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Isolation, amino acid sequence and biological characterization of an "aspartic-49" phospholipase A_2 from *Bothrops (Rhinocerophis) ammodytoides* venom

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A B S T R A C T

A phospholipase enzyme was separated by chromatography from the venom of the snake *Bothrops* (*Rhinocerophis*) *ammodytoides* and characterized. The experimentally determined molecular weight was 13,853.65 Da, and the full primary structure was determined by Edman degradation and mass spectrometry analysis. The enzyme contains 122 amino acids residues closely stabilized by 7 disulfide bridges with an isoelectric point of 6.13. Sequence comparison with other known secretory PLA2 shows that the enzyme isolated belongs to the group II, presenting an aspartic acid residue at position 48 (numbered by convention as Asp49) of the active site, and accordingly displaying enzymatic activity. The enzyme corresponds to 3% of the total mass of the venom. The enzyme is mildly toxic to mice. The intravenous LD_{50} of this phospholipase in CD-1 mice was around 6 μg/g of mouse body weight (more exactly 117 μg/mouse of 20 g) and the minimal mortal dose (MMD) was estimated to be close to 10 μg/g. In contrast, the LD_{50} of the venom was *circa* 2 μg/g mouse body weight. Toxicological analyses of the purified enzyme were performed *in vitro* and *in vivo* using experimental animals (mice and rats). The enzyme at high doses caused pulmonary congestion, intraperitoneal bleeding, inhibition of clot retraction and muscle tissue alterations with increasing of creatine kinase levels.

1. Introduction

Bothrops ammodytoides is the most Southern situated viper in the world. This snake inhabits a geographical region that goes from the warm desert regions to the cold Patagonia, in the South of the American Continent. This viper was reclassified under the genus *Rhinocerophis* (Fenwick et al., 2009). Carrasco et al. (2012) proposed calling this snake as belonging to the genus *Rhinocerophis*, synonymizing *Bothrops*, however according to the present nomenclature accepted it is known by the name of *Bothrops ammodytoides*. It is a small viper that usually does not surpass 70 cm in length, but can cause the typical problems

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of bothropic envenoming (Martino et al., 1979; de Roodt et al., 2000; Ministerio de Salud, 2007).

Its vulgar name in Argentina is "yarará ñata" or "ñata". The word "ñata" means "flat nose" (from *ñato* in *quechua* language) due to the characteristic of the nasal plaques of some specimens that gives the aspect of a "flat nose". This snake is different from other *Bothrops* species of Argentina, because its diet commonly includes lizards (Martins et al., 2002).

Some preliminary characteristics of the venom from this snake indicate that it is lethal and can cause hemorrhage, dermonecrosis, and edema in mice. It is also myotoxic to mice, and shows procoagulant activity on human plasma, but low or absent thrombin like activity over bovine fibrinogen (de Roodt, 2002). The enzymatic activities described were proteolysis over gelatin, casein and p-toluensulfonil arginine (TAME), phospholipase and indirect hemolytic activities and DNAse activity (de Roodt, 2002; de Roodt et al., 1998, 2003). Although partial separation of the venom demonstrated that some fractions are lethal to mice, *per se* (de Roodt et al., 1998) there are no reports on the identity of their venom components. In this work we describe the fractionation of the venom using several chromatographic steps and the isolation, biochemical and biological characterization of an aspartic-49 (D49) phospholipase A_2. As in other enzymes of this group, the aspartic acid residue is situated at position 48, but conventionally referred to as D49. The enzyme has 122 amino acid residues, packed by 7 disulfide bridges with a molecular weight of 13,853 Da and a theoretical isoelectric point of 6.13. A general characterization of this enzyme is described, but also includes a phylogenetic tree, where this phospholipase segregates closely to other phospholipases from Brazilian snakes of the genus *Bothrops* (*B).*

2. Materials and methods

2.1. Venom

The venom used was a pool of adult specimens of *B. ammodytoides* from different regions of Argentina: the provinces of La Pampa, Chubut and the Central region and South of the province of Buenos Aires.

Adult snakes in good health conditions were kept in plastic cages, at 27 °C of constant temperature. Snakes were fed with 1 or 2 white mice weekly and received filtered water *ad libitum*. The light/darkness cycles were kept at 12 h each.

Venom was obtained by manual extraction. Immediately after extraction the venom was vacuum dried and stored at −20 °C until use.

2.2. Purification of the enzyme

Various chromatographic steps were used to obtain in homogeneous form the enzyme. Initially, 100 mg of venom diluted in 20 mM ammonium acetate buffer pH 4.7 was separated by gel filtration into a Sephadex G-75 column (200 × 1 cm) at a flow rate of 14.7 mL/h. Fractions of 2 ml were collected and absorbance recorded at 280 nm. A second fractionation was obtained by separating the fraction III of the gel filtration by means of cation exchange chromatography in a Fast Protein Liquid Chromatography System (Pharmacia LKB) in a Mono-S column (HR 5/5 cation interchange 0.5 × 5 cm of Phamacia). Buffer A was 20 mM ammonium acetate, pH 4.7 and buffer B was same buffer containing 2 M NaCl. The gradient was run from initial buffer A to 30% buffer B, run for 50 min at flow rate of 1 mL/min. Various fractions were collected, pooling the tubes that correspond to the same chromatographic peak and lyophilized. A final C18 analytical reverse-phase column (Vydac 218 TP104) was used by HPLC in order to obtain the enzyme in pure form. For this separation buffer A was 0.12% trifluoroacetic acid (TFA) in water, and buffer B was 0.10% TFA in acetonitrile. The gradient was run from buffer A to 60% buffer B for 60 min at flow rate of 1 mL/min.

2.3. Sequence determination

A sample of purified enzyme was directly submitted to automatic Edman degradation with a Beckman LF3000 protein sequencer (Palo Alto, CA). Additional sequence data was obtained using reduced and alkylated enzyme (Brune, 1992) subjected to enzymatic hydrolysis with trypsin, chymotrypsin and endoproteinase Glu-C (Roche Diagnostics, GmbH, Penzberg, Germany) using procedures earlier described by our group (Diego-García et al., 2005; Caliskan et al., 2006; Batista et al., 2006). Sub-peptides obtained from the digested PLA_2 were separated by HPLC (Supplementary material Table 1 and Figs. 1–3) and completion of the sequence at the C-terminal residues was obtained by mass spectrometry fragmentation analysis (collision induced dissociation), using a mass spectrometer LTQ-Orbitrap XL (Thermo-Fisher Co., San Jose, CA). The same equipment was used for determination of the exact molecular mass of the PLA_2 described here.

2.4. Phospholipase activity

The enzymatic activity of the purified enzyme was determined by the hydrolysis of egg yolk phospholipids and titration of fatty acids with NaOH (Shiloah et al., 1973). The activity was expressed as μEq of NaOH/min (units) by mg of enzyme.

2.5. Toxicity assays

2.5.1. Lethality

CD-1 mice (18–22 g body weight) were injected with 10, 50, 100, 200, 250 and 300 μg of the purified PLA_2 by the intravenous route. Four animals per each dose of enzyme were used. Immediately after death, the necropsy was performed annotating the pathological findings for each animal studied. In all cases samples of several organs (lungs, hearth, kidneys and liver) were taken for the histopathological analysis.

The challenge dose and deaths after 24 h were plotted and analyzed by non linear regression. The median lethal dose (LD_{50}) was estimated as the dose of enzyme that killed half of injected mice. The minimal mortal dose (MMD) was defined as the lowest concentration of enzyme that killed 100% of injected mice.

As control, the LD_{50} of the venom was determined in the same type of mice (5 mice per dose level) according to suggestions of the WHO (2010).

2.5.2. Creatine kinase activity

Wistar rats ($n = 7$) were slight anaesthetized with ether and injected intramuscularly (i.m.) into the *tibialis* anterior muscle with 50 μl containing 5 or 10 μg of the isolated PLA_2 in a final volume of 50 μl solution made with 0.15 M NaCl. Rats injected in the same muscles with 0.15 M NaCl were used as control. All the animals were slightly anesthetized with ether and bled by the retroocular plexus in hours 2, 6 and 24 h after injection. The values were expressed as U/L (units/liter).

The creatine kinase (CK) activity was determined by means of the kit CK-NAC (Laboratory Wiener, Rosario, Argentina). This procedure permits estimation of ATP levels from ADP and creatine phosphate. The catalytic concentrations are determined using the reactions coupled to hexoquinase and glucose 5-P deshidrogenase based on the levels of NADPH formation by reading at 340 nm.

2.5.3. In vitro *anticoagulant activity*

Different doses (5–80 μg) of the purified PLA_2 in 0.15 M NaCl in a final volume of 100 μl were added to 0.4 ml of human plasma with sodium citrate at 0.4%. After incubation at 37 °C for 1 min, 100 μl of 50 mM $CaCl_2$ was added and the time of clot formation was estimated. Samples of plasma treated in the same manner, but without addition of the PLA_2, were used as control. Experiments were performed by at least triplicate.

2.5.4. Coagulant activity

The coagulant activity on plasma and bovine fibrinogen were determined as described by Theakston and Reid (1983), by triplicate measurements.

2.5.5. Defibrinogenating activity

The ability of venoms to cause blood incoagulability *in vivo* was measured according to Theakston and Reid (1983). Briefly, CF-1 mice (18–22 g, 4 by level dose) were injected intraperitoneally with increasing amounts of enzyme (starting with 12.5 μg and up to 100 μg because venom doses in the order of 200 μg kills the mice). After 1 h of injection, animals were bled and samples allowed clotting for 30 min. Inspection for the presence of blood clots were conducted.

2.5.6. Clot retraction

Clot retraction is a function of platelets (BCSH, 1988) and can be affected by snake venoms (Dodds and Pickering, 1972; Chow et al., 1998). We studied the clot retraction of blood treated with the enzyme, as function of the platelets functionality (Kitchen et al., 2010). Human blood of healthy no smoker volunteers (Hematocrit 43%, platelets 320.000 mm^3) was treated with several doses of the enzyme (from 0.56 μg/ml to 28.57 μg/ml) and the samples were incubated 2 h at 37 °C. After incubation, the volume of exudates of serum was compared with the initial volume of blood and the difference was expressed as percentage. This difference in volume after treatment is representative of the clot retraction (Kitchen et al., 2010). Tubes without the addition of the enzyme and treated with 0.15 M NaCl were used as control.

2.5.7. Edema formation

As a measure of the local activity of the venom, the occurrence of edema, in injected limbs was estimated. Limbs of rats (Wistar, 6 animals by time of determination) injected with venom as described for CK activity, were excised in the hip joint and weighed. The difference in weight regarding the controls (contralateral limbs injected with the same volume of NaCl 0.15 M alone) was expressed as percentage.

2.5.8. Hemorrhagic activity

To detect the presence of hemorrhagic activity in addition to the macroscopic observation, CF-1 mice ($n = 5$) and Wistar rats ($n = 5$) were injected (intradermal route) with 100 μg of the enzyme. After 3 h (mice) or 24 h (rats), animals were killed and the presence of hemorrhage in the dermal side of the skin was observed.

2.6. Histopathology

2.6.1. Systemic study

Necropsy of mice (CD-1) injected by the i.v. route were processed immediately after death and samples of lungs, hearth, kidneys, liver and striated muscle were obtained and conserved in buffered formaldehyde 10%.

2.6.2. Study of myotoxicity

For the myotoxicity assays after 24 h of venom injection as described, rats (Wistar) were anaesthetized (diethilic ether), killed by an overdose of anesthesia and the necropsy was immediately performed. Samples of muscles injected with venom, control limbs and kidneys of injected animals and controls, were conserved in 10% formaldehyde buffered. Samples were embebed in paraffin and stained with hematoxiliyn–eosine.

2.7. Statistics

Results were expressed as mean and standard deviation, or as mean with their 95% confidence intervals. To establish the normal distribution of the variables the Kolmogorv–Smirnov test was used. When necessary the *t* Student or Mann–Whitney tests were used for comparison between groups. For all the statistical methods the software Prism 4.0 (Graph Pad Inc., San Diego, CA) was utilized.

2.8. Phylogenetic analysis

A multiple alignment of 40 secretory PLA_2 sequences representative of group II was performed using the program CLUSTALX (Larkin et al., 2007). The phylogenetic analysis was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and performed using the Molecular Evolutionary Genetics Analysis version 5 (MEGA5) (Tamura et al., 2007). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as

those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset.

The sequences selected and downloaded from GenBank, were from *Bothropoides diporus* (AFJ79207, AFJ79208), *Bothrops erythromelas* (ABC96692), *Bothrops pictus* (AAF91498; Q9I8F8), *Bothrops moojeni* (ADQ08654), *Bothrops pirajai* (C9DPL5), *Bothrops jararacussu* (AAN37410, 1Z76_B), *Bothrops alternatus* (P86456), *Sistrurus catenatus catenatus* (ABY77921), *Sistrurus catenatus edwardsi* (ABG26993), *Sistrurus catenatus tergeminus* (AAS79430), *Bothriechis schlegelii* (AAW92118; AAW92117), *Crotalus viridis viridis* (AAO93140, AAO93137), *Crotalus atrox* (P00624), *Lachesis muta* (ADB77855), *Gloydius halys* (O42190, O42192), *Gloydius shedaoensis* (AAR11860), *Trimeresurus gramineus* (P81479, BAA06552, P20476), *Viridovipera stejnegeri* (AAP48897), *Trimeresurus flavoviridis* (BAA01568), *Trimeresurus puniceus* (AAR14168), *Vipera aspis aspis* (CAE47225), *Daboia russellii* (AAZ53183), *Bothropoides pauloensis* (3MLM_A), *Bothrops atrox* (Q6JK69), *Protobothrops elegans* (BAE72890), *Crotalus durissus terrificus* (2QOG_A), *Sistrurus catenatus tergeminus* (AAR14164, AAR14163), *Deinagkistrodon acutus* (Q1ZY03), *Cerrophidion godmani* (AAR14161), *Sistrurus miliarius* (AAR14160), *Homo sapiens* (AAH05919). The PLA_2 sequence from snake *Naja sputatrix* (AAF82186) that belong to group I, was used as outgroup.

2.9. Animals

The rats (Wistar strain) utilized in this study were kindly obtained from Gema Biotech (Buenos Aires, Argentina). The mice were provided by the animal facilities of the National Institute of Production of Biologicals (Buenos Aires, Argentina) (CF-1 strain) or by the Institute of Biotechnology of the Autonomous National University of Mexico (Morelos, Cuernavaca, Mexico) (CD-1 strain). Animals were kept in plastic cages receiving water and food *ad libitum* under controlled temperature conditions. Ethical guidelines regarding the management of animals were those recommended by the National Research Council (2002).

3. Results and discussion

3.1. Isolation and sequence determination of the PLA_2 enzyme

Venom of *B. ammodytoides* was loaded into a Sephadex G-75 column separating at least six distinct fractions (Fig. 1), in which the phospholipasic activity was present in fraction III. The inset of Fig. 1 shows the SDS-PAGE (Laemmli, 1970) resolution of the various components eluted from the column. Lane III contains proteins on the range of the expected molecular weight for this PLA_2, smaller than 16 kDa but higher than 6.5 kDa.

Fraction III was applied into an FPLC system using a Mono S column from which *circa* 12 different components were recovered (Fig. 2). Fraction number 4 contained the enzymatic activity, which was finally purified to homogeneity applying this fraction into a C18 reverse phase column of the HPLC apparatus and resolved a major component (labeled with asterisk in the inset of Fig. 2) displaying the enzymatic activity. The purity of this protein was verified by mass spectrometry: only one component with molecular weight of 13,853.65 Da. Additional confirmation that the protein was pure was obtained by loading a sample into the Beckman Sequencer. The N-terminal sequence showed only one amino acid residue per cycle and allowed the identification of the first 26 amino acid residues. Samples of the enzyme were reduced and alkylated as described in the Materials and methods section and digested with three different enzymes (trypsin, chymotrypsin and endoproteinase Glu-C) in independent experiments. Several sub-peptides were obtained (Supplementary Material Figs. 1–3 and Table 1) and sequenced. Fig. 3 shows the full sequence obtained after application and identification of 11 different peptides, labeled Tryp1 to Tryp6, Chym 1 to 4 and Glu-C 1, which allowed preparing an overlapping map of the full amino acid sequence. In order to fully complete the sequence the most C-terminal situated peptide was submitted to collision induced dissociation into the Orbitrap spectrometer, which allowed the identification of the three last residues Glu-Pro-Cys (see details in Table 1 of Supplementary Material).

The experimentally determined molecular weight was 13,853.65 Da which is in agreement with the isotope-averaged theoretically expected mass estimated from the sequence shown in Fig. 3. The 14 cysteine residues are all forming 7 disulfide bridges. These data unequivocally supports the conclusion that the determination of the primary structure of the PLA_2 is correct. A sample of pure enzyme was applied in gels prepared with ampholites for isoelectric focusing (Fig. 4 of Supplementary Material), showing that the isoelectric point is 6.13, the same as the theoretically calculated using the “compute pI/MX tool” of SIB Bioinformatics Resource Portal of ExPASy. The amount of enzyme in the whole venom was estimated to be in the order of 3%.

The phospholipasic activity of the enzyme was obtained by the technique that uses egg yolk (Shiloah et al., 1973). In these conditions the enzymatic activity of the isolated enzyme was of 159 U/mg and that of the whole venom of 36 U/mg.

The enzyme has 122 amino acid residues, showing important sequence similarities with other known phospholipases A_2, as it will be discussed later in section 3.3. Close analysis of the sequence shows that this enzyme belongs to the D49 group of Type II PLA_2. Due to high sequence identity with other known phospholipase A_2 from snake venom origin, the enzyme purified and described here was assumed to hydrolyze fatty acids in position 2 of the phospholipids, defined as phospholipase A_2 type.

The protein sequence data reported of this enzyme will appear in the UniProt Knowledgebase under the accession number 86907.

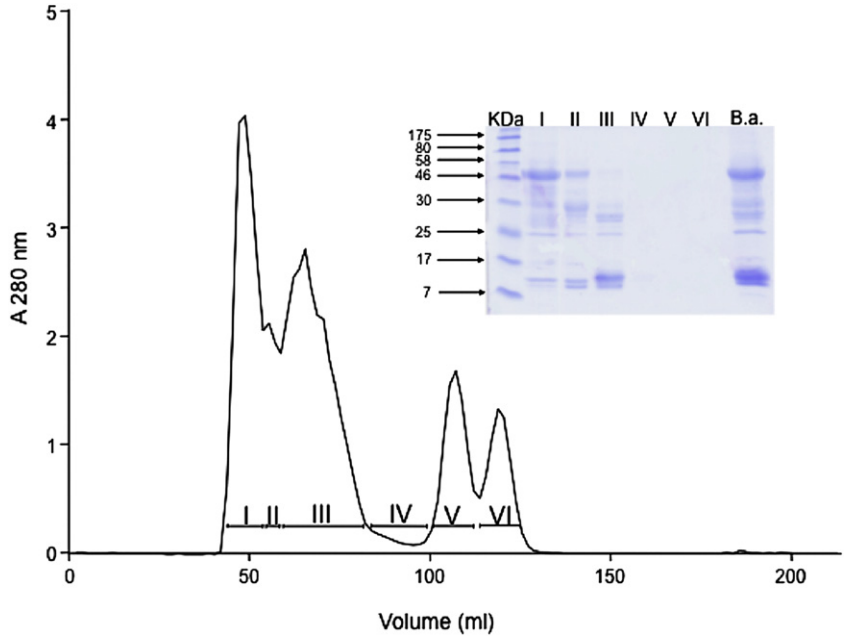

Fig. 1. Sephadex G-75 gel filtration and SDS-gel electrophoresis. Venom (100 mg) was applied to the column (200 × 1 cm) and eluted with 20 mM ammonium acetate buffer, pH 4.7. Fractions of 2 ml each were collected and pooled according to the main chromatographic peaks, indicated by roman numbers I to VI. Final recovery was approximately 98%. A sample of each sub-fraction (20 μg) was subjected to electrophoretic separation on polyacrylamide gels (Laemmli, 1970) showing the type of molecular weight proteins contained in each fraction. Left lane is molecular weight markers and *B.a* means venom of *Bothrops ammodytoides*.

3.2. Toxicological and biochemical observations due to PLA_2 application in experimental animals

A series of experiments were conducted to characterize the lethality of the enzyme in experimental animals as well as to study clinical aspects caused by the enzyme when injected into mice and rats. Toxicity assays showed relatively low lethal potency. The LD_{50} was exactly 5.85 μg/g (117 μg per 20 g mouse weight) and the MMD was estimated to be close to 10 μg/g. In contrast, the LD_{50} of the venom was

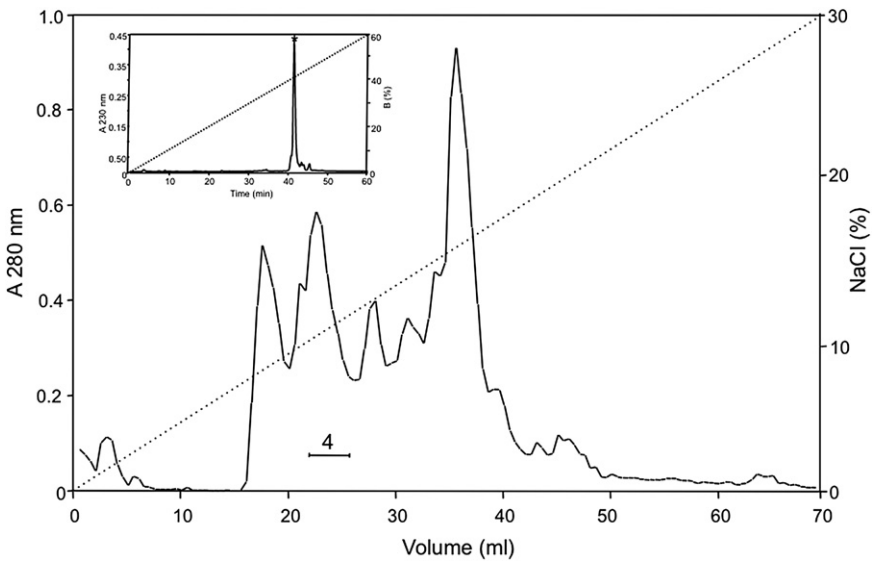

Fig. 2. Ion-exchange separation of sub-fraction III. A fast protein liquid chromatography (FPLC) system from Pharmacia LKB, using a Mono-S column, was load and run with 33 mg protein of sub-fraction III in 20 mM ammonium acetate pH 4.7 (buffer A). For elution a gradient of buffer A up to 30% buffer B (same buffer containing 2 M NaCl) was run at 1 mL/min flow rate. Sub-fraction 4 (about 2.5 mg of protein) displayed phospholipase activity and was further separated in a C18 reverse-phase column of the HPLC system, using gradient indicated in Material and methods. Fraction labeled with asterisk corresponds to pure enzyme.

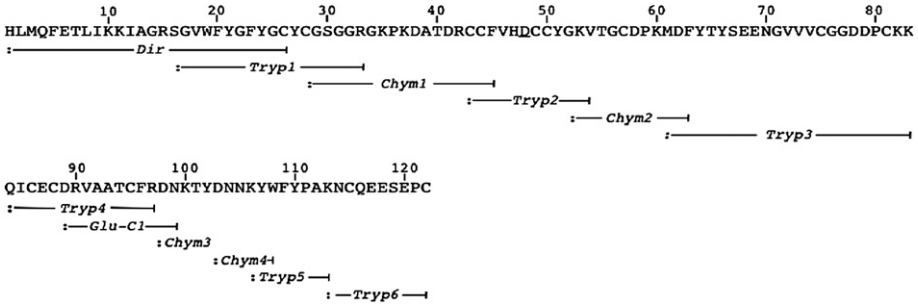

Fig. 3. Amino acid sequence of the PLA_2 enzyme. A complete overlapping mapping was obtained for the 122 amino acid residues. Details are on Figs. 1–3 and Table of Supplementary Materials. Direct Edman degradation allowed the identification of the first 26 residues and 11 sub-peptides obtained by enzymatic hydrolysis with Try (trypsin), Chym (chymotripsin) and endopeptidase Glu-c (Glu-C1) permitted to obtain the full amino acid sequence. The last peptide was sequenced by mass spectrometry analysis. The protein sequence data reported in this paper will appear in the UniProt Knowledgebase under the accession number 86907.

2.15 μg/g (varying from 2.0 to 2.25 μg/g body weight), which shows the low systemic toxicity of the enzyme.

The gross pathological examination showed pulmonary congestion and some hemorrhagic spots in lungs and, hemorrhages in abdominal cavity represented by intraperitoneal bleeding. Nevertheless the enzyme did not affect *in vitro* or *in vivo* the coagulation following the assay described. The PLA_2 did not show coagulant activity nor delayed the coagulation time in human normal plasma in the conditions of this study.

The histological study of the organs from mice injected with 100 μg of venom showed slight edema and congestion in lungs with no alterations in the remaining organs. Mice injected with 200 μg showed congestion and occasionally septal edema in lungs, hepatic congestion and focal injury of kidney tubular epithelium. Mice injected with 300 μg showed congestion and acute edema of lungs with emphysema, atelectasis and thrombosis (Fig. 4).

The enzyme showed a slight dose dependent edematogenic activity. Intramuscular injection of 2.0 μg into limbs

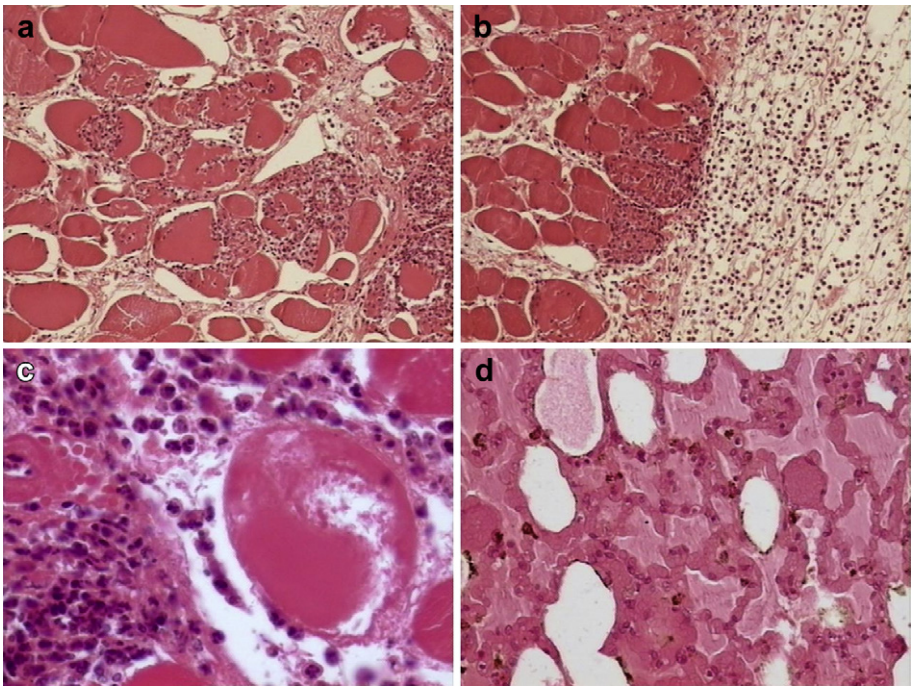

Fig. 4. Histopathological lesions of muscle and lung. a: Muscle of rat injected with 10 μg of venom shows acute extensive leukocyte infiltration with compromise inter and extrafibrillar (enhancement: 250×). b: Right segment shows marked edema and acute leukocyte infiltration. In the left it can be observed muscular necrosis with inflammation (enhancement: 250×). c: Severe lesion of muscular fiber, hemorrhage with arterial lesions and presence of numerous neutrophils (enhanced 400×). d: Lung of mouse injected with a lethal dose of venom. The figure shows septal congestion, edema and presence of alveolar macrophages with content of pigments (enhancement: 250×).

showed differences regarding the controls only after 120 min ($p = 0.008$), which was persistent at least up to 240 min post inoculation ($p = 0.013$). The injection of 20 μg of enzyme caused an elevation of the edema after 30 min injection, when compared to controls ($p = 0.037$). The inflammation started decreasing after 240 min post inoculation, and the injected limbs after 24 h experiment were normal compared to controls in both cases ($p > 0.2$), (see Fig. 5).

Although the levels of CK increased after inoculation (Fig. 6), the values were low, very close with the controls in both experimental models despite its statistical significance ($p < 0.05$). The CK levels after 2 h were increased ($p = 0.004$) and the highest level of plasmatic CK activity was found at 6 h post injection of the enzyme ($p = 0.029$).

The histological study of the muscles showed lesions of different intensity depending on the dose of enzyme injected. After 2 h of injecting muscles with 5 μg of enzyme some interfibrillar edema was observed and a mild process of leukocyte recruitment begun. After 6 h, muscles showed interfibrillar edema and a clear leukocyte recruitment, but after 24 h showed slight interfibrillar edema and occasional inflammatory infiltrate in the interphase of fat muscle tissue. When using 10 μg of enzyme after 2 h muscles showed acute lesion, moderate edema and focal necrosis of fibers, leukocyte marginalization and low acute inflammatory changes. After 6 h moderate interfibrillar edema, occasional leukocyte infiltrate and leukocyte marginalization, with light inflammatory response were observed. After 24 h injected muscles show intense inflammation of mononuclear type affecting vessels, inflammatory interfibrillar compromise and focal necrosis of muscular fibers.

The enzyme isolated did not show important systemic toxicity. The lethality was only observed with very high doses (LD_{50} 117 μg and MMD of 200 μg) and only slight muscular lesions were observed after their intramuscular injection. Despite these findings, the intraperitoneal bleeding and some hemorrhagic spots in lungs, confirmed the presence of systemic lesions at high dose of enzyme injection. High doses of the enzyme caused acute pulmonary edema, which is congruent with observations of the participation of PLA_2 enzymes in the mechanisms of pulmonary edema in some envenomations (Cher et al., 2003).

The study of coagulation did not show coagulant activity on plasma or fibrinogen *in vitro* or defibrinogenating activity *in vivo*. The recalcification time did not show differences regarding the controls, which indicate no anticoagulant activity on plasma. These results show absence or minimal activity on humoral hemostatic system.

Although hemorrhagic spots and peritoneal bleeding was observed under necropsy with highest doses, no

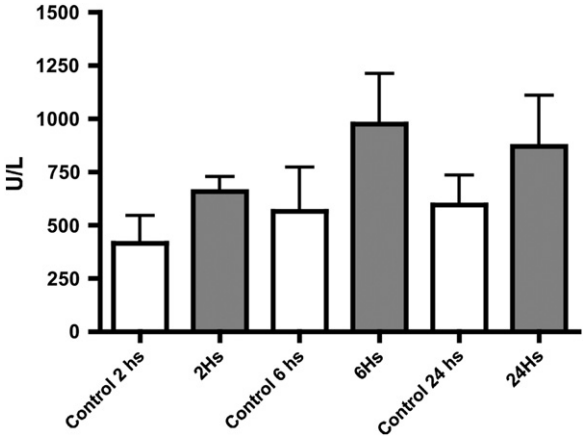

Fig. 6. Plasmatic creatine kinase (CK) activity in rats. The values of CK were obtained after injecting pure enzyme by intramuscular route. Elevation of activity was observed after 2 h (p 0.004), reaching the highest values after 6 h (p 0.029) of inoculation, maintaining high levels after 24 h although without differences with statistical significance ($p = 0.389$). White bars are values obtained from rats used as controls, injected with NaCl 0.15 M. Dark grey bars are the values measured after injection with pure enzyme. Results are expressed as units of CK activity by liter of plasma.

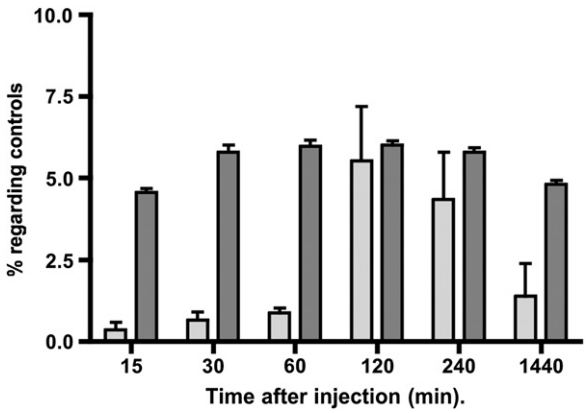

Fig. 5. Inflammation and edema produced by intramuscular injection. The figure shows the edematogenic activity in rats after intramuscular injection of the enzyme, measured at distinct intervals of time. The difference between limbs injected with 2 μg (white bars) or 20 μg (grey bars) of the enzyme regarding the contralateral limb injected with NaCl 0.15 M is expressed as percentage. Differences regarding controls turn significant ($p < 0.05$) after 30 min using 20 μg of enzyme whereas with 2 μg differences were observed after 120. After 24 h, differences were not observed with either 2 μg ($p > 0.5$) or 20 μg ($p > 0.2$).

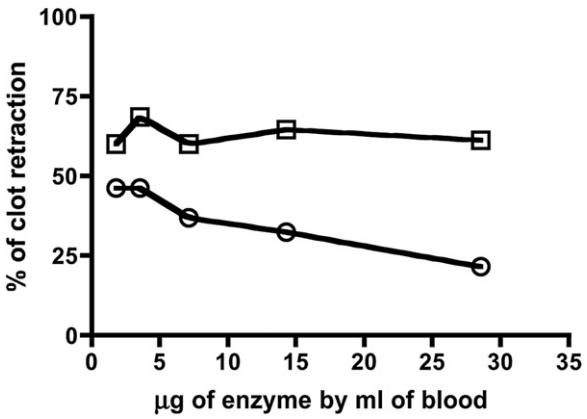

Fig. 7. Inhibition of clot retraction. Percentages of clot retraction are shown for blood treated with the enzyme (circles) or with a control using NaCl 0.15 M (squares). Differences were observed with all the doses tested, from 1.79 μg of enzyme by mL of blood, and the inhibition was dose dependent.

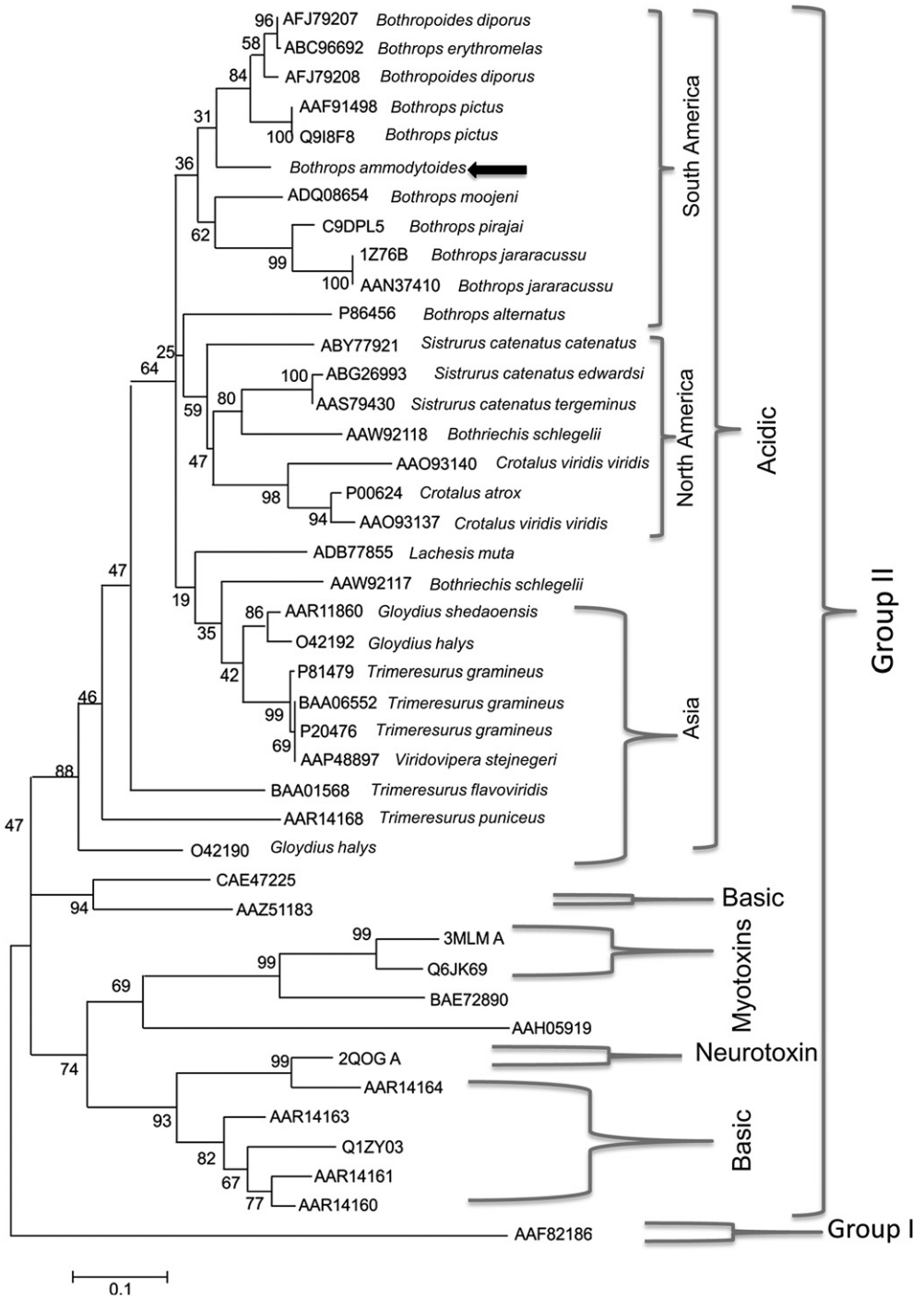

Fig. 8. Phylogenetic tree of secretory phospholipases. Phylogenetic tree constructed using the Neighbor-Joining method (Saitou and Nei, 1987) for secretory PLA_2's, based on their amino acid sequences. The numerals at the nodes represent the bootstrap confidence (1000 replicates). The horizontal branch lengths represent the numbers of nucleotide substitution per site. Lengths are in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The phylogenetic tree was generated using PLA_2 sequences from the snakes of the genera: *Bothropoides*, *Bothrops*, *Sistrurus*, *Bothriechis*, *Crotalus*, *Lachesis Gloydius*, *Trimeresurus*, *Viridovipera*, *Vipera*, *Daboia*, *Protobothrops*, *Deinagkistrodon* and *Cerrophidion*. Those PLA_2 were compared with PLA_2 from *Homo sapiens* (AAH05919) from group II, and the PLA_2 sequence from *Naja sputatrix* from group I, was used as outgroup. In order to avoid the crowding names within the figure-tree, only related sequences with the acidic PLA_2 described here are indicated with the accession numbers followed by the species name of the animal. An arrow indicates PLA_2 from this work. Accession number identified the other sequences.

hemorrhage could be observed in the skin assay. The blood extravasation does not seem related with lesions in the extracellular matrix of blood vessels. However, significant differences regarding the clot retraction of the samples of blood treated with the PLA_2 were observed compared to controls ($p = 0.0004$, $t = 6.3$). In addition the inhibition of clot retraction showed a dose dependent pattern (Fig. 7). The findings in abdominal cavity and lungs could be related with some interference of the enzyme in the platelet aggregation (since it was possible to observe a dose dependent clot retraction) and/or on coagulation system that could not be detected by means of the techniques used here. Regarding the activity on platelets, from the venom of *Bothrops erythromelas* was isolated an acidic PLA_2 that inhibit the platelet aggregation (de Albuquerque et al., 2006), and this PLA_2 is strongly related with the enzyme isolated in this work (Fig. 8).

Further studies on the activity over the coagulation system and action on platelets are required to fully understand the possible mechanism on hemostatic abnormalities observed in experimental animals.

3.3. Phylogenetic analysis of the PLA_2 studied

Fig. 8 shows the results of conducting a phylogenetic analysis of the PLA_2 described here, in comparison with 40 secretory PLA_2's from different snake venoms belonging to the group II, including representative sequences of acidic and basic subgroups, and covering species from Old and New World. Genera like *Bothropoides*, *Bothrops*, *Sistrurus*, *Bothriechis*, *Crotalus*, *Lachesis*, *Trimeresurus*, *Viridovipera*, *Vipera*, *Protobothrops*, among others, were considered. The PLA_2 from the mammalian (*Homo*) belonging to group II was also considered. The PLA_2 sequence from elapid *N. sputatrix* that belongs to the group I, was used as outgroup. The strategy described in Section 2.8 was repeated several times in order to obtain a robust phylogenetic result. As it can be observed the PLA_2 here reported was segregated very close with PLA_2 from *Bothrops erythromelas*, *B. pictus*, *B. diporus* and *B. pirajai*. Particularly, *B. erythromelas* and *B. ammodytoides* feed on reptiles, 30% of the diet for *B. erythromelas* and about 40% for *B. ammodytoides* are lizards (Martins et al., 2002). If this enzyme possesses some differential toxicological or enzymatic activities on reptile tissues, should be further investigated.

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Appendix A. Supplementary material

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2012.08.019.

Conflict of interest statement

The authors declare that there is no competing interest in this work.

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