide gel electroforesis (PAGE)

In order to assay the isolated enzyme for purity, electrophoresis was performed on 15% polyacrylamide slab gels following the method of Laemmli (1970). Samples were pretreated in reducing conditions (SDS plus β-mercaptoethanol) at 100ºC for 5 min. Runs were of 1 h with 40 mA, and bromophenol blue was used as a tracking dye. Gel was stained with silver. Molecular weight markers run in parallel were bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehydes-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14,20 kDa).

Hemolytic activity

In order to evaluate the phospholipase A_2 activity of collected fractions or isolated enzyme, the indirect hemolytic activity was assayed as described by Gutiérrez *et al*. (1988). Three hundred microlitters of packed sheep erythrocytes washed four times with saline solution, 300 µl of 1:3 egg yolk solution in saline solution and 250 µl of 0.01 M $CaCl_2$ solution were added to 25 ml of 1% (w/v) of agar at 50ºC dissolved in PBS pH 7.2. The mixture was applied to plastic plates (135 x 80 mm) and allowed to gel. Then, 3 mm diameter wells were filled with 15 µl of samples. After 20 h of incubation at 37ºC, the diameters of hemolytic halos were measured. To determine the minimum hemolytic dose (MHD) of isolated enzyme 15 µl of solutions containing different amounts of isolated phospholipase A_2 (from 0.26 to 8.16 µg), were applied into the wells. After 20 h of incubation at 37ºC, the diameters of hemolytic halos were measured and dose-response curves plotted. The minimum hemolytic dose (MHD) was defined as the amount of enzyme that induced a hemolytic halo of 15-mm diameter.

Edema- forming activity

The method of Yamakawa *et al*. (1976) was used. Five groups of four mice were injected s.c. in the right foot pad with 50 µl of solutions containing different amounts of isolated PLA_2 (from 0.45 to 7.2 µg), whereas the left foot pad was injected with 50 µl of phosphate buffered saline solution (pH 7.2) as control. The mice were anesthetized with chloral hydrate i.p. 300 mg/kg and killed by cervical dislocation 1 hr after injection. Both feet were cut-off at exactly the same level and weighted individually. The edema was expressed as the percentage increase in weight of the right foot compared to that of the left one. The values of percentage edema were estimated and the minimum edema dose (MED) was determined. The MED is defined as the least quantity of PLA_2 causing 30% increase in the weight, compared to the control.

Myotoxic activity

Groups of 4 mice (CF1, 18–22 g) were injected i.m. in the right gastrocnemius with 20 µg of crude venom or isolated enzyme dissolved in 0.1 ml of phosphate buffered saline solution, pH 7.2. Four mice were used as control samples, receiving each of them 0.1 ml of phosphate buffered saline solution (pH 7.2). After 1, 3, 6, 12 and 24 h of PLA_2 enzyme injection, mice were anesthetized with chloral hydrate i.p. 300 mg/kg to collect blood samples from the tail without using anticoagulant. Serum was obtained for the enzymatic determination of creatine kinase (CK) levels using a kinetic method U. V. (Randox). The CK activity was expressed in international units per liter, with one unit defined as the amount of enzyme that transfers 1.0 mM of phosphate from phosphocreatine to ADP per min at pH 7.4 at 30ºC. Creatine kinase activity was expressed in units per liter.

In order to have a histological assessment of myotoxicity, mice were sacrificed by cervical dislocation 1, 3, 6, 12 and 24 h after enzyme injection and samples of injected muscle were taken and fixed with Bouin solution for 24–48 h. Thereafter, the muscle was dehydrated in a graded alcohol series and embedded in paraffin. Sections 5 mm thick were stained with Haematoxylin and Eosin (H & E). Control muscles were processed in an identical manner. Necrosis was classified according to the method of Homma and Tu (1971), based on the morphology of the necrotic fibers. The myolytic type was characterized by fibrilar material, alterning with clear areas. In the coagulative necrosis type, the fibers acquired a hyaline appearance and its distribution was homogeneous.

Anticoagulant activity

Sheep citrated plasma was centrifuged at 1000 ∞ *g* at 4ºC and the anticoagulant effect was assessed as described by Gutiérrez *et al*. (1986a). Subsequently, 0.25 ml aliquots of plasma were incubated with 50 µl of solutions containing the following quantities of PLA_2,from 0.39; 0.78; 1.56; 3.13; 6.25; 12.5 and 25µg) dissolved in phosphate buffered saline (PBS, pH 7.5) for 10 min at 37ºC. Then, 0.1 ml of a solution of 0.25 M $CaCl_2$ was added and the clotting time determined. Control tubes contained only PBS incubated plasma, while $CaCl_2$ was added as described above. Observations were carried out for a maximum period of 60 min.

Lethality

Lethality was assayed by injecting i.p. different amounts of the enzyme PLA_2 (from 12.5 to 300 µg), dissolved in 500 µl of PBS, to groups of six mice (18-20 g). Deaths were recorded during 48 h (Sperman-Karber, 1981).

Hemorrhagic activity assay

The hemorrhagic activity in the fractions collected from Sephadex G-75 column was detected by the skin method of Kondo *et al*. (1960) and modified by Gutierrez *et al*. (1985). Instead of mice (CF1, 18–22 g) rabbits were used (as described by Kondo). They were shaved on the backs and intradermally (i.d) injected with 100 µl of samples. Skins were removed 2 h after and the diameters of the hemorrhagic spots were measured on the inside surfaces. Skins controls were injected with 100 µl of phosphate buffered saline solution (pH 7.2) which should not show halo.

Thrombin-like activity

In order to detect coagulant activity in fractions collected from Sephadex G-75 chromatography, the test

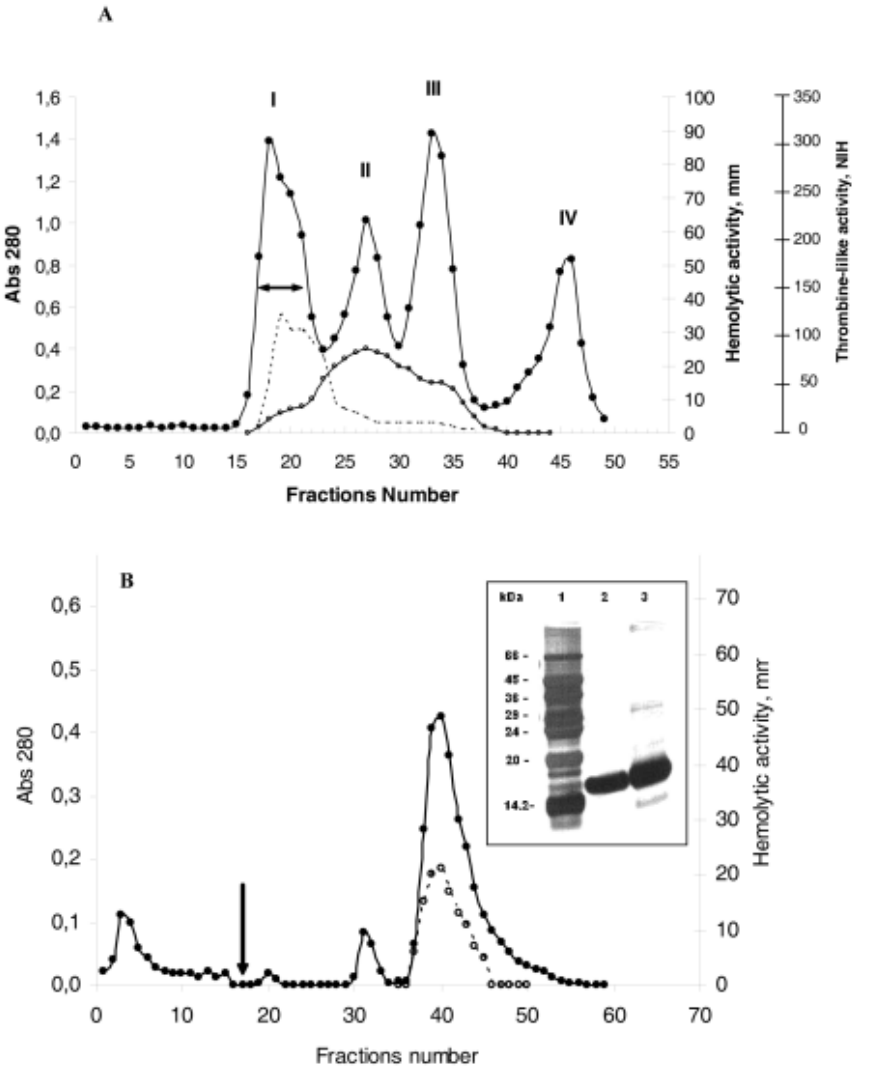

FIGURE 1. Isolation of phospholipase A_2 from *B. jararacussu* venom. (A) Twenty milligram of the venom dissolved in 250 µl of 10 mM sodium phosphate buffer, pH 7.2 was applied to a 1.0 x 50 cm column of Sephadex G-75 equilibrated with the buffer. The column was eluted with the same buffer and 0.75 ml fractions were collected. -●-, Protein (Abs_{280} nm); -○-, hemolytic activity (diameter of the halo, mm); ---, thrombin-like activity (NIH). Double arrow indicates fractions that showed hemorrhagic activity. (B) Ion exchange chromatography of the pooled fraction (SIII) on column SP Sephadex C-25 previously equilibrated with 50 mM Tris, 100 mM KCl, pH 7.0. The column was eluted with a linear gradient of KCl from 0.1 to 0.5 M in the same buffer, pH 7. Arrow indicates change in elution buffer -●-, Protein (Abs_{280} nm); -○-, hemolytic activity (diameter of the halo, mm). Insert SDS-PAGE at 15% (w/v), after reducing conditions. Molecular Mass Estimation. Lane 1, molecular mass markers: bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehydes-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), and a-lactalbumin (14,20 kDa). Lane 2, purified phospholipase A_2 obtained from the major peak of SP chromatography; Lane 3, fractions pool SIII. The gel was silver stained by the procedure of Blum *et al*. (1987).

described by Raw *et al*. (1986) was assayed. Clotting for 0.2 ml of diluted bovine plasma (3:1) with 20 mM Tris HCl buffer, pH 8.0) with 0.1 ml of chromatographied venom fractions at 37ºC, was recorded. The clotting times obtained were transformed into NIH units comparing clotting times of the test against a NIH Standard curve for Thrombin clotting times versus NIH units of Thrombin/ ml. (Lundblad *et al*., 1976).

Statistical analysis

The edema-forming activity, anticoagulant activity and indirect hemolytic activity of PLA_2, were estimated by linear regression, adjusted to the minimum square method. The results of the enzymatic determination of the serum CK levels were expressed as the arithmetic mean ± standard deviation (S.D.). The significance of differences between means was assessed by ANOVA followed by Dunnet´s test for multiple comparisons among to be statistically significant. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Isolation of Phospholipase A_2

In this work, a Phospholipase A_2 was purified from the venom of the snake *Bothrops jararacussu* in two steps. The fractionation of the crude venom on Sephadex G-75 showed four major peaks (Fig. 1A). The third peak (SIII) showed phospholipase A_2 activity, weak coagulant activity and no hemorrhagic activity, so it was submitted to purification. The second step, on a SP-Sephadex C-25 column (Fig. 1B), showed a small amount of non-bounded proteins and a major peak belonging to proteins retained by the cation exchanger and then eluted with a concentration gradient of KCl. This peak exhibited only phospholipase activity and showed a single band on SDS-PAGE, under reducing (insert Fig. 1B) and non- reducing conditions (not shown) corroborating a very efficient purification method. The molecular mass of the isolated enzyme was 15.6 kDa.

Characterization of Phospholipase A_2

Indirect hemolytic activity of PLA_2

Phospholipase A_2 activity is evidenced by the formation of haemolytic halos in the agarose-erythrocyte-egg yolk gels. In contrast, saline solution did not induce haemolysis. When egg yolk was not added to the gels there was no hemolysis, indicating that hemolysis was only of the indirect type, i.e. due to PLA_2 activity in this venom. The hemolytic halos were evaluated 20 h after enzyme incubation into agar gel and were proportional to the amount of enzyme loaded; the obtained linear relationship ($r = 0.991$) let us determine the MHD. The isolated phospholipase exhibited a hemolytic activity with a minimal hemolytic dose of 3.6 µg. MHD obtained for crude venom was 0.66 µg and the correlation coefficient ($r = 0.994$) obtained as a measure for hemolityc halos at different venom concentrations was appropriated for determining the corresponding MHD for *B. jararacussu* crude venom (Fig. 2).

Edema-forming activity

The isolated enzyme from *Bothrops jararacussu* snake venom exhibited intense edematogenic activity when tested by the foot pad assay. The percentage increases in weight of the right foot compared to that of the left were evaluated 1 h after injection and were proportional to the amount of solution injected; the obtained linear relationship for the isolated phospholipase A_2 (r =0,995) let us determine the corresponding MED. Compared to mouse foot pads injected with phosphate buff-

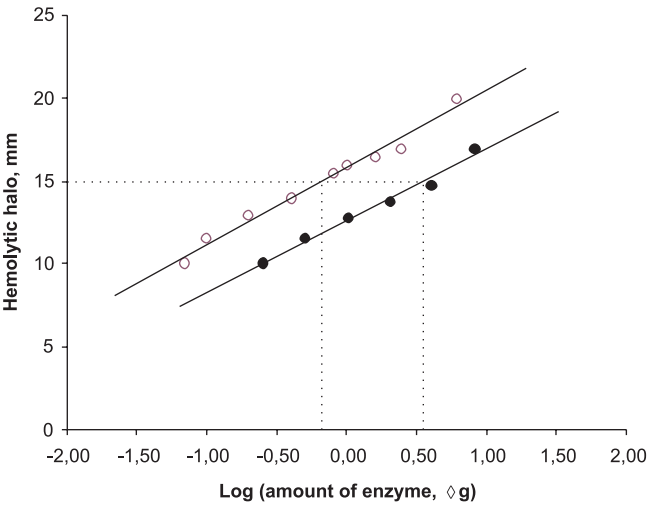

FIGURE 2. Effect of dose of *B. jararacussu* venom (-○-) and PLA_2 enzyme (-●-) on the indirect hemolytic activity *in vitro*. The DHM obtained from venom (0.66 mg) and from enzyme (3.66 mg) corresponding to the amount of venom or enzyme that induced a hemolytic halo of 15-mm diameter. Values are means of three determinations.

ered saline solution, a 30% increase in the weight was produced by injecting 0.61µg of the purified enzyme (Fig. 3).

Anticoagulant activity

The recalcification time of sheep platelet-poor plasma was prolonged after incubation with isolated phospholipase A_2. Clotting time showed a direct dependence according with the amount of enzyme considered, from 0.0078 to 0.500 mg/ml (Fig. 4). Enzyme concentrations larger than 0.031 mg/ml produced a significant effect (P<0.01) over the prolonged clotting time.

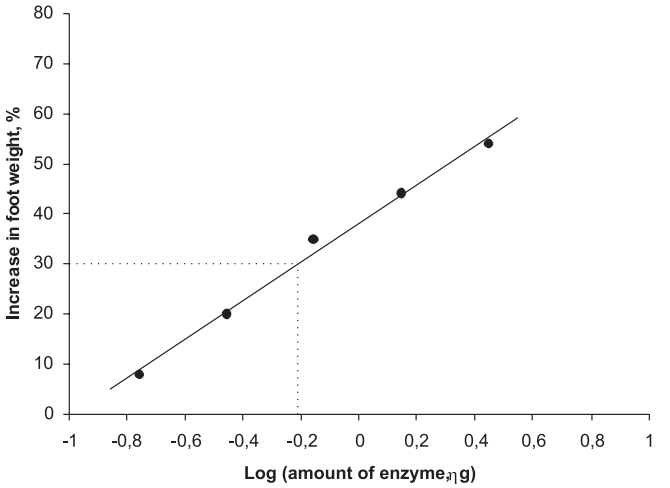

FIGURE 3. Effect of dose of PLA_2 enzyme isolated from *B. jararacussu* venom on edematogenic activity. The DEM obtained (0.61 µg) is defined as the quantity of PLA_2 causing 30% increase in the weight of the right foot, compared to the contralateral foot (control). Values are means of three determinations.

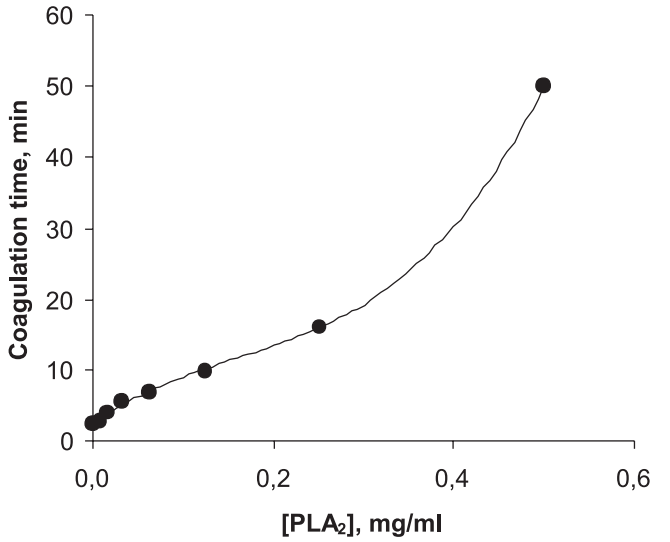

FIGURE 4. Delay in the clotting time of mice plasma previously incubated with purified enzyme from *B. jararacussu* venom. The clotting time of plasma by 0.25 M $CaCl_2$ corresponded to 2.5 minutes. Data show mean ± SD (n= 3) *P < 0.01 versus control plasma.

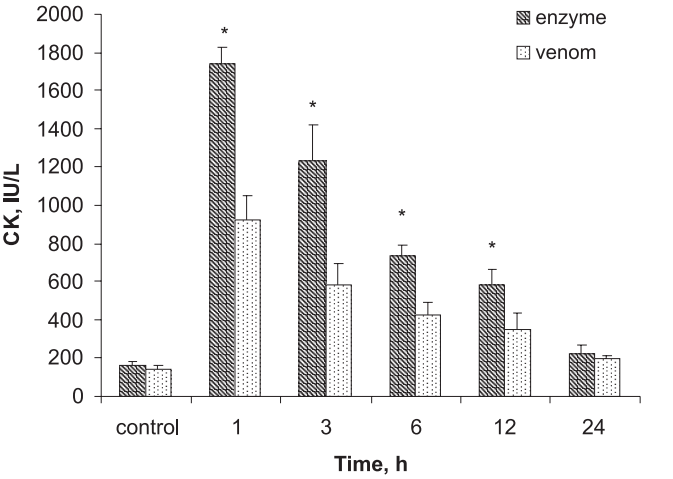

FIGURE 5. Changes in serum creatine kinase (CK) levels of mice after i.m. injection of 20 µg PLA_2 isolated or *Bothrops jararacussu* venom. At various time intervals mice were bled and serum CK levels determined. CK activity is expressed in international units/l. Results are presented as means ± SD (n = 4). All times were statistically different of the control (*P < 0.01) except at 24 hours.

Lethality

Groups of six mice were injected i.p. with different doses of 12.5 µg to 300 µg. Smaller doses than 100 µg were no lethal after 48 h, and doses of 300, 222, 164 and 122 µg produced 100, 83, 67 and 33% of deaths respectively. Sperman-Karber method was use for estimating the medial lethal dose which causes the death of 50% of a group of test animals. So then, the LD_{50} value obtained was 148.60 µg.

Myotoxic activity

After i.m. injection the enzyme induced a rapid and drastic increase in plasma creatine kinase levels, which is a specific marker for muscle damage. Maximum levels were observed at the 1st h, and decreased after 24h (Fig. 5).

Histological observations in samples of gastrocnemius muscle obtained after 1h of 20 µg PLA_2 injection showed intense destruction of muscular fibers accompanied by a light exudate inflammatory. Necrosis belonging to myolytic and coagulative type reached its highest levels at this time. Three hours later, inflammatory infiltrate, polymorphonuclear neutrophils migra-

tion and intense necrotic muscular fibers were observed. Results obtained 6 h after venom injections were not significantly different from those of 3 h later. Twelve hours later, edema, polymorphonuclear neutrophil inflammatory infiltrate and necrotic muscular fibers were observed (Fig. 6). Twenty four hours later abundant amount of rests of cellular membrane, resulting of the phagocytic process carried out by neutrophils, was observed. Necrosis of muscular fibers was more intense with the time of exhibition to the venom, arriving to its maximum expression between the 12 and 24 hours. Haemorrhage was not observed in all the cases.

The histological observations of samples of gastrocnemius muscle obtained 60 min after injection with whole venom, showed intense haemorrhage and muscular fibers destroyed as well as myonecrosis belonging to myolytic and coagulative type, accompanied with a slight edema. Necrosis in muscular fibers became more intense for the time of exposure to whole venom, reaching a peak between 6 hours (not show) and 24 hours. The inflammatory infiltrate was observed since 3 hours, predominantly polymorphonuclear neutrophil. Phagocytosis of death muscular fibers was active after 12 hours. At 24 hours, intensity of haemorrhage decreased.

On the contrary, histological observations of samples of gastrocnemius muscle injected with PLA_2 showed a mild necrosis of muscular fibers accompanied by inflammatory infiltrate of interfascicular polymorphonuclear. Haemorrhage was not detected. Among 6, 12 and 24 hours myonecrosis did not increase its intensity however; the inflammatory infiltrate began to be evident within 6 hours, raising the maximum level at 24 hours, which was an interfibrilar kind. These results are coincident with CK values observed, so that serum rate of animals injected with PLA_2 was double of that of the corresponding value for animals treated with the whole venom. In both cases, the major peak occurred between 1 and 3 hours, decreasing up to their normal values after 6, 12 and 24 hours.

Discussion

Previous studies have shown the presence of several PLA_2s in *Bothrops jararacussu* venom corresponding to animals kept in Brazilian areas (Díaz *et al*., 1992; Ketelhut *et al*., 2003; Roberto *et al*., 2004). This article reported the isolation of a basic phospholipase A_2 from

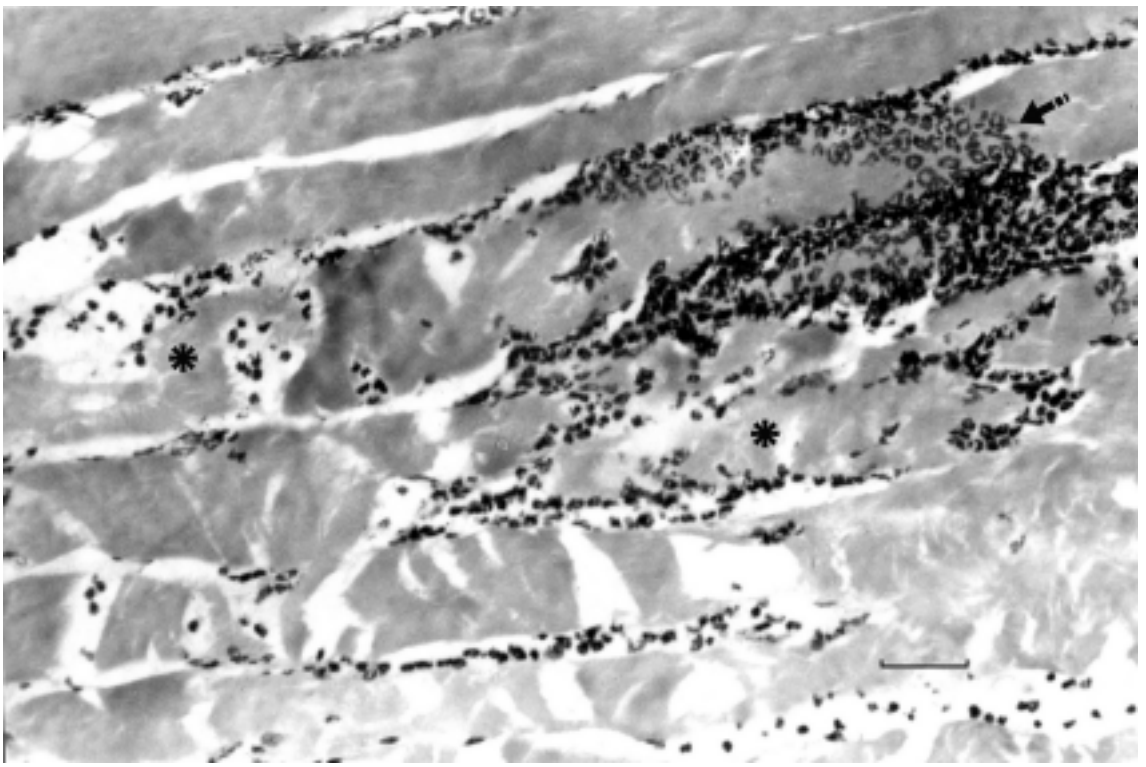

FIGURE 6. Histological cut of mouse gastrocnemius muscle. Mouse was injected with 20 μg of PLA_2 i.m. Time of exibition 12 hours. Intense inflammatory infiltrate, mainly polymorphonuclear (↑) and necrotic muscular fibers (*) were observed. Hematoxylin-Eosin. Bar represents 10 μm.

the venom of *B. jararacussu* of Argentina using a combination of gel filtration and ion-exchange chromatography. This enzyme showed to be a basic protein eluting from cation exchange column using salt gradient at neutral pH. The SP- Shephadex C25 purification procedure described here results in the isolation of homogeneous protein with a molecular mass of 15.6 kDa similar to other isolated phospholipases of *B. jararacussu* from Brazil 13.7 kDa (Roberto *et al*., 2004) and 15 kDa (Ketelhut *et al*., 2003).

PLA_2s snake venom usually revealed indirect hemolytic activity because promote hydrolysis of lecithins to lysolecithins able to lyse red blood cell membranes (Ketelhut *et al*., 2003). The isolated phospholipase A_2 showed indirect haemolytic activity as well as Bth TXII, in opposite to BthTX-I and PLA_2s acidic from *Bothrops jararacussu* that did not exhibit this activity. MHD for both the purified enzyme and the whole venom comparison (Table 1) suggests that probably other highly active PLA_2s could be present in this snake secretion.

The purified PLA_2 also induced edema, an activity known to be characteristic of other PLA_2venom (Selistre *et al*., 1990; Daniele *et al*., 1995). Development of edema is a common feature of the cutaneous inflammatory response and is dependent on a synergism between endogenous mediators that increase vascular permeability and those that increase blood flow (Ketelhut *et al*., 2003). Edematogenic activity exhibited by isolated enzyme was higher than the activity for the whole venom, indeed for BthTX-II a basic phospholipase A_2 from a brazilian *B. jararacussu* venom (Table 1), then it resulted in a significant low value assayed by MED.

With reference to the anticoagulant activity of PLA_2, we observed that when increasing the concentration of the enzyme the clotting time is prolonged. Díaz and coworkers (1991) suggest that the anticoagulant effect of myotoxic phospholipases A_2 from Bothrops venoms is dependent on enzymatic degradation of phospholipids necessary for the adherence and activation of coagulation protein factors. This hypothesis is supported by the observation that myotoxins prolong both recalcification time and prothrombin time, but not thrombin time, since only the former two depend on plasma phospholipids (Stefansson *et al*., 1989).

Most of the PLA_2 of venom snakes of the genus Bothrops possesses an action mechanism that consists on interact and to disorganize the phopholipids of the cellular membrane; followed by alterations in intracellular structures, with increment of cytosolic calcium what generates an ionic disorder favouring the entrance of water and macromolecules to the cell, the one that culminates dying (Gutiérrez *et al*., 1991). The histological observations of samples of gastrocnemius muscle obtained 1, 3, 6, 12 and 24 h after i.m. injection corroborated edema-forming activity and important myotoxicity. As early as this latter showed a rapid increase in plasma creatine ki-

TABLE 1.

Biological activities of whole venom and basic PLA_2s isolated from *Bothrops jararacussu* venom.

	MHD (μg)	MED (μg)	CK[a] (UI/L)	DL_{50} (μg/20g mouse -i.p.)	Reference
Venom (*Bj* A)[b]	0.66	1.5	579[d]	43.5	this work Acosta *et al.*, 1998 Maruñak *et al.*, 2006
Venom (*Bj* B)[c]		0.63	3197	74.7	Adrião-Escarso *et al*., 2000 Sanchez *et al*., 1992
PLA_2 isolated	3.6	0.61	1230[d]	148.6	this work
BthTX-I	(-) [e]	31.3	2279	170	Adrião-Escarso *et al*., 2000
BthTX-II	(+)[f]	23.8	3556	140	Adrião-Escarso *et al*., 2000

[a] 3 h after 50 μg injection, CK control 189 UI/l; [b] *Bj* A: *Botrhops jararacussu* from Argentina; [c] *Bj* B: *Botrhops jararacussu* from Brazil; [d] 3 h after 20 μg injection, CK control 189 UI/l; [e] no activity detected; [f] activity detected.

nase levels 1 h after injection, typical of myotoxic PLA_2 from Bothrops venoms. Tissue damage shows an increase of creatine kinase levels in plasma, since this enzyme is abundant in the cytosol of skeletal muscle fibers. This finding suggests that the plasmatic membrane of these cells has rapidly lost its ability to regulate the permeability to this enzyme (Gutiérrez *et al*., 1991). Similar observations have been made with other myotoxins from Bothrops venoms, such as *B. asper* myotoxin I (Gutiérrez *et al*., 1984a, b, 1986b) and *B. nummifer* myotoxin (Gutiérrez *et al*., 1989).

On the other hand, the endothelial wall is not affected by the enzyme, so that haemorrhage is not detected in histological observations, on the contrary injuries are produced by the whole venom, where other components like proteolytic enzymes are capable of degrade proteins from the basal membrane, allowing red blood cells extravation followed by haemorrhage. Such alterations are similar to those generated by venom that results in muscular necrosis (Gutiérrez and Lomonte, 1995; Acosta de Pérez *et al*., 1998).

The purified enzyme has a high LD_{50} value showing that little contributes to the lethality exhibited by the whole venom (43.5 µg). This result does agree with observations described for Brazilian *B. jararacussu* venom. Adrião-Escarso *et al*. (2000) showed that it was necessary a high dose of 140 µg for Bth-TX II or 170 µg for Bth-TX I to induce death in 50 percent of mice by i.p. injection while the medial lethal dose for the whole venom was 74.7 µg (Sanchez *et al.*, 1992).

The isolated enzyme possesses common characteristics with Bth-TX II isolated from *B. jararacussu* of Brazil. However, they do not show the identical behavior. Both phospholipases presented similar myotoxicity and lethality (Table 1), and even showed catalytic activity exhibited through indirect haemolytic action, although PLA_2 from Argentinean species is much more edematogenic than Bth-TX II. Further studies will be carried out to reveal if the found differences are due to geographic origin or molecularly distinct proteins.

In conclusion we isolated a basic phospholipase A_2 from *B. jararacussu* snake venom that inhabits the north-east region of Argentina. It was purified to homogeneity by only two simple chromatographic steps. This enzyme showed low lethality and the LD_{50} value was higher than the obtained for the whole venom (Table 1). On the contrary, this phospholipase exhibited high edematogenic and myotoxic activities, demonstrating its capacity to contribute to tissue damage after snakebite or envenoming produced by this *Bothrops yararacussu* snake.

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