Purification procedure for the isolation of a P-I metalloprotease and an acidic phospholipase A2 from Bothrops atrox snake venom

Danilo L. Menaldo, Anna L. Jacob-Ferreira, Carolina P. Bernardes, Adélia C. O. Cintra and Suely V. Sampaio*

Abstract

Background: Snake venoms are complex mixtures of inorganic and organic components, mainly proteins and peptides. Standardization of methods for isolating bioactive molecules from snake venoms is extremely difficult due to the complex and highly variable composition of venoms, which can be influenced by factors such as age and geographic location of the specimen. Therefore, this study aimed to standardize a simple purification methodology for obtaining a P-I class metalloprotease (MP) and an acidic phospholipase A2 (PLA2) from Bothrops atrox venom, and biochemically characterize these molecules to enable future functional studies.

Methods: To obtain the toxins of interest, a method has been standardized using consecutive isolation steps. The purity level of the molecules was confirmed by RP-HPLC and SDS-PAGE. The enzymes were characterized by determining their molecular masses, isoelectric points, specific functional activity and partial amino acid sequencing.

Results: The metalloprotease presented molecular mass of 22.9 kDa and pI 7.4, with hemorrhagic and fibrin(ogen)olytic activities, and its partial amino acid sequence revealed high similarity with other P-I class metalloproteases. These results suggest that the isolated metalloprotease is Batroxase, a P-I metalloprotease previously described by our research group. The phospholipase A2 showed molecular mass of 13.7 kDa and pI 6.5, with high phospholipase activity and similarity to other acidic PLA2s from snake venoms. These data suggest that the acidic PLA2 is a novel enzyme from B. atrox venom, being denominated BatroxPLA2.

Conclusions: The present study successfully standardized a simple methodology to isolate the metalloprotease Batroxase and the acidic PLA2 BatroxPLA2 from the venom of B. atrox, consisting mainly of classical chromatographic processes. These two enzymes will be used in future studies to evaluate their effects on the complement system and the inflammatory process, in addition to the thrombolytic potential of the metalloprotease.

Keywords: Snake venoms, Bothrops atrox, Toxins, Metalloprotease, Phospholipase A2, Isolation, Characterization, Chromatography
other components of snake venoms can act independently or synergistically to cause local or systemic tissue damage and various other toxic effects [8, 9].

In order to isolate specific proteins from snake venoms, which are highly complex and may present more than 100 protein components [10], usually two or more chromatographic steps are needed, which may include steps of molecular exclusion, ion exchange, affinity, reverse phase, among others. The choice of chromatography type depends on the specific characteristics of each protein to be isolated.

The composition of snake venoms results from the interaction of several factors such as genetics, age, sex, feeding and geographic location of the specimen [11, 12]. Thus, standardization of methods for the isolation of bioactive molecules from these venoms is extremely difficult to achieve since they may vary widely in their compositions, even within the same snake species. Proteomic studies on venoms of Bothrops atrox, for example, showed significant variations in their protein compositions when venoms were from specimens in different stages of maturation or different geographic locations [13–15].

The snake species B. atrox is responsible for the majority of snakebites in the Brazilian Amazon region. In humans, envenomations by this snake cause local effects such as edema, necrosis and local hemorrhage, as well as systemic effects, including changes in blood coagulation and various bleeding sites along the bite [13]. Proteomic analyses of venoms from specimens located in Brazil have shown that metalloproteases account for more than 70 % of their protein content (~23 % of the P-I class and 49 % of the P-III class), followed by PLA₂ with approximately 14 % (~12 % of Asp₄₉ PLA₅S and ~2 % of Lys₄₉ PLA₅S) [14].

In this context, the present study aimed to standardize a method of isolation to obtain a metalloprotease of the P-I class and an acidic phospholipase A₂ from the crude venom of B. atrox, as well as to characterize and identify these molecules to enable future functional studies.

**Materials and methods**

**Venom and other materials**

The venom of B. atrox, collected from specimens found in the region of Peri Mirim, state of Maranhão, was acquired from the Center for Extraction of Animal Toxins (CETA, Morungaba, SP). Equipment and other materials used in this study are described in each specific section of the article, and reagents not otherwise specified were of analytical grade.

**Animals**

Male BALB/c mice (18–22 g) were provided by the animal facilities at the University of São Paulo (USP), Ribeirão Preto, SP, Brazil, and maintained on a 12 hour-cycle at room temperature (22–25 °C) with free access to standard chow and water. Animal care procedures were performed according to the Brazilian College of Animal Experimentation (COBEA) guidelines and the experimental protocols were approved by the Committee for Ethics on Animal Use (CEUA) from FCFRP-USP (protocol number: 13.1.336.53.4).

**Isolation of toxins from Bothrops atrox venom**

Chromatographic fractionation of B. atrox venom to obtain the toxins of interest began with a molecular exclusion step on Sephacryl S-200, followed by anion exchange chromatography on DEAE Sepharose. The fraction containing the metalloprotease (MP) was then ultrafiltered in a concentrator tube with membrane of MWCO 3,000, Vivaspin® 20 (Sartorius, Germany), while the fraction containing the phospholipase A₂ (PLA₂) was subjected to a C18 reverse phase column using ÄKTA™ purifier system. The classical chromatography resins as well as the reverse phase column and the ÄKTA™ system were obtained from GE Healthcare (USA).

The absorbance of the chromatographic fractions were measured at a wavelength of 280 nm, using a spectrophotometer Thermo Scientific™ GENESYS 10 UV (Thermo Fisher Scientific, Inc., USA) or the UNICORN™ 5.11 software for the ÄKTA™ purifier system (GE Healthcare, USA). Then, data were plotted on graphs using Origin 8 software for the obtainment and analysis of the chromatographic profiles.

**Molecular exclusion chromatography on Sephacryl S-200**

Crude and crystallized venom from B. atrox (350 mg) was suspended in 2 mL of 0.2 M ammonium bicarbonate buffer (AMBIC), pH 7.8, followed by centrifugation at 10,000 × g for ten minutes at room temperature. The clear supernatant obtained was applied to a chromatography column containing Sephacryl S-200 resin (100 × 2.6 cm), previously equilibrated and eluted with 0.2 M AMBIC buffer, pH 7.8. Fractions of 3 mL were collected per test tube, at a flow rate of 20 mL/hour at room temperature. All eluted fractions were assessed for their hemorrhagic activity and on SDS-PAGE, as described below. Chromatographic fraction S3 was selected based on its protein profile in gel and by presenting hemorrhagic activity, being lyophilized and submitted to the next chromatographic step.

**Ion exchange chromatography on DEAE Sepharose**

Fraction S3 from Sephacryl S-200 was diluted in 3 mL of 0.05 M AMBIC buffer, pH 7.8, and applied to a chromatography column containing DEAE Sepharose resin (15 × 2 cm), previously equilibrated with the same buffer. Elution of fractions was performed using three steps: 50 mL of 0.05 M AMBIC, pH 7.8; continuous
concentration gradient of AMBIC from 0.05 M to 0.5 M, pH 7.8 (150 mL), and finally 100 mL of 1 M AMBIC, pH 7.8. Fractions of 3 mL were collected per test tube at a flow rate of 30 mL/hour at room temperature. All eluted fractions were assessed for their hemorrhagic and phospholipase activities and on SDS-PAGE, as described below. The chromatographic fraction that showed hemorrhagic activity (D4) was selected and lyophilized, and then subjected to ultrafiltration on Vivaspin® 20. The fraction with phospholipase activity (D3) was lyophilized and subjected to a third chromatographic step on a C18 reverse phase column.

**Ultrafiltration on Vivaspin® 20**

A pool of D4 fractions obtained in the chromatographic step on DEAE Sepharose was diluted in 15 mL of Milli-Q water, and desalinated by ultrafiltration on Vivaspin® 20 system, with polyethersulfone membrane with 3,000 MWCO cutoff, by centrifugation at 8,000 × g (5804R centrifuge, Eppendorf, Germany) for 20 minutes. The pool was ultrafiltered until the material passing through the ultrafiltration membrane showed an optical reading lower than 0.1 Abs at 280 nm, thereby freeing the sample of salts, peptides and other low molecular mass components. Then, the sample had its protein concentration measured by the method of Bradford as described below, and was separated in 1.5 mL conical tubes in volumes equivalent to 1 mg/tube and lyophilized.

**Reverse phase chromatography (RP-HPLC) on C18 column**

Fraction D3 obtained in the chromatographic step on DEAE Sepharose was subjected to a C18 reverse phase column (4.6 mm ID × 25 cm, CLC-ODS, Shimadzu, Japan) using ÄKTA™ purifier system (GE Healthcare, USA) for 20 minutes. The pool was ultrafiltered until the material passing through the ultrafiltration membrane showed an optical reading lower than 0.1 Abs at 280 nm, thereby freeing the sample of salts, peptides and other low molecular mass components. Then, the sample had its protein concentration measured by the method of Bradford as described below, and was separated in 1.5 mL conical tubes in volumes equivalent to 1 mg/tube and lyophilized.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**

Chromatographic fractions and isolated toxins were evaluated by SDS-PAGE, performed on 12 % gels using a Mini VE 10 × 10 cm Vertical Gel Electrophoresis System (GE Healthcare, USA), according to Laemmli [16]. Samples were prepared using reducing buffer containing SDS and β-mercaptoethanol, followed by heating at 100 °C for three minutes. After running (15 A, 120 V), the gels were stained with Coomassie brilliant blue R250. The molecular mass standard used was either Spectra Multicolor Broad Range Protein Ladder (10–260 kDa, Thermo Fisher Scientific, Inc., USA) or Unstained Protein Molecular Weight Marker (14.4-116 kDa, Thermo Fisher Scientific, Inc., USA).

**Protein quantification**

Dosages of proteins were performed using Bradford reagent (Sigma-Aldrich, USA), according to the manufacturer instructions, whereas the absorbance of samples was determined at 595 nm in a microplate reader (PowerWave XS2, BioTek, USA). The standard curve was determined from different concentrations (0.1 to 1.5 mg/mL) of bovine serum albumin (BSA).

**Characterization of B. atrox toxins**

**Molecular mass determination**

The molecular masses of B. atrox toxins were initially estimated according to their SDS-PAGE profile, by interpolating a linear logarithmic curve of the relative molecular mass of standard proteins versus the distance of migration of sample proteins in the gel.

MALDI-TOF mass spectrometry analyses were also performed to determine the molecular mass of intact proteins, using an AXIMA Performance MALDI-TOF/TOF mass spectrometer (Shimadzu, Japan) previously calibrated with known molecular mass standards. Mass spectra were acquired in linear mode, evaluating the range from 5,000 to 50,000 m/z. The samples were diluted in 50 μL of Milli-Q water, mixed in a 1:1 ratio with a matrix consisting of sinapinic acid (10 mg/mL) in 50 % acetonitrile and 0.1 % TFA, and applied on the MALDI plate using the dried-droplet method.

**Isoelectric focusing**

The pl of the purified toxins was determined by isoelectric focusing as described by Arantes et al. [17]. Briefly, the isoelectric focusing was carried out on a 7 % polyacrylamide gel containing carrier ampholytes (pH 3–10, Sigma-Aldrich). After prefocusing for 30 minutes (settings: 100 V, 30 mA, 5 W), the samples were applied as drops of liquid on the surface of the gel. Standards of isoelectric focusing (IEF Standards, pl range of 4.45 to 9.6, Bio-Rad, USA) were run in parallel to the samples under
the same conditions. The isoelectric focusing was performed for approximately four hours (settings: 1500 V, 30 mA, 5 W). Focusing was completed when the voltage reached 1500 V and the current was 2 mA or less. The pH gradient was determined after the current was switched off by cutting sections of the gel (1 x 2 cm) along the gel sides, immersing them individually in 0.5 mL of Milli-Q water for two hours, and measuring their pH. The remaining gel containing the proteins was stained with Coomasie brilliant blue G250. The pl of samples was calculated from the curve of pH versus the distance of migration in the gel.

**In situ gel digestion and mass spectrometry analysis**

MP bands separated by 12 % SDS-PAGE were subjected to *in situ* gel digestion with 0.5 μg of modified trypsin (Promega Co., USA) [18]. The tryptic peptides obtained were desalted in a microtip filled with POROS R2 (Perseptive Biosystems, USA) and eluted with 5 % formic acid in 60 % methanol for analysis. Samples were dried and re-dissolved in 5 μL of α-cyano-4-hydroxycinnamic acid (10 mg/mL), then 2 μL was applied to the MALDI target using the dried-droplet method, followed by analysis by MALDI-TOF MS (AXIMA Performance, Shimadzu Biotech, UK) in the automatic data acquisition mode.

**N-terminal amino acid sequencing**

PLA₂ sample from RP-HPLC was lyophilized and submitted to Edman degradation [19]. N-terminal amino acid sequencing was performed using a PPSQ-33A automatic sequencer (Shimadzu, Japan). Phenylthiohydantoin (PTH) derivatives of amino acids were identified using an online RP-HPLC by comparison with the retention times of PTH-amino acids of a standard mixture.

**Amino acid sequence alignment**

The amino acid sequences obtained by MALDI-TOF MS and Edman degradation were compared using multiple sequence alignment with other sequences obtained from the NCBI database (http://blast.ncbi.nlm.nih.gov/), using the software ClustalX version 2.0.11. (http://www.clustal.org/).

**Fibrinolytic activity**

The fibrinolytic activity of MP was assessed on fibrin clots formed in Petri dishes, prepared with 0.95 % agarose solution containing 0.3 % fibrinogen and 1 mg/mL thrombin in 50 mM Barbital buffer, pH 7.8, according to Leitão et al. [20]. Samples (25 μL) of phosphate-buffered saline (PBS, negative control), *B. atrox* crude venom (20 μg, positive control) or MP (4, 6, 8 and 10 μg) were added to cavities (5 mm diameter) made on the fibrin gel, and incubated at 37 °C for 24 hours. The fibrinolytic activity was evaluated visually and quantified according to the halo diameter (mm).

**Fibrinogenolytic activity**

The ability of MP to digest fibrinogen was evaluated according to the method published by Edgar and Prentice [21], with modifications. Briefly, 25 μL of fibrinogen solution (3 mg/mL in 2 mM Tris–HCl, pH 7.4) was incubated with MP (1 μg) at 37 °C for one hour. The reaction was stopped with 15 μL of 50 mM Tris–HCl, pH 6.8, containing 10 % glycerol (v/v), 4 % SDS (w/v), 0.05 % bromophenol blue (v/v) and 4 % β-mercaptoethanol (v/v), followed by heating at 100 °C for five minutes. After denaturation, one third of the samples (final volume of 75 μL) was assayed by 10 % SDS-PAGE.

**Hemorrhagic activity**

The hemorrhagic activity of chromatographic fractions and the isolated metalloprotease was evaluated by the method described by Nikai et al. [22]. Briefly, 50 μL of samples or PBS (negative control) was injected intradermally into the back of BALB/c mice. Inhibition of this activity was evaluated by pre-incubation of enzyme with 5 mM EDTA (ethylenediamine tetraacetic acid) for 30 minutes at 37 °C. After three hours, the animals were euthanized in a CO₂ chamber, and had their skins removed in order to observe the presence or absence of hemorrhagic halos. The minimum hemorrhagic dose (MHD) was defined as the minimum dose of sample required to induce the formation of a halo with 10 mm diameter.

**Phospholipase activity**

The phospholipase activity of chromatographic fractions and of the isolated phospholipase A₂ was evaluated on plates, as described by Gutiérrez et al. [23], changing the agarose for agar and without using erythrocytes. Briefly, a gel containing 0.01 M CaCl₂, egg yolk diluted in PBS (pH 7.2) in the ratio 1:3 (v/v), 1 % bacteriological agar and 0.005 % sodium azide was formed in Petri dishes. Then, holes of approximately 5 mm in diameter were made in the gel, and samples were applied at a final volume of 40 μL, followed by incubation at 37 °C overnight. The formation of translucent halos around the holes in the gel was considered to be indicative of phospholipase activity, which was quantified by the measurement of each hole in millimeters. The minimum phospholipase dose (MPD) was defined as the minimum dose of the sample required to induce the formation of a halo with 20 mm diameter.

Modification of residue His48 of the PLA₂ with 4-bromophenacyl bromide (BPB) was carried out based on previously described methodologies [24]. Briefly, the PLA₂ (100 μg) was dissolved in 90 μL of 0.1 M ammonium
bicarbonate, pH 8.0, and 10 μL of BPB (1 mg/mL in ethanol) was added. The mixture was incubated for 24 hours at 25 °C. After that period, the phospholipase activity of BPB-PLA<sub>2</sub> (2 μg) was evaluated as described above.

Results and Discussion

Purification of B. atrox toxins

The toxins of interest, a P-I class metalloprotease (MP) and an acidic phospholipase A<sub>2</sub> (PLA<sub>2</sub>), were isolated from B. atrox venom by consecutive chromatographic steps, starting the process by performing chromatography on Sephacryl S-200. The chromatographic profile obtained showed six well-defined fractions, identified as S1a, S1b, S2, S3, S4 and S5 (Fig. 1a). Analysis of these fractions by SDS-PAGE showed that fractions S1a, S1b and S2 mainly displayed protein components with molecular masses above 30 kDa. The protein profile of fraction S3 presented bands with molecular masses around 25 kDa and 15 kDa, while fractions S4 and S5 seemed to consist only of components with molecular masses below 15 kDa (Fig. 1b). Fraction S3 was chosen based on its protein profile in SDS-PAGE and the hemorrhagic activity observed, being then subjected to an anion exchange chromatography.

DEAE Sepharose chromatography of fraction S3 resulted in six fractions, denominated D1, D2, D3, D4, D5a and D5b (Fig. 2a). Several of these fractions (D1, D5a and D5b) showed no visible bands on SDS-PAGE, indicating the presence of only low-molecular-mass compounds. Fraction D2 showed a protein band with molecular mass slightly above 25 kDa, whereas fractions D3 and D4 showed single bands around 15 kDa and 25 kDa, respectively (Fig. 2b). Based on these protein profiles and the hemorrhagic and phospholipase activities, it was determined that the PLA<sub>2</sub> was in fraction D3 and the MP in fraction D4. Thereafter, two separate isolation procedures were used to obtain each enzyme.

Fraction D3, which contained the PLA<sub>2</sub>, was applied to a C18 reverse phase column, resulting in six major fractions (Fig. 3a). This chromatographic step enabled the separation of the acidic and catalytically active PLA<sub>2</sub>, from other fractions containing low molecular mass peptides (fractions 1, 4 and 5) which do not appear on 12 % SDS-PAGE, and from phospholipases A<sub>2</sub> without catalytic activity (fractions 2 and 3) (Fig. 3b) The fraction that showed phospholipase activity (fraction 6), indicative of catalytic activity related to the residue Asp49, was then rechromatographed on the same column to assess its purity level. The chromatographic profile shows a fraction eluted around 65 % solvent B (Fig. 4a), which appeared as a single band of approximately 14 kDa on SDS-PAGE (Fig. 4b).

Fraction D4 containing the MP was ultrafiltered on a Vivaspin® 20 system, which was used as a third step of isolation and may be considered a molecular exclusion step, since it enabled clearance of the fraction of low-molecular-mass compounds (such as peptides) and salts from the anion exchange chromatographic step. After this ultrafiltration step, the MP purity level was evidenced by RP-HPLC using a C18 column, eluting with ~90 % solvent B (Fig. 5a), and appearing as a single band of molecular mass around 25 kDa on SDS-PAGE (Fig. 5b). Usually, reverse phase chromatographic steps are used only to verify the purity levels of metalloproteases, since these enzymes lose their proteolytic activity when exposed to organic solvents such as TFA and acetonitrile, possibly due to denaturation of the molecules promoted by the low pH of solvents.

Purification of P-I class metalloproteases is commonly performed using two to three chromatographic steps,
with a predominance of molecular exclusion and ion exchange steps. A purification process employing a single chromatographic step was described for neuwiedase from \textit{B. neuwiedi} venom, nevertheless, most procedures usually comprise two steps, as described for BaP1 from \textit{B. asper}, leucurolysin-a from \textit{B. leucurus}, atroxlysin-I from \textit{B. atrox} and BjussuMP-II from \textit{B. jararacussu} venom [25–29]. Some studies also show the isolation of P-I metalloproteases using three chromatographic steps, as described for BmooMP-\(\alpha\) from \textit{B. moojeni} and for BpirMP from \textit{B. pirajai} venom, which were isolated by combining molecular exclusion, ion exchange and affinity steps [30, 31].

Although our research group had already proposed a method for the isolation of a P-I metalloprotease denominated Batroxase from \textit{B. atrox} venom using a molecular exclusion step on Sephadex G-75 and an anion exchange chromatography on ES-502 N 7C column [32], the new method described in the present study was standardized so that an acidic phospholipase A\(_2\) could also be obtained from this venom. By using this novel methodology, a P-I metalloprotease and an acidic PLA\(_2\) were successfully isolated from \textit{B. atrox} venom using the same two initial chromatographic steps and a third distinct one for each enzyme.

Acidic PLA\(_2\)s from \textit{Bothrops} venoms are commonly purified by a combination of chromatographic methods, including molecular exclusion, ion exchange, RP-HPLC and hydrophobic steps. Cogo \textit{et al.} [33] isolated two acidic PLA\(_2\)s from \textit{B. insularis} venom using a single RP-HPLC step. Other enzymes were isolated using two chromatographic steps, as described for BthA-I-PLA\(_2\) from \textit{B. jararacussu}, BpirPLA\(_2\)-I from \textit{B. pirajai}, BL-PLA\(_2\) from \textit{B. leucurus}, BmooPLA\(_2\) from \textit{B. moojeni} and

![Chromatographic profile of fraction S3 on a DEAE Sepharose anion exchange column. Elution was initiated with 0.05 AMBIC, pH 7.8, followed by a gradient of AMBIC from 0.05 M to 0.5 M, pH 7.8, and finally 1 M AMBIC, pH 7.8. Fractions of 3 mL/tube were collected at a flow rate of 30 mL/hour. b 12 % SDS-PAGE. Lanes: 1 – tube 15 (D1), 2 – tube 28 (D2), 3 – tube 36 (D3), 4 – tube 40 (D3), 5 – molecular mass standard (260, 140, 100, 70, 50, 40, 35, 25, 15, 10 kDa), 6 – tube 44 (D3-D4 valley), 7 – tube 49 (D4), 8 – tube 55 (D4), 9 – tube 71 (D5a), 10 – tube 77 (D5b)](image)

![Chromatographic profile of fraction D3 on a C18 reverse phase column. Elution was performed using a RP-HPLC system at a flow rate of 0.5 mL/minute with a linear concentration gradient of 0-100 % solvent B (70 % acetonitrile and 0.1 % TFA) in ten column volumes. b 12 % SDS-PAGE. Lanes: 1 – fraction 1; 2 – fraction 2; 3 – fraction 3; 4 – fraction 4; 5 – fraction 5; 6 – fraction 6; 7 – molecular mass standard (116, 66.4, 45, 35, 25, 18, 4, 14.4 kDa)](image)
BaSPIIRP4 from *B. alternatus* venom [24, 34–37]. There are also reports of the combination of three or four chromatographic steps for obtaining some acidic PLA$_2$s from *Bothrops* venoms [38–40].

After the isolation of *B. atrox* toxins, some biochemical and functional experiments were performed in order to identify the enzymes of interest, including the determination of molecular masses, isoelectric points, partial amino acid sequences and evaluation of characteristic functional activities for metalloproteases and phospholipases A$_2$.

**Fig. 4 a** Chromatographic profile of fraction 6 on a C18 reverse phase column. Elution was performed using a RP-HPLC system at a flow rate of 0.5 mL/minute using a segmented concentration gradient of 0-60 % solvent B in three column volumes, 60-80 % in five column volumes and 80-100 % in one column volume. **b** 12 % SDS-PAGE. Lanes: 1 – PLA$_2$, 2 – molecular mass standard (116, 66.4, 45, 35, 25, 18.4, 14.4 kDa)

**Characterization of the MP**

MP showed molecular mass of 22.9 kDa by MALDI-TOF MS and 26.2 kDa when estimated by SDS-PAGE. P-I SVMPs present variable molecular masses ranging from 20 to approximately 30 kDa, e.g. neuwiedase (20 kDa), BthMP (23 kDa), leucurolysin-a (23 kDa), atroxlysin-I (23 kDa), BpirMP (23 kDa), BaP1 (24 kDa), BnP1 (24 kDa), BH2 (26 kDa), Batroxase (27 kDa by SDS-PAGE and 22.9 kDa by MALDI-TOF MS) and BaltMP-I (29 kDa) [25–28, 31, 32, 41–44]. Nevertheless, it should be taken into account that some of those

**Fig. 5 a** Evaluation of the purity level of fraction D4 after Vivaspin® 20 on a C18 reverse phase column. After having been subjected to ultrafiltration on a Vivaspin® 20 system, fraction D4 was evaluated by RP-HPLC using a C18 column with a linear concentration gradient of 0-100 % solvent B in five column volumes. **b** 12 % SDS-PAGE. Lanes: 1 – MP, 2 – molecular mass standard (260, 140, 100, 70, 50, 40, 35, 25, 15, 10 kDa)
differences in the molecular masses could be attributable to the different sensitivity of the methodologies used for their resolution, e.g. SDS-PAGE or MALDI-TOF MS.

The isoelectric focusing showed that MP is a neutral protein with pl of approximately 7.4 (Fig. 6). P-I metalloproteases may present pl values ranging between 5 and 8, and thus may show acidic character such as neuwiedase, BjussuMP-II and BH2, neutral character as Batroxase and BthMP or basic character as BJ-P12 [25, 29, 32, 41, 43, 45].

Partial amino acid sequencing revealed high similarity between MP and other P-I metalloproteases previously isolated from *B. atrox* venom, such as atroxlysin-I and Batroxase, with 100 % identity with the latter enzyme (Fig. 7) [28, 32]. SVMPs are classified according to their structural domains: P-I class, presenting only the metalloprotease domain; P-II class, presenting the metalloprotease domain and the disintegrin domain; P-III class, containing the disintegrin domain, cysteine rich domain and the metalloprotease domain [46]. Thus, P-I SVMPs belong to the simplest class of metalloproteases, with lower molecular masses and an average of 200–210 amino acid residues [47]. The zinc binding catalytic site of these molecules is formed by the consensus sequence HExxHxxGxxH, with the conserved Met-turn sequence CI/VM adjacent to the site [48, 49]. Although these portions were not determined in the partial sequencing of the MP from *B. atrox* venom, the multiple alignment (Fig. 7) and the biochemical and functional characteristics confirm that it is a P-I class metalloprotease.

In relation to the functional characterization, MP showed high fibrin(ogen)lytic activity, with low doses inducing significant fibrinolysis halos (Fig. 8a) and preferential degradation of the Aα chains of fibrinogen, although it also induced degradation of the Bβ chains (Fig. 8b).

Most P-I SVMPs are fibrinogenolytic enzymes that preferentially degrade the Aα chains of fibrinogen, while also degrading the Bβ chains at slower ratios [6]. Examples of fibrinogenolytic metalloproteases from *Bothrops* venoms include BthMP, BmooMP-α, atroxlysin-I, Batroxase, BaltMP-I, BaP1, neuwiedase and BpirMP [25, 26, 28, 29, 32, 41, 43, 45].

Fig. 6 Isoelectric focusing of the toxins isolated from *B. atrox* venom on a 7 % polyacrylamide gel. Samples: (1) isoelectric focusing standard (IEF Standards, pl ranges from 4.45 to 9.6, Bio-Rad, USA), (2) PLA2 (pl ~6.5) and (3) MP (pl ~7.4). The pl of samples was calculated from the curve of pH versus the migration distance in the gel.
Some SVMPs also showed fibrinolytic activity, including BnP1, bothrojaractivase, BthMP, BpirMP, atroxylisin-I and Batroxase [28, 31, 32, 41, 42, 50].

Recent studies have been exploring the potential of fibrin(ogen)olytic SVMPs as thrombolytic agents, since these enzymes may act directly on fibrin clots, and also promote depletion of fibrinogen molecules and thus prevent the formation of new clots. For this reason, fibrin(ogen)olytic SVMPs with low or no hemorrhagic activity, such as fibrinase and its recombinant form alfimeprase, have been evaluated as potential drugs for the treatment of patients with vascular diseases [51].

The MP from *B. atrox* venom showed high hemorrhagic activity at doses up to 20 μg, with MHD close to 5 μg and significant inhibition observed after incubation with 5 mM EDTA (Fig. 9). This inhibition promoted by chelating agents such as EDTA and 1,10-phenanthroline on the proteolytic and hemorrhagic activities of SVMPs is associated with the chelation of the zinc ion, which is essential for the catalytic activity of these molecules [26, 43].

The hemorrhage induced by SVMPs is related to the hydrolysis of basal membrane of capillaries [52]. In relation to hemorrhagic potential, P-III SVMPs are the most potent among the three classes, being capable of inducing...
not only local but also systemic hemorrhages, while P-I SVMPs induce mostly local hemorrhaging [53]. Additionally, differences in the hemorrhagic potential of P-I SVMPs can also be observed [54, 55], with some enzymes presenting this activity [26, 28, 32, 41, 56] while others do not [25, 29, 30]. The comparison between structures of hemorrhagic and non-hemorrhagic P-I SVMPs showed small variations in loop regions around the catalytic site, suggesting that the variations of hemorrhagic potential may be related to such areas [57].

Some P-I metalloproteases have already been described from B. atrox venom, e.g. HI-5, atroxlysin-I, BaTx-I and Batroxase [28, 32, 56, 58]. As not all of these enzymes had their amino acid sequences elucidated, it is possible that some of them are the same molecule described by different authors. It is also possible that the different geographical locations of B. atrox specimens used as venom sources in these different studies (i.e. Brazil, Peru and Colombia) exerted some type of influence on the structural and functional differences reported for these enzymes, as shown by proteome studies on venoms of this snake species [14].

Considering the characteristics found for the MP from B. atrox described in the present study, it was noted that this enzyme showed significant similarities to Batroxase, including partial amino acid sequence, molecular mass (determined by both SDS-PAGE and MALDI-TOF MS methods), neutral pl and fibrin(ogen)olytic and hemorrhagic activities [32]. Thus, our findings suggest that the isolated MP can be identified as Batroxase.

**Characterization of the PLA2**

B. atrox PLA2 presented a molecular mass of 13.7 kDa by MALDI-TOF MS and 14.4 kDa by SDS-PAGE, and was revealed to be an acidic enzyme with pl ~ 6.5 (Fig. 6). Acidic snake venom PLA$_2$s present molecular masses around 14 kDa and pl varying from 4.0 to 5.5. Some examples are BthA-I-PLA$_2$ with 13.7 kDa and pl 4.5, BpirPLA$_2$-I with 13.7 kDa and pl 4.8, Bp-PLA$_2$ with 15.8 kDa and pl 4.3, BmooPLA$_2$ with 13.6 kDa and pl 5.2 and BL-PLA$_2$ with 15 kDa and pl 5.4 [24, 34–36, 59]. None of these acidic PLA$_2$s showed a pl value superior to 5.5 as shown for the PLA$_2$ isolated in this study (pl ~ 6.5). Although this pl value is close to neutral, alignment with other PLA$_2$s confirmed its high similarity to acidic proteins, including PA2-II from Gloydius blomhoffii venom (gi 129420) and BpPLA$_2$-TX-I from B. pauloensis venom [40] (Fig. 10).

PLA$_2$s are classified into 15 different subgroups according to specific features, with snake venom PLA$_2$s belonging to groups I and II [60, 61]. Group IIA PLA$_2$s are further divided into Asp49 PLA$_2$s, enzymes that usually exhibit high catalytic activity, and Lys49 PLA$_2$s with low or no enzymatic activity on artificial substrates [62, 63]. The catalytic site of PLA$_2$s is composed of the residues His$_{48}$, Asp$_{49}$, Tyr$_{52}$ and Asp$_{99}$, and the majority of these enzymes have conserved His$_{48}$ and Asp$_{49}$ residues that present a catalytically essential water molecule attached to their side-chains by hydrogen bonds [64, 65]. Besides the catalytic site, the calcium binding site (X27CGXGG32) is also present in all group II catalytically active PLA$_2$s [66].
Three active site residues (His48, Asp49 and Tyr52), the calcium binding site and six conserved cysteine residues related to the formation of disulfide bonds were determined in the partial amino acid sequencing of *B. atrox* PLA₂ (Fig. 10).

The PLA₂ showed high phospholipase activity at doses up to 8 μg, with MPD of 2 μg (Table 1). These results show a high catalytic activity for this enzyme, which is consistent with other acidic PLA₂s from *Bothrops* venoms, such as BmooTX-I, with MPD of 1 μg, and BpirPLA₂-I, with MPD of 4 μg [34, 38]. Some studies describe the acidic Asp49 PLA₂s as catalytically more active than the basic isoforms [24, 34, 38, 39, 59]. Additionally, treatment with BPB significantly inhibited the phospholipase activity of the PLA₂ from *B. atrox* (Table 1), as already described for other acidic PLA₂s [34, 38].

Some phospholipase A₂ enzymes were previously described from *B. atrox* venom, e.g. a myotoxin with phospholipase and anticoagulant activity, basic and neutral Asp49 PLA₂s, named BaPLA₂-I and BaPLA₂-II, and a Lys49 PLA₂ called myotoxin I [67–69]. Recently, a study published by Furtado *et al.* [70] described the isolation of three PLA₂s from *B. atrox* venom: a Lys49 (BaTX-I), an Asp49 (BaTX-II) and an acidic Asp49 myotoxin (BaPLA₂). However, as the authors did not perform amino acid sequencing of the molecules, it is not possible to determine whether these enzymes are novel or the same ones previously described in the literature. Thus, as there are no reports in the literature of acidic PLA₂s isolated from *B. atrox* venom, we denominated the enzyme described in the present study BatroxPLA₂, an unprecedented acidic phospholipase A₂.

### Synergism

It is well known that snake toxins can act independently or synergistically [8]. To assess this possible synergism between the MP and the PLA₂ from *B. atrox*, the hemorrhagic and phospholipase activities were evaluated using a mixture of the enzymes at the molar ratio of 1:1, which resulted in no significant changes compared to the isolated activities of each enzyme (Fig. 9 and Table 1), suggesting that those activities were not enhanced or impaired by the combination of both toxins. Future studies will further evaluate the

### Table 1

| Samples                          | Halo diameter (mm) (mean ± SEM) (n = 3) |
|----------------------------------|----------------------------------------|
| PBS                              | 0                                      |
| *B. atrox* (5 μg)                | 17.5 ± 0.5                              |
| *B. atrox* (10 μg)               | 20.0 ± 0.5                              |
| PLA₂ (0.5 μg)                    | 14.5 ± 0.5                              |
| PLA₂ (1 μg)                      | 16.5 ± 0.5                              |
| PLA₂ (2 μg) *                    | 20.0                                    |
| PLA₂ (4 μg)                      | 21.0 ± 0.5                              |
| PLA₂ (8 μg)                      | 22.0                                    |
| PLA₂ (2 μg) + BPB                | 6.5 ± 0.5                               |
| PLA₂ (2 μg) + MP (1:1)           | 20.5 ± 0.5                              |

* Determined as the minimum phospholipase dose (MPD)
possible synergistic effects of the two toxins on different physiological processes, e.g. inflammation.

Conclusions
The interest in the biochemical and functional characterization of toxins isolated from snake venoms is due not only to their relevance in envenomations, but also to their potential use as valuable research tools in different areas of knowledge. Pharmacological and biochemical studies conducted in recent decades have shown the presence of a variety of enzymes, toxins and biologically active compounds in these venoms, as well as the great diversity of their actions. Consequently, numerous attempts have been made to use these compounds as tools for research and for applications in the medical field, and as such, the purification and characterization of snake toxins are of utmost importance.

In this context, the present study successfully standardized a purification procedure, mainly composed of classical chromatographic techniques, for the isolation of a P-I metalloprotease identified as Batroxase and a new acidic PLP₂ denominated BatroxPLP₂ from B. atrox venom. These two enzymes will be used in future studies to evaluate their effects on the complement system and the inflammatory process, in addition to the thrombotic-lytic potential of the metalloprotease.

Ethics committee approval
Animal care procedures were performed according to the Brazilian College of Animal Experimentation (COBEA) guidelines and the experimental protocols were approved by the Committee for Ethics on Animal Use (CEUA) from FCFRP-USP (protocol number: 13.1.336.53.4).

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
DLM and ALJF contributed equally to the accomplishment of this study and wrote the manuscript. CPB and ACOC assisted in the biochemical and functional experiments, while SSV supervised and critically discussed the study. All authors read and approved the final manuscript.

Authors' Information
1Department of Clinical Analyses, Toxicology and Food Sciences, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo (USP), Ribeirão Preto, SP, Brazil.

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References
1. Gutiérrez JM, Williams DJ, Fan HW, Warrell DA. Snakebite envenoming from a global perspective: towards an integrated approach. Toxicon. 2010;56(7):1223–35.
2. Habib A. Public health aspects of snakebite care in West Africa: perspectives from Nigeria. J Venom Anim Toxins incl Trop Dis. 2013;19(1):27.
3. Gutiérrez JM. Current challenges for confronting the public health problem of snakebite envenoming in Central America. J Venom Anim Toxins incl Trop Dis. 2014;20(1):7.
4. Bochner R. The international view of envenoming in Brazil: myths and realities. J Venom Anim Toxins incl Trop Dis. 2013;19:29.
5. Sistema de Informação de Agravos de Notificação - SINAN. Brasília: Ministério da Saúde. http://dtr.2004.saude.gov.br/sinanweb/index.php. (2012). Access 25 Jan. 2015.
6. Markland FS. Snake venoms and the hemostatic system. Toxicon. 1998;36:1749–800.
7. Mels D. Snake venom composition and evolution of vipers. Kaupiatzt. 1999;8:145–8.
8. Doley R, Kini RM. Protein complexes in snake venom. Cell Mol Life Sci. 2009;66(17):2851–71.
9. Vonk FJ, Jackson K, Doley R, Madaras F, Mirtschin PJ, Vidul N. Snake venoms: from fieldwork to the clinic: recent insights into snake biology, together with new technology allowing high-throughput screening of venom, bring new hope for drug discovery. Biogeochemicals. 2011;133(4):269–79.
10. Calvete JJ, Jaurez P, Sanz L. Snake venomics. Strategy and applications. J Mass Spectrom. 2007;42(11):1402–14.
11. Mackessy SP. The field of reptile toxicology: snakes, lizards, and their venoms, Handbook of venoms and toxins of reptiles. Boca Raton: CRC Press; 2009. p. 3–23.
12. Lourenço Junior A, Zunzela Cestre CF, de Barros LC, Delaúzari dos Santos L, Arruda DC, Baraviera B, et al. Individual venom profiling of Crotalus durissus terrificus specimen from a geographically limited region: crassine assessment and captivity evaluation on the biological activities. Toxicon. 2013;69:75–81.
13. Guerrio RC, Shevchenko A, Shevchenko A, López-Lozano JL, Paba J, Sousa MV, et al. Ontogenetic variations in the venom proteome of the Amazonian snake Bothrops atrox. Proteome Sci. 2006;4:11.
14. Núñez V, Cid P, Sanz L, De La Torre P, Angulo Y, Lomonte B, et al. Snake venomics and antivenomics of Bothrops atrox venoms from Colombia and the Amazon regions of Brazil, Perú and Ecuador suggest the occurrence of geographic variation of venom phenotype by a trend towards paedomorphism. J Proteomics. 2009;73(1):57–78.
15. Calvete JJ, Sanz L, Pérez A, Borges A, Vargas AM, Lomonte B, et al. Snake population venomics and antivenomics of Bothrops atrox: Paedomorphism along its transamazonian dispersal and implications of geographic venom variability on snakebite management. J Proteomics. 2011;74(4):1510–27.
16. Lenné ML. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970/227(5259):680–5.
17. Arantes EC, Prado WA, Sampaio SV, Giglio JR. A simplified procedure for the fractionation of Tityus sumatus venom: isolation and partial characterization of TSTX-V, a new neurotoxin. Toxicon. 1989;27(9):907–16.
18. Williams KR, Stone KL. Enzymatic cleavage and HPLC peptide mapping of proteins. Mol Biotechnol. 1989;2(2):115–67.
19. Edman P, Begg G. A protein sequenator. Eur J Biochem. 1967;1(1):80–91.
20. Leitão DP, Polizello AC, Rothschild Z. Coagulation and fibrinolysis in Costa Rica. Comp Biochem Physiol A Mol Integr Physiol. 2005;146(1):113–20.
21. Edgar W, Prentice CRM. The proteolytic action of ancrod on human fibrinogen and its polypeptide chains. Thromb Res. 1972;3(2):185–95.
22. Nikai T, Mori N, Kishida M, Sugihara H, Tu AT. Isolation and biochemical characterization of cappybara (Hydrochaeris hydrochaeris), a close relative of the guinea-pig (Cavia porcellus). Comp Biochem Physiol A Mol Integ Physiol. 2000;125(1):113–20.
23. Arthur B, Prentice CRM. The proteolytic action of ancrod on human fibrinogen and its polypeptide chains. Thromb Res. 1972;3(2):185–95.
24. Nikai T, Mori N, Kishida M, Sugihara H, Tu AT. Isolation and biochemical characterization of hemorhagic toxin f from the venom of Crotalus atrox (western diamondback rattlesnake). Arch Biochem Biophys. 1984;231(3):309–19.
25. Gutiérrez JM, Avila C, Rojas E, Cerdas L. Alternative characterization of an acidic platelet aggregation inhibitor and hypotensive phospholipase A₂ from Bothrops jararacussu snake venom. Biochim Pharmacol. 2002;64(4):723–32.

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Bothrops neuwiedi snake venom acts both on blood vessel ECM and platelets. J Devic JM, Romero M, Diaz C, Borkow G, Ovadía M. Isolation and characterization of a metalloproteinase with weak hemorrhagic activity from the venom of the snake Bothrops asper (terciopelo). Toxicon. 1993;33(3):119–29.

Gremski LH, Chaim OM, Paludo Ks, Sade YB, Otsuki MF, Richardsen M, et al. Cytotoxic, thrombolytic and edematogenic activities of leucocytolisin-α, a metalloproteinase from Bothrops leucurus snake venom. Toxicon. 2007;50(1):29–34.

Sanchez EF, Schneider FS, Yarique A, Borges MH, Richardson M, et al. Molecular and functional characterization of a new non-hemorrhagic metalloproteinase from Bothrops jararacussu snake venom with antiplatelet activity. Peptides. 2007;28(7):2328–39.

Bermudes CP, Satoshi-Filho NA, Mazzoni MW, Oliveira CZ, Izidoro LF, et al. Molecular characterization of a new fibrinogen-binding metalloproteinase from Bothrops moojeni snake venom. Toxicon. 2008;51(14):574–84.

Bermudes CP, Menaldo DL, Camacho E, Rosa JC, Escalante T, Rucavado A, et al. Characterization of the new myotoxic acidic phospholipase A2 from Bothrops jararacussu snake venom. J Venom Anim Toxins incl Trop Dis. 2009;15(4):745–57.

Cintra AC, De Toni LG, Sartim MA, Franco JJ, Caetano RC, Murakami MT, et al. Batroxase, a new fibrinolytic enzyme from B. atrox (Jergón) snake venom acts both on blood vessel ECM and platelets. Arch Biochem Biophys. 2010;496(1):9–20.

Marcusi S, Bermudes CP, Satoshi-Filho NA, Mazzoni MW, Oliveira CZ, Izidoro LF, et al. Molecular and functional characterization of a new non-hemorrhagic metalloproteinase from Bothrops jararacussu snake venom with antiplatelet activity. Peptides. 2007;28(7):2328–39.

Bermudes CP, Satoshi-Filho NA, Costa TR, Gomes MS, Torres FS, Costa J, et al. Isolation and structural characterization of a new fibrinogen-binding metalloproteinase from Bothrops moojeni snake venom. Toxicon. 2008;51(14):574–84.

Cintra AC, De Toni LG, Sartim MA, Franco JJ, Caetano RC, Murakami MT, et al. Batroxase, a new fibrinolysin from B. atrox snake venom with strong fibrinolytic activity. Toxicon. 2012;60(1):70–82.

Cogo JC, Lila S, Souza GH, Hypolito S, De Nucci G. Purification, sequencing and structural analysis of two acidic phospholipases A2, from the venom of Bothrops insularis (jararaca ilhoa). Biochimie. 2006;88(12):1947–59.

Teixeira SS, Silveira LB, da Silva FM, Marchi-Salvador DP, Silva JR FP, Izidoro LF, et al. Molecular characterization of an acidic phospholipase A2 from Bothrops jararacussu snake venom: synthetic C-terminus peptide identifies its antiplatelet region. Arch Biochem. 2011;510(1):1219–33.

Nunes DC, Rodrigues RS, Lucena MN, Cologna CT, Oliveira AC, Hamaguchi A, et al. Isolation and functional characterization of proinflammatory acidic phospholipases A2 from Bothrops leucurus snake venom. Comp Biochem Physiol C Toxicol Pharmacol. 2011;154(3):226–33.

Silveira LB, Marchi-Salvador DP, Satoshi-Filho NA, Silva Jr FP, Marcussi S, Fuly AL, et al. Isolation and expression of a hypertensive and anti-platelet acidic phospholipase A2 from Bothrops moojeni snake venom. J Pharm Biomed Anal. 2011;53(3):235–43.

Garcia Denegri ME, Acouta DC, Huacahuaire-Vega S, Martins-de-Souza D, Marangoni S, Marahukl S, et al. Isolation and functional characterization of a new acidic PLA2 Ba SpII RP4 of the snake venom of the snake Bothrops alternatus. J Venom Anim Toxins incl Trop Dis. 2008;51(1):54–65.

Sanchez EF, Schneider FS, Yarleque A, Borges MH, Ovadía M, et al. Purification and functional characterization of a prothrombin-activating, a prothrombin-activating metalloproteinase isolated from Bothrops jararaca snake venom. Toxicon. 2008;51(4):488–501.

Dechter SR, Toombs CF. Non-clinical and clinical characterization of a novel acting thrombolytic: alfimeprase. Pathophysiolog Haemost Thromb. 2005;34(4):215–20.

Escalante T, Shannon J, Moura-da-Silva AM, Gutié rm JM, Fox JW. Novel insights into capillary vessel basement membrane damage by snake venom hemorrhagic metalloproteinases: A biochemical and immunohistochemical study. Arch Biochem Biophys. 2006;455(2):144–53.

Gutiérrez JM, Rucavado A, Escalante T, Diaz C. Hemorrhage induced by snake venom metalloproteinases: biochemical and biophysical mechanisms involved in microvesSEL damage. Toxicon. 2005;45(8):997–1011.

Ramos OH, Selistre-de-Araujo HS. Comparative analysis of the catalytic domain of hemorrhagic and non-hemorrhagic snake venom metalloproteinases using bioinformatic tools. Toxicon. 2004;44(5):529–38.

Escalante T, Rucavado A, Fox JW, Gutiérrez JM. Key events in microvascular damage induced by snake venom hemorrhagic metalloproteinases. J Proteomics. 2011;74(8):1781–94.

Patrío AC, Perea-Blanco J, Núñez V, Benjumea DM, Fernandez M, Rucavado A, et al. Isolation and biological characterization of Bat-Ix, a weak hemorrhagic and fibrinogenolytic P-I metalloproteinase from Colombian Bothrops atrox venom. Toxicon. 2010;55(6):936–43.

Lingtott S, Schleberger C, Gutiérrez JM, Merfort I. High-resolution crystal structure of the snake venom metalloproteinase BaP1 complexed with a peptidomimetic: insight into inhibitor binding. Biochemistry. 2009;48(26):6166–74.

Petretski JK, Kanashiro MM, Rodrigues FG, Alves EW, Machado OL, Kipnis TL. Purification and identification of a 25 kDa hemorrhagin from B. atrox venom. Protein Peptide Lett. 2001;8(3):187–92.

Rodrigues RS, Izidoro LF, Teixeira SS, Silveira LB, Hamaguchi A, Homi-Brandebugo MJ, et al. Isolation and functional characterization of a new myotoxic acidic phospholipase A2 from Bothrops pauloensis snake venom. Toxicon. 2007;50(1):153–65.

Six DA, Dennis EA. The expanding superfamily of phospholipase A2 enzymes: classification and characterization. Biochim Biophys Acta. 2000;1488(1–2):1–19.

Burke JE, Dennis EA. Phospholipase A2 structure/function, mechanism, and signaling. J Lipid Res. 2009;50(suppl):S237–42.

Gutiérrez JM, Lomonte B. Phospholipase A2 myotoxins from Bothrops snake venoms. Toxicon. 1995;33(11):1405–24.

Guarnieri MC, Molinero E, Valsecchi L, Gatti AR, Rádis-Baptista G. Cloning of a novel acidic phospholipase A2 from the venom gland of Bothrops jararca (Brazilian eastern rattlesnake). J Venom Anim Toxins incl Trop Dis. 2009;15(4):745–61.

Arn RK, Ward RJ. Phospholipase A2 enzymes – a structural review. Toxicon. 1996;34(8):827–41.

Kang TS, Georgiev D, Genov N, Murakumi MT, Sinha M, Kumar RP, et al. Enzymatic toxins from snake venom: structural characterization and mechanism of catalysis. FEMS J. 2011;278(3):4544–76.

Murakumi M, Kudo I. Phospholipase A2 enzymes – a structural review. J Biochem. 2002;131(3):285–92.
67. Lomonte B, Gutiérrez JM, Furtado MF, Otero R, Rosso JP, Vargas O, et al. Isolation of basic myotoxins from Bothrops moojeni and Bothrops atrox snake venoms. Toxicon. 1990;28(10):1137–46.

68. Kanashiro MM, Escocard RCM, Petretski JH, Prates MV, Alves EW, Machado OLT, et al. Biochemical and biological properties of phospholipases A2 from Bothrops atrox snake venom. Biochem Pharmacol. 2002;64(7):1179–86.

69. Núñez V, Arce V, Gutiérrez JM, Lomonte B. Structural and functional characterization of myotoxin I, a Lys49 phospholipase A2 homologue from the venom of the snake Bothrops atrox. Toxicon. 2004;44(1):91–101.

70. Furtado JL, Oliveira GA, Pontes AS, Setúbal Sda S, Xavier CV, Lacouth-Silva F, et al. Activation of J77A.1 macrophages by three phospholipases A2 isolated from Bothrops atrox snake venom. Biomed Res Int. 2014;2014:683123.