Research Article

Bp-13 PLA₂: Purification and Neuromuscular Activity of a New Asp49 Toxin Isolated from *Bothrops pauloensis* Snake Venom

Georgina Sucasaca-Monzón,¹ Priscila Randazzo-Moura,¹ Thalita Rocha,^{2,3}
Frank Denis Torres-Huaco,² Augusto Vilca-Quispe,² Luis Alberto Ponce-Soto,²
Sérgio Marangoni,² Maria Alice da Cruz-Höfling,² and Léa Rodrigues-Simioni¹

¹Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas (UNICAMP),
13083-881 Campinas, SP, Brazil

²Department of Biochemistry and Tissue Biology, Institute of Biology, State University of Campinas (UNICAMP),
13083-365 Campinas, SP, Brazil

³Multidisciplinary Research Laboratory, São Francisco University, 12916-350 Bragança Paulista, SP, Brazil

Correspondence should be addressed to Léa Rodrigues-Simioni; simioni@fcm.unicamp.br

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A new PLA₂ (Bp-13) was purified from *Bothrops pauloensis* snake venom after a single chromatographic step of RP-HPLC on μ -Bondapak C-18. Amino acid analysis showed a high content of hydrophobic and basic amino acids and 14 half-cysteine residues. The N-terminal sequence showed a high degree of homology with basic Asp49 PLA₂ myotoxins from other *Bothrops* venoms. Bp-13 showed allosteric enzymatic behavior and maximal activity at pH 8.1, 36°–45°C. Full Bp-13 PLA₂ activity required Ca²⁺; its PLA₂ activity was inhibited by Mg²⁺, Mn²⁺, Sr²⁺, and Cd²⁺ in the presence and absence of 1 mM Ca²⁺. In the mouse phrenic nerve-diaphragm (PND) preparation, the time for 50% paralysis was concentration-dependent ($P < 0.05$). Both the replacement of Ca²⁺ by Sr²⁺ and temperature lowering (24°C) inhibited the Bp-13 PLA₂-induced twitch-tension blockade. Bp-13 PLA₂ inhibited the contractile response to direct electrical stimulation in curarized mouse PND preparation corroborating its contracture effect. In biventer cervicis preparations, Bp-13 induced irreversible twitch-tension blockade and the KCl evoked contracture was partially, but significantly, inhibited ($P > 0.05$). The main effect of this new Asp49 PLA₂ of *Bothrops pauloensis* venom is on muscle fiber sarcolemma, with avian preparation being less responsive than rodent preparation. The study enhances biochemical and pharmacological characterization of *B. pauloensis* venom.

1. Introduction

Phospholipase A₂ belongs to an expanding superfamily of enzymes that catalyzes ester bond hydrolysis at the sn-2 position of 1,2-diacyl-sn-3-phosphoglycerides and generates arachidonic acid. Depending on the molecular taxonomy, intracellular and secretory PLA₂s are currently classified in six to twelve groups [1]. Secretory PLA₂s are enzymes of 13–18 kDa with 5–8 disulfide bonds whose activity requires millimolar Ca²⁺ concentration [2].

Despite the variety of the local and systemic pathophysiological effect, such as myotoxicity, neurotoxicity, anticoagulation, hemolysis, hypotension, platelet aggregation inhibition, and bactericidal and proinflammatory activities, PLA₂

groups show highly conserved molecular regions and similar three-dimensional structure [3]. The majority of such local and systemic effects caused by *Bothrops* sp. envenomation is often due to the PLA₂ activity [3–5]. Although generally the neurotoxic effects are unnoticed clinically, they can be observed during *in vitro* experiments and are frequently associated with PLA₂ of bothropic venoms [6–14].

B. pauloensis is found in humid and cool regions in the central and Southwest of the state of São Paulo [15, 16] and in seasonally dry savannas of the Brazilian Cerrado [17]. From the 292 notified accidents caused by *Bothrops* snakes, 18% (52 cases) were caused by *B. pauloensis* [18], thus evidencing that the study of the venom of this snake species can be of medical relevance. In this work, we describe the isolation and

enzymatic characterization of a highly basic PLA₂ from the venom of *B. pauloensis*. We also investigated whether this isolated PLA₂ possesses neurotoxic activity.

2. Material and Methods

2.1. Venom and Reagents. Venom was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Solvents (HPLC grade), 4-nitro-3-octanoyloxy-benzoic acid, sequencing grade bovine pancreatic trypsin and other reagents were also obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Reverse Phase HPLC (RP-HPLC). Bp-13 PLA₂ from *B. pauloensis* venom was purified by reverse phase HPLC, according to the method described by Ponce-Soto et al. [19], with minor changes. Briefly, 5 mg of the whole venom was dissolved in 200 μ L of buffer A (0.1% TFA) and centrifuged at 4500 g; the supernatant was then applied to a μ -Bondapak C18 column (0.78 $\times$ 30 cm; Waters 991-PDA system), previously equilibrated in buffer A for 15 min. The protein elution was then conducted using a linear gradient (0–100%, v/v) of buffer B (66.5% acetonitrile in buffer A) at a constant flow rate of 1.0 mL/min. The chromatographic run was monitored at 280 nm of absorbance. The purity and PLA₂ activity were monitored according to Sections 2.3 and 2.6. All fractions eluted were lyophilized and then stored at –20°C for further biochemical and pharmacological assays.

2.3. Electrophoresis. Tricine SDS-PAGE in a discontinuous gel and buffer system was used to estimate the molecular mass of Bp-13 PLA₂, under reducing and nonreducing conditions [20]. The used molecular weight markers in kDa were phosphorylase B: ~ 94, albumin: 67, ovalbumin: 43, carbonic anhydrase: 30, soybean trypsin inhibitor: 20, and α -lactalbumin: 14 (GE Healthcare).

2.4. Amino Acid Analysis. Amino acid analysis was performed on a Pico-Tag Analyzer (Waters Systems), as described by Heinrikson and Meredith [21], with minor changes. Bp-13 PLA₂ sample (30 μ g) was hydrolyzed at 105°C for 24 hours, in 6 M HCl (Pierce sequencing grade) containing 1% phenol (w/v). Hydrolyzates were reacted with 20 μ L of derivatized solution (ethanol : triethylamine : water : phenylisothiocyanate, 7 : 1 : 1 : 1, v/v) for one hour at room temperature. Afterwards, PTC-amino acids were identified and quantified by HPLC, by comparing their retention times and peak areas with those from a standard amino acid mixture (Sigma-Aldrich).

2.5. Mass Spectrometry. Molecular mass of intact native and alkylated Bp-13 PLA₂ was analyzed by MALDI-TOF mass spectrometry using a Voyager-DE PRO MALDI-TOF apparatus (Applied Biosystems, Foster City, CA, USA) equipped with a pulsed nitrogen laser (337 nm, pulse with 4 ns). The amount of 1 μ L of sample in 0.1% TFA was mixed with 2 μ L of sinapinic acid matrix (3, 5-dimethoxy-4-hydroxycinnamic acid). The matrix was prepared with 30% acetonitrile and 0.1% TFA and its mass analyzed under the

following conditions: 25 kV accelerating voltage, the laser fixed at 2890 μ J/cm², 300 ns delay, and linear analysis mode [22].

For de novo sequencing of N-terminal, the first 52 amino acids from Bp-13 PLA₂, alkylated tryptic peptides were fractionated by RP-HPLC, manually collected, lyophilized, and resuspended in 80% H₂O, 20% acetonitrile and 0.1% TFA. One peptide was introduced separately into the mass spectrometer source using a syringe pump at a 500 nl/min flow rate. Before performing a tandem mass spectrum, an ESI/MS mass spectrum (TOF MS mode) was acquired for each HPLC fraction over the mass range of 400–2000 *m/z*, aiming to select the ion of interest. Subsequently, these ions were fragmented in the collision cell (TOF MS/MS mode). Different collision energies were used depending on the mass and charge state of the ions. The resulting product-ion spectra were acquired with the TOF analyzer and deconvoluted using the MassLynx-MaxEnt 3 algorithm (Waters). Singly charged spectra were manually processed using the PepSeq application included in MassLynx.

2.6. PLA₂ Activity. PLA₂ activity was measured using the assay described by Cho and Kézdy [23] and Holzer and Mackessy [24] modified for 96-well plates. The standard assay mixture contained 200 μ L of buffer (10 mM Tris-HCl, 10 mM CaCl₂, and 100 mM NaCl, pH 8.0), 20 μ L of synthetic chromogenic substrate 4-nitro-3-(octanoyloxy) benzoic acid 3 mM, 20 μ L of water, and 20 μ L of PLA₂ fractions (1 mg/mL) or whole venom (1 mg/mL) in a final volume of 260 μ L. After adding the samples, the mixture was incubated for up to 40 min at 37°C, absorbance reading at intervals of 10 min. The enzyme activity, expressed as the initial velocity of the reaction (*V*₀), was calculated based on the increase of absorbance after 20 min.

The pH and optimal temperature of PLA₂ were determined by incubating the four reaction buffers with different pH ranging from 4 to 10 and at different temperatures, respectively. The effect of substrate concentration (40, 20, 10, 5, 2.5, 1.0, 0.5, 0.3, 0.2, and 0.1 mM) on enzyme activity was determined by measuring the increase of absorbance after 20 min in optimum pH and temperature. The effect of different concentration of Ca²⁺ on Bp-13 PLA₂ enzymatic activity was tested by preincubating the enzyme with different ion concentrations (0.005, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 M) at 37°C for 30 minutes prior to standard experiment. Also, the effects of different divalent ions (Sr²⁺, Mg²⁺, Mn²⁺, and Cd²⁺, 5 mM) were tested in presence (1 mM) or absence of Ca²⁺. Finally the effect of urea (4 M) on the enzymatic activity was tested by preincubating Bp-13 for 30 minutes at 37°C.

All assays were done in triplicate and the absorbance at 425 nm was measured using a VersaMax 190 multiwell plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.7. BC, PND, and EDL Nerve-Muscle Preparations. Male Swiss mice (*Mus musculus*) weighing 20–30 g and 8-day-old young chick (Hy Line W36) were used for twitch-tension studies in presence of Bp-13 PLA₂. Mice and young chick were sacrificed by halothane inhalation. The hemidiaphragm

FIGURE 1: RP-HPLC chromatography of *Bothrops pauloensis* venom on μ -Bondapak C 18 column (0.78 cm $\times$ 30 cm; Waters 991-PDA system Waters). A sample of 20 mg from venom was eluted with solvent B (acetonitrile, 0–66%) at 25°C. The elution profile was monitored at 280 nm. The main fractions obtained are identified as Bp-1–Bp-18.

(PND) and extensor digitorum longus (EDL) muscles isolated from mice and biventer cervicis muscle (BC) isolated from chick were mounted according to Bülbring [25] and Ginsborg and Warriner [26], respectively.

The PND and EDL mammal preparations were suspended under a constant resting tension of 5 g/cm and 0.5 g/cm, respectively, in a 5 mL (for PND) and 3.5 mL (for EDL) organ bath containing aerated (95% O₂–5% CO₂) Tyrode solution, whose composition in mM was NaCl 137, KCl 2.7, CaCl₂ 1.8, MgCl₂ 0.49, NaH₂PO₄ 0.42, NaHCO₃ 11.9, and glucose 11.1, pH 7.4, 37°C. A supramaximal pulse (0.1 Hz, 0.2 ms) delivered by a Grass S48 electronic stimulator (Grass Instrument Co., Quincy, MA, USA) was applied through electrodes placed around the motor phrenic nerve (PND) and tendon (EDL). For both preparations, the isometric muscle tension was recorded using a force-displacement transducer Load Cell BG 50 g (Kulite Semiconductor Products Inc., Leonia, NJ, USA) coupled to a physiograph (Gould RS 3400, Cleveland, OH, USA). The PND preparations were allowed to stabilize for at least 20 min before the addition of Bp-13 toxin at different concentrations: 0.71 μ M (10 μ g/mL), 1.42 μ M (20 μ g/mL), 3.56 μ M (50 μ g/mL), and 7.12 μ M (100 μ g/mL). The EDL preparations were incubated with Bp-13 toxin at 3.56 μ M.

Some curarized PND preparations (d-tubocurarine, 10 μ M) were incubated with Bp-13 toxin at 1.42 μ M and 3.56 μ M concentrations and the twitch response was recorded under direct muscle stimulation at supramaximal pulses of 70 V, 0.1 Hz at 2 ms duration. The effect of divalent ions, Mg²⁺, Mn²⁺, Sr²⁺, and Cd²⁺ (10 mM), on the Bp-13 PLA₂ activity was done by replacement of Ca²⁺ (1.8 mM) in the nutritive Tyrode solution; replacement of Ca²⁺ by Sr²⁺ (4 mM) was also assayed. The effect of temperature (5 to 60°C) during 20 min was read at 425 nm.

The biventer cervicis (BC) preparations were suspended in a 5 mL organ bath containing Krebs solution (composition in mM: NaCl 118.6, KCl 4.69, CaCl₂ 1.88, KH₂PO₄ 1.17, MgSO₄ 1.17, NaHCO₃ 25.0, and glucose 11.65), aerated with carbogen (95% O₂–5% CO₂) at 37°C. A bipolar platinum ring electrode was placed around the muscle tendon, within which run the motor nerve trunk. Field stimulation using a Grass S48 stimulator set at 0.1 Hz, 0.2 ms, and 4–6 V was applied and the muscle contractions and contractures were recorded isometrically via force-displacement transducer coupled to a physiograph. The muscle responsiveness to exogenously applied acetylcholine (ACh, 110 μ M) and KCl (13.4 mM) was recorded in the absence of field stimulation both prior to toxin addition and at the end of the experiment (120 min). The BC preparation was stabilized for at least 15 min before addition of Bp-13 at concentration of 3.56 (50 μ g/mL) and 7.12 μ M (100 μ g/mL). The results were compared with control BC preparations incubated with Krebs solution alone.

2.8. Statistical Analyses. Results were reported as mean $\pm$ SEM. Differences among means was assessed by one-way ANOVA and followed by Mann-Whitney test for comparison between two groups. Differences were considered statistically significant if $P < 0.05$.

3. Results

3.1. Purification and Characterization. Fractionation of *B. pauloensis* venom by reverse phase HPLC (Figure 1) showed the elution of 18 main fractions: Bp-1 to Bp-18. The Bp-13, which was eluted at 39 min, was characterized as a not yet described PLA₂-active toxin (Figure 2(a)). The purity of this

FIGURE 2: (a) PLA₂ activity of *Bothrops pauloensis* whole venom (WV), Bp-13 PLA₂. ANOVA and followed by Mann-Whitney test for comparison between two groups. Data are represented as mean $\pm$ SEM. (b) SDS-PAGE profile of Bp-13 PLA₂, Mm, molecular mass markers ($\times 10^{-3}$).

FIGURE 3: Mass determination of *Bothrops pauloensis* Bp-13 by MALDI-TOF mass spectrometry.

peak was confirmed through rechromatography on an analytical RP-HPLC μ -Bondapak C18 column and showing a single peak (Figure 1, insert).

Tricine SDS-PAGE and MALDI-TOF mass spectrometry showed that Bp-13 PLA₂ presented a molecular mass of ~14 kDa (Figure 2(b)) and 14035.628 Da (Figure 3), respectively. The amino acid analysis revealed the following composition: Asx/9, Glx/5, Ser/4, Gly/13, His/2, Arg/5, Thr/7, Ala/6, Pro/11, Tyr/14, Val/4, Cys/14, Ile/3, Leu/11, Phe/3, and Lys/12 with a high content of basic and hydrophobic amino acids and 14 half-cystine residues. Its N-terminal sequence of the 52 initial residues was as follows: DLWQFGKMIL KENGK-SPFSF YGAYGCGYGGW GRRKPKDKT TDRCCFVHDC CR. Such amino acid sequencing of Bp-13 PLA₂ shares 95% sequence identity with other bothropic PLA₂s (Figure 4).

3.2. Enzymatic Characterization of Bp-13 PLA₂. The Bp-13 PLA₂ activity measured through synthetic substrate 4-nitro-3-octanoyloxy-benzoic acid and 1–20 mM Ca²⁺ showed that the catalytic activity was expressed after 2 mM Ca²⁺, but the maximal PLA₂ activity was reached with 10 mM Ca²⁺ (Figure 5(a)). For the conditions tested, Bp-13 PLA₂ showed

a discrete allosteric-like behavior, mainly at low substrate concentrations (Figure 5(b)). Estimated $V_{\max}$ was 11.6 nmol/min/mg and K_m was 11.8 mM (Figure 5(c)). The optimal pH and temperature for development of the maximum enzymatic activity were 8.3 (Figure 5(d)) and ~38°C (Figure 5(e)), respectively. The addition of Mn²⁺, Mg²⁺, Sr²⁺, and Cd²⁺ (10 mM) in the presence of low Ca²⁺ concentration (1 mM) decreased enzyme's activity; the substitution of Ca²⁺ by Mn²⁺, Mg²⁺, Sr²⁺, or Cd²⁺ (10 mM) in the absence of Ca²⁺ (0 mM) also reduced PLA₂ activity (Figure 5(f)). In addition, preincubation with urea (4 M) did not affect significantly the enzymatic activity of Bp-13 (data not shown).

3.3. Neuromuscular Activity. Assays to study the neuromuscular activity of Bp-13 PLA₂ were performed using avian BC preparation and rodent's PND and EDL preparations. The toxin induced a time- and concentration-dependent and irreversible twitch-tension blockade. In the PND preparations, the time needed for 50% paralysis in response to 7.12 μ M ($n = 3$), 3.56 μ M ($n = 6$), and 1.42 μ M ($n = 3$) of Bp-13 PLA₂ was 18 ± 1 min, 28 ± 3 min, and 120 ± 4 min, respectively ($P < 0.05$); Bp13 at 0.71 μ M concentration induced a 25% paralysis only after 120 ± 2 min relative to control ($P < 0.05$) (Figure 6(a)). The catalytic activity of Bp-13 PLA₂ was similar both in the presence of 1 or 10 mM in the nutritive bath of PND preparation. The addition of Mg²⁺, Mn²⁺, Sr²⁺, and Cd²⁺ (10 mM) in the nutritive Tyrode solution in the absence of Ca²⁺ or presence of 1 mM Ca²⁺ showed significant loss of the catalytic activity of the toxin indicating that these divalent ions cannot replace the Ca²⁺ for the development of the PLA₂ catalytic activity. The replacement of 1.8 mM Ca²⁺ by 4 mM Sr²⁺ in the Tyrode solution prevented the blocking effect of Bp13 PLA₂ (3.56 μ M) since the twitch-tension response showed an amplitude of $83.7 \pm 14\%$ after 120 min incubation which was not different from baseline of the control preparations (Figure 6(b)). The finding indicates that the neuromuscular blocking effect of the Bp-13 is calcium-dependent.

The effect of temperature (5°C–60°C) on the catalytic activity of Bp-13 PLA₂ showed that the optimal enzymatic

	10	20	30	40	50
Bp-13	D L W Q F G K M I L K E N G K S P F S F Y G A Y G C Y C G W G G R R K P K D K T T D R C C F V H D C C R				
BnpTX-I	D L W Q F G K M I L K V A G K L P F P Y Y G A Y G C Y C G W G G R G K P K D P T D R C C F V H D C C				
Bj6-1	D L W Q F G Q M I L K E T G K I P F P Y Y G A Y G C Y C G W G G R G G K P K D G T D R C C V V H D C C Y ...				
Bj6-2	D L W Q F G Q M I L K E T G K I P F P Y Y G A Y G C Y C G W G G R G G K P K D G T D R C C V V H D C C Y ...				
BthTX-II	D L W Q F G Q M I L K E T G K L P F P Y Y T T Y G C Y C G W G G Q G Q - P K D A T D R C C F V H D C C Y ...				
NeuTX-I	D L V Q F G Q M I L K V A G R S L P K S Y G A Y G C Y C G W G G R G K - - - - - - - - - - - - - - - -				
PrtTX III	D L W Q F G Q M I L K E T G K L P F P Y Y T Y G G C Y C G V G G R R G - L G T K D D R C C Y V H D C C Y ...				
BnpTX-II	S L W E F A Q M I L E E T K R L P F P Y Y G A Y G C Y C G W G G Q G Q - - - - - - - - - - - - - - - -				

FIGURE 4: The amino acid sequence N-terminal, alignment of Bp-13 with selected PLA₂ sequences obtained from the BLAST protein data bank (PubMed-Medline). PrtTX III from *Bothrops pirajai* [55], PLA₂Bj p from *Bothrops jararacussu* [30], PLA₂ 6-1 and PLA₂6-2 [22] and BthTx-II, bothropstoxin II, from *Bothrops jararacussu* [56], BnpTX-I and BnpTX-II [32], and NeuTX-I [12].

activity occurred around 38°C (Figure 6(c), insert). The neuromuscular blockade was prevented when the temperature incubation was set at 24°C; after 120 min, the twitch-tension response was 37.4% compared with the 90% seen at 37°C, Figure 6(c). The PND preparations previously treated with d-Tc (10 µM) and under direct electrical stimulation showed that Bp-13 (1.42 and 3.56 µM) was able to cause a significant contracture followed by blockade of the contractile response (73 ± 7% and 14 ± 6%, respectively, $n = 3-6$, $P < 0.05$, Figure 6(d)).

In the EDL preparations, the time needed for 50% paralysis at a 3.56 µM Bp13 PLA₂ concentration was 120 min ± 2 min (Figure 7). As displayed in the Figure 7, the contractile response of the EDL preparation was maintained steady during the 60 min period, regardless of whether the EDL was incubated in normal Tyrode solution or in Tyrode solution whose Ca²⁺ (1.8 mM) was replaced by Sr²⁺ (4 mM). The Bp-13 (3.56 µM) addition caused a significant blockade of the twitch tension which achieved 90% after 80 min of toxin addition in the normal Tyrode solution. The replacement of 1.8 mM Ca²⁺ by 4 mM Sr²⁺ also prevented completely such neuromuscular blockade induced by the toxin (3.56 µM) which was sustained until the end of observation (180 min, $n = 6$, $P < 0.05$). Similarly, the lowering of temperature to 24°C prevented the blockade of twitch tension in the EDL preparation (not shown).

In relation to avian preparations, it was shown that they exhibited lower sensibility to Bp-13 PLA₂ than the mammalian preparations. Bp-13 PLA₂ (3.56 and 7.12 µM) induced an irreversible but mild decrease of the twitch tension of 21 ± 6% and 28 ± 2% after 120 min, respectively (Figure 8(a)). The response to acetylcholine (ACh, 110 µM) was significant just when Bp-13 PLA₂ concentration was set at 7.12 µM ($P > 0.05$). In contrast, Bp-13 PLA₂ regardless of the concentration, 3.56 or 7.12 µM, did not interfere in the KCl (13.4 mM) induced contracture (Figure 8(b)).

4. Discussion

The presence of Bp-10 and Bp-11 (K49 PLA₂ homologous Bnp 6 and Bnp7) [8], Bp-14 and Bp-15 (Asp49 PLA₂ NeuTX-I and NeuTX-II) [12], and Bp-12 (Lys49 PLA₂) [11] has been already demonstrated in the *B. pauloensis* snake crude venom. Interestingly, another Asp49 PLA₂, the Bp13, of the same *Bothrops* species venom was now isolated and characterized biochemically and pharmacologically. Such a

diversity of PLA₂ isoforms in the venom of a same species evidences the necessity of developing efficient methodologies to purify and identify different isoforms in venom fractions otherwise considered homogeneous. The RP-HPLC was more suitable to purify Bp-13 PLA₂ than other conventional methods previously described for other toxins of the same venom, since it required just a single chromatographic step [19, 22, 27].

The purity of Bp-13 PLA₂ was confirmed by rechromatography on an analytical RP-HPLC µ-Bondapak C18 column. SDS-PAGE showed the monomeric nature of Bp-13 and a relative molecular mass of ~14 kDa and it was confirmed by MALDI-TOF mass spectrometry with a molecular mass of 14035.628 Da. MALDI-TOF/MS has a precision in measuring protein molecular mass of 0.1% Da; thus, this characteristic allows us to demonstrate that Bp13 is another PLA₂ isoform present in the *Bothrops pauloensis* venom, as was for other bothropic PLA₂ isoforms [19, 22, 27–32].

The amino acid composition of Bp-13 PLA₂ suggests that this PLA₂ is a basic protein because it possesses more basic residues (Arg, His, and Lys, total 19) than acid residues (Asx/Gnx, total 13), 14 half- Cys, and also because this protein showed high content of residues Tyr, Pro, Gly, and Lys which was a composition featured by other catalytic active bothropic myotoxins such as the 6-1 PLA₂ and 6-2 PLA₂ isoforms from *B. jararacussu* or the BaTX, a basic PLA₂ from *B. alternatus* [22, 27].

In Asp49 PLA₂, a conserved N-terminal helix region forms a hydrophobic channel involving L2, Q4, F5, and I9. Conversely, the level of identity between PLA₂s is very high in Ca²⁺-loop sequence (residues 24–34 YGCXCGXGGRG) and in the active site (residues 42–54 DRCCFVHDCYXK) [22, 33–35]. The conserved residues Y28, G30, G32, Asp49, H48, and Y52 are directly or indirectly linked to Bp-13 PLA₂ catalysis. The N-terminal amino acid sequence of the first 52 residues from Bp-13 PLA₂ shows these regions highly conserved and directly linked to catalytic activity.

Bp-13 PLA₂ herein analyzed showed the presence of some important mutations in N-terminal sequence (up to the 52nd residue). Thus, Bp-13 PLA₂ shows K7 -> Q7, N13 -> T13, F20 -> Y20, R34 -> G34, and F46 -> Y46, which are strategic positions for expression of the catalytic activity. The presence of K7 in Bp-13 PLA₂ shows that this residue can contribute to keep hydrophobic cavity conformation of the N-terminal region. The N-terminal channel present in PLA₂ enzymes is highly conserved and provides access to the lipid substrate to

FIGURE 5: Kinetic analysis of Bp-13 PLA₂ activity. (a) Influence of calcium ion on PLA₂ activity; (b) effect of substrate concentration on the kinetics of Bp-13 PLA₂; (c) Lineweaver-Burk (double-reciprocal) plot of Bp-13 PLA₂; (d) optimal pH for PLA₂ activity; (e) optimal temperature for PLA₂ activity; (f) influence of ions (10 mM each) on PLA₂ activity in the absence or presence of 1 mM and 10 mM Ca^{2+} . The results are the mean $\pm$ SEM of five experiments. * $P < 0.05$ when compared with control values. ANOVA and followed by Mann-Whitney test for comparison between two groups.

FIGURE 6: Twitch-tension response of direct and indirect stimulated PND preparations for 120 min. (a) The preparations were incubated with Tyrode (control) or Bp-13 PLA₂ (0.71–7.12 μ M) at 37°C ($n = 3-6$ experiments); (b) twitch-tension response of PND preparations incubated with Bp-13 PLA₂ in Tyrode solution and in Tyrode whose 1.8 mM Ca²⁺ was replaced by 4 mM Sr²⁺ at 37°C ($n = 4-6$); (c) twitch-tension response of indirectly stimulated PND preparations incubated with Tyrode (control) or Bp-13 (3.56 μ M) at 24°C ($n = 4-6$ experiments); (d) twitch-tension response of directly stimulated PND preparations incubated with Tyrode (control) or Bp-13 (1.42 and 3.56 μ M) at 37°C ($n = 4-6$ experiments). Twin arrows represent the time of toxin addition. Each point represents the mean $\pm$ SEM; * $P < 0.05$ compared to control values. ANOVA and followed by Mann-Whitney test for comparison between two groups.

the PLA₂ catalytic site. Also, Bp-13 shows at position 13 the lack of Thr residue usually found in other PLA₂s enzymes; however, both BnpTX-I (A13 -> T13) and NeuTx-I (A13 -> T13) also showed mutation in this position for polar residue, suggesting that this position is not as well conserved and that it could be a structural feature for the PLA₂ of *B. pauloensis*. The same was observed for the position 16, but in this particular case this position conserved its hydrophobic nature. Despite the change, Bp-13 still maintains catalytic activity and indicates that these residues have no important role in Bp-13 activity [33, 36].

Breithaupt [37] reported that *Crotalus* PLA₂ shows classic Michaelis-Menten behavior. However, PLA₂ activity of Bp-13 from *B. pauloensis* shows a discrete allosteric-like behavior,

and this activity is enhanced by the presence of even low Ca²⁺ concentrations. The PLA₂ from *C. durissus terrificus* venom exhibits a typical PLA₂ activity, since it hydrolyzes synthetic substrates at position 2 and preferentially attacks substrates in their micellar state [24, 37]. Similarly, Bp-13 PLA₂ exhibits allosteric behavior with a V_{max} of 11.6 nmol/min and a K_m of 11.8 mM.

However, at low concentrations of the synthetic substrate, Bp-13 PLA₂ showed a sigmoidal kinetic behavior; this phenomenon was observed for other Crotalinae PLA₂s [2, 22, 38–40], suggesting an allosteric behavior for these enzymes. The allosteric term was originally used for enzymes with altered kinetic properties in the presence of ligands (effectors) that do not show any structural similarity to the substrate. Allosteric

FIGURE 7: Twitch-tension response of indirectly stimulated EDL preparations incubated with $3.56 \mu\text{M}$ of Bp-13 in Tyrode and Tyrode Sr^{2+} . Each point represents the mean $\pm$ SEM of 3–6 experiments; * $P < 0.05$ compared to control values. ANOVA and followed by Mann-Whitney test for comparison between two groups.

(a)

(b)

FIGURE 8: Twitch-tension response curve of indirectly stimulated BC preparations incubated with 3.56 and $7.12 \mu\text{M}$ (50 and $100 \mu\text{g/mL}$, resp.) at 37°C . (a) Each point represents the mean $\pm$ SEM of 3–6 experiments. (b) Percentage of ACh ($110 \mu\text{M}$) and KCl (20 mM) evoked contracture after Bp-13 administration; * $P < 0.05$ compared to control values. ANOVA and followed by Mann-Whitney test for comparison between two groups.

enzymes show a number of properties that distinguish them from the nonallosteric ones. Sigmoidal kinetics in the velocity substrate curve, the existence of effectors, and a polymeric structure are some of the properties of a genuine allosteric enzyme. In the case of *Crotalinae* D49-PLA₂, SDS-PAGE without reducing agents showed a weak band at $\sim 28 \text{ kDa}$ [2, 40] indicating that some molecule populations of these enzymes exist in a dimeric form, which could be responsible for the observed enzymatic behavior.

Bp-13 PLA₂ was resistant to heat and acid like PLA₂s from *Crotalus d. cascavella* [38, 41], *C. d. collineatus*, and *Lachesis*

muta muta [42] venoms; Bp-13 optimal activity was at pH 8.3 and was inactivated at pH higher than 9, like the PLA₂ from *C. mitchelli pyrrhus* [24], *C. d. terrificus* [43], and *B. neuwiedi* [44] snake venoms. The maximal enzymatic activity occurred at $\sim 38^\circ\text{C}$ and persisted at 60°C , indicating a heat-stable enzyme.

Pharmacologically, as for other types of PLA₂, the activity of Bp-13 PLA₂ was shown to be completely Ca^{2+} -dependent [45]. The coinubation of Bp-13 PLA₂ with other divalent ions (Mg^{2+} , Mn^{2+} , Sr^{2+} , and Cd^{2+}) in the presence of 1 mM Ca^{2+} or in Ca^{2+} absence reduced or abolished the enzymatic activity.

The Ca^{2+} replacement by 4 mM Sr^{2+} abolished the neuromuscular blockade. It is well known that calcium ions are essential cofactors for the enzymatic activity of both toxic and nontoxic phospholipases A_2 [46]. Several divalent ions, including Sr^{2+} , can bind to the same site of the Ca^{2+} , allowing neuromuscular transmission; nevertheless, this ion does not substitute Ca^{2+} in the catalysis processes [47–50]. Thus, the observation that neuromuscular effect of Bp-13 was Ca^{2+} -dependent indicates that the enzymatic activity might contribute for such effect as for neuwieditoxin I and neuwieditoxin II, an Asp49 PLA_2 from *B. pauloensis* [12]. Also, the lowering of the temperature of incubation bath to 24°C abolishing the neuromuscular effect of Bp-13 indicates that the enzymatic activity has a role on the neuromuscular action in PND and EDL preparations. Such finding is similar to the one observed by Galbiatti et al. [14] with Bmaj-9 PLA_2 from *B. marajoensis* venom, also an Asp49 PLA_2 . Likewise, a high catalytic activity was found in neuwieditoxin-I and -II Asp49 PLA_2 s, whose neuromuscular blockade was also temperature-dependent [12]. Both the dependence of Ca^{2+} and the need of temperature equal or above 30°C for enzymatic activity of Bp-13 identify the toxin as a typical Asp49 PLA_2 .

Bothropic envenomation does not cause neurotoxic clinical signs, but some experimental studies have shown that the venom of several species causes neuromuscular blockade *in vitro* [8, 11–14, 32, 51, 52] and induces peripheral muscular weakness signs [6, 53].

Borja-Oliveira et al. [54] reported that *B. pauloensis* crude venom causes partial blockade of directly evoked muscular contractions in BC preparations; in 2007, the authors suggested that neuwieditoxin-I and -II (Asp49 PLA_2 s) from *B. pauloensis* venom were probably responsible for the venom presynaptic neurotoxicity *in vitro*. However, herein it was shown that BC preparations showed low responsiveness to the Bp-13 (less than 30% of neuromuscular blockade) when compared to the crude venom. This could mean that this new Asp49 PLA_2 from *B. neuwiedi* venom has preponderantly a muscular action, thus differing from the presynaptic neurotoxic action of neuwieditoxin-I and -II Asp49 PLA_2 s referred by Borja-Oliveira et al. [12].

Taken together, these results identify Bp-13 isolated from *Bothrops pauloensis* snake venom, as a new member of the Asp49 PLA_2 family. It is suggested that the Bp-13 PLA_2 catalytic activity may contribute to the neuromuscular effect already reported for the crude venom on rodent preparations. It is suggested that the main effect of the Bp-13 toxin seems to be on the fiber sarcolemma, with prominence in the mice preparation. In BC preparations, the Bp-13 PLA_2 showed little neuromuscular effect. This study is an additional contribution for understanding the toxic effect caused by the *Bothrops pauloensis* crude venom.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

- [1] M. Murakami and I. Kudo, "Phospholipase A_2 ," *Journal of Biochemistry*, vol. 131, no. 3, pp. 285–292, 2002.
- [2] V. L. Bonfim, M. H. Toyama, J. C. Novello et al., "Isolation and enzymatic characterization of a basic phospholipase A_2 from *Bothrops jararacussu* snake venom," *Protein Journal*, vol. 20, no. 3, pp. 239–245, 2001.
- [3] R. M. Kini, "Excitement ahead: structure, function and mechanism of snake venom phospholipase A_2 enzymes," *Toxicon*, vol. 42, no. 8, pp. 827–840, 2003.
- [4] J. M. Gutiérrez and C. L. Ownby, "Skeletal muscle degeneration induced by venom phospholipases A_2 : insights into the mechanisms of local and systemic myotoxicity," *Toxicon*, vol. 42, no. 8, pp. 915–931, 2003.
- [5] B. Lomonte, Y. Angulo, and L. Calderón, "An overview of lysine-49 phospholipase A_2 myotoxins from crotalid snake venoms and their structural determinants of myotoxic action," *Toxicon*, vol. 42, no. 8, pp. 885–901, 2003.
- [6] J. C. Cogo, J. Prado-Franceschi, J. R. Giglio et al., "An unusual presynaptic action of *Bothrops insularis* snake venom mediated by phospholipase A_2 fraction," *Toxicon*, vol. 36, no. 10, pp. 1323–1332, 1998.
- [7] J. C. Cogo, S. Lilla, G. H. M. F. Souza, S. Hyslop, and G. de Nucci, "Purification, sequencing and structural analysis of two acidic phospholipases A_2 from the venom of *Bothrops insularis* (jararaca ilhoa)," *Biochimie*, vol. 88, no. 12, pp. 1947–1959, 2006.
- [8] A. M. Soares, R. Guerra-Sá, C. R. Borja-Oliveira et al., "Structural and functional characterization of BnSP-7, a Lys49 myotoxic phospholipase A_2 homologue from *Bothrops neuwiedi pauloensis* venom," *Archives of Biochemistry and Biophysics*, vol. 378, no. 2, pp. 201–209, 2000.
- [9] Y. Oshima-Franco, G. B. Leite, C. A. Dal Belo et al., "The presynaptic activity of bothropstoxin-I, a myotoxin from *Bothrops jararacussu* snake venom," *Basic and Clinical Pharmacology and Toxicology*, vol. 95, no. 4, pp. 175–182, 2004.
- [10] P. Randazzo-Moura, G. B. Leite, G. H. Silva et al., "A study of the myotoxicity of Bothropstoxin-I using manganese in mouse phrenic nerve-diaphragm and extensor digitorum longus preparations," *Brazilian Journal of Morphological Sciences*, vol. 23, pp. 171–184, 2006.
- [11] P. Randazzo-Moura, L. A. Ponce-Soto, L. Rodrigues-Simioni, and S. Marangoni, "Structural characterization and neuromuscular activity of a new Lys49 phospholipase A_2 homologue (Bp-12) isolated from *Bothrops pauloensis* snake venom," *Protein Journal*, vol. 27, no. 6, pp. 355–362, 2008.
- [12] C. R. Borja-Oliveira, B. H. Kassab, A. M. Soares et al., "Purification and N-terminal sequencing of two presynaptic neurotoxic PLA_2 , neuwieditoxin-I and neuwieditoxin-II, from *Bothrops neuwiedi pauloensis* (Jararaca pintada) venom," *Journal of Venomous Animals and Toxins Including Tropical Diseases*, vol. 13, no. 1, pp. 103–121, 2007.

- [13] W. L. Cavalcante, S. Hernandez-Oliveira, C. Galbiatti et al., "Biological characterization of *Bothrops marajoensis* snake venom," *Journal of Venom Research*, vol. 2, pp. 37–41, 2011.
- [14] C. Galbiatti, T. Rocha, P. Randazzo-Moura et al., "Pharmacological and partial biochemical characterization of Bmaj-9 isolated from *Bothrops marajoensis* snake venom," *Journal of Venomous Animals and Toxins Including Tropical Diseases*, vol. 18, no. 1, pp. 62–72, 2012.
- [15] A. R. Hoge, "Preliminary account on neotropical Crotalinae (Serpentes: Viperidae)," *Memórias do Instituto Butantan*, vol. 32, pp. 109–184, 1965.
- [16] J. A. Campbell and W. W. Lamar, *The Venomous Reptiles of Latin America*, Cornell University Press, Ithaca, NY, USA, 1989.
- [17] P. H. Valdujo, C. Nogueira, and M. Martins, "Ecology of *Bothrops neuwiedi pauloensis* (Serpentes: Viperidae: Crotalinae) in the Brazilian cerrado," *Journal of Herpetology*, vol. 36, no. 2, pp. 169–176, 2002.
- [18] S. D. A. Nishioka and P. V. P. Silveira, "A clinical and epidemiologic study of 292 cases of lance-headed viper bite in a Brazilian teaching hospital," *American Journal of Tropical Medicine and Hygiene*, vol. 47, no. 6, pp. 805–810, 1992.
- [19] L. A. Ponce-Soto, B. Lomonte, J. M. Gutiérrez, L. Rodrigues-Simioni, J. C. Novello, and S. Marangoni, "Structural and functional properties of BaTX, a new Lys49 phospholipase A₂ homologue isolated from the venom of the snake *Bothrops alternatus*," *Biochimica et Biophysica Acta—General Subjects*, vol. 1770, no. 4, pp. 585–593, 2007.
- [20] H. Schagger and G. von Jagow, "Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa," *Analytical Biochemistry*, vol. 166, no. 2, pp. 368–379, 1987.
- [21] R. L. Heinrikson and S. C. Meredith, "Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivatization with phenylisothiocyanate," *Analytical Biochemistry*, vol. 136, no. 1, pp. 65–74, 1984.
- [22] L. A. Ponce-Soto, V. L. Bonfim, L. Rodrigues-Simioni, J. C. Novello, and S. Marangoni, "Determination of primary structure of two isoforms 6-1 and 6-2 PLA₂D49 from *Bothrops jararacussu* snake venom and neurotoxic characterization using in vitro neuromuscular preparation," *Protein Journal*, vol. 25, no. 2, pp. 147–155, 2006.
- [23] W. Cho and F. J. Kézdy, "Chromogenic substrates and assay of phospholipases A₂," *Methods in Enzymology*, vol. 197, pp. 75–79, 1991.
- [24] M. Holzer and S. P. Mackessy, "An aqueous endpoint assay of snake venom phospholipase A₂," *Toxicon*, vol. 34, no. 10, pp. 1149–1155, 1996.
- [25] E. Bülbring, "Observations on the isolated phrenic nerve diaphragm preparation of the rat," *British Journal of Pharmacology*, vol. 120, supplement 1, pp. 1–2, 1997.
- [26] B. L. Ginsborg and J. Warriner, "The isolated chick biventer cervicis nerve-muscle preparation," *British Journal of Pharmacology*, vol. 15, pp. 410–415, 1960.
- [27] L. A. Ponce-Soto, J. C. Barros, S. Marangoni et al., "Neuromuscular activity of BaTX, a presynaptic basic PLA₂ isolated from *Bothrops alternatus* snake venom," *Comparative Biochemistry and Physiology—C Toxicology and Pharmacology*, vol. 150, no. 2, pp. 291–297, 2009.
- [28] J. M. Gutiérrez and B. Lomonte, "Phospholipase A₂ myotoxins from *Bothrops* snake venoms," *Toxicon*, vol. 33, no. 11, pp. 1405–1424, 1995.
- [29] K. Kemparaju, T. P. Krishnakanth, and T. V. Gowda, "Purification and characterization of a platelet aggregation inhibitor acidic phospholipase A₂ from Indian saw-scaled viper (*Echis carinatus*) venom," *Toxicon*, vol. 37, no. 12, pp. 1659–1671, 1999.
- [30] A. M. Moura-Da-Silva, H. Desmond, G. Laing, and R. D. G. Theakston, "Isolation and comparison of myotoxins isolated from venoms of different species of *Bothrops* snakes," *Toxicon*, vol. 29, no. 6, pp. 713–723, 1991.
- [31] R. S. Rodrigues, L. F. M. Izidoro, S. S. Teixeira et al., "Isolation and functional characterization of a new myotoxic acidic phospholipase A₂ from *Bothrops pauloensis* snake venom," *Toxicon*, vol. 50, no. 1, pp. 153–165, 2007.
- [32] V. M. Rodrigues, S. Marcussi, R. S. Cambraia et al., "Bactericidal and neurotoxic activities of two myotoxic phospholipases A₂ from *Bothrops neuwiedi pauloensis* snake venom," *Toxicon*, vol. 44, no. 3, pp. 305–314, 2004.
- [33] R. K. Arni and R. J. Ward, "Phospholipase A₂—a structural review," *Toxicon*, vol. 34, no. 8, pp. 827–841, 1996.
- [34] E. Valentin and G. Lambeau, "What can venom phospholipases A₂ tell us about the functional diversity of mammalian secreted phospholipases A₂?" *Biochimie*, vol. 82, no. 9–10, pp. 815–831, 2000.
- [35] J. M. Gutiérrez, L. A. Ponce-Soto, S. Marangoni, and B. Lomonte, "Systemic and local myotoxicity induced by snake venom group II phospholipases A₂: comparison between crotoxin, crotoxin B and a Lys49 PLA₂ homologue," *Toxicon*, vol. 51, no. 1, pp. 80–92, 2008.
- [36] D. L. Scott, Z. Otwinowski, M. H. Gelb, and P. B. Sigler, "Crystal structure of bee-venom phospholipase A₂ in a complex with a transition-state analogue," *Science*, vol. 250, no. 4987, pp. 1563–1566, 1990.
- [37] H. Breithaupt, "Enzymatic characteristics of crotalus phospholipase A₂ and the crotoxin complex," *Toxicon*, vol. 14, no. 3, pp. 221–233, 1976.
- [38] L. A. Ponce-Soto, M. H. Toyama, S. Hyslop, J. C. Novello, and S. Marangoni, "Isolation and preliminary enzymatic characterization of a novel PLA₂ from *Crotalus durissus collilineatus* venom," *Journal of Protein Chemistry*, vol. 21, no. 3, pp. 131–136, 2002.
- [39] A. K. Calgarotto, D. C. S. Damico, L. A. Ponce-Soto et al., "Biological and biochemical characterization of new basic phospholipase A₂ BmTX-I isolated from *Bothrops moojeni* snake venom," *Toxicon*, vol. 51, no. 8, pp. 1509–1519, 2008.
- [40] S. Huancahuire-Vega, L. A. Ponce-Soto, D. Martins-de-Souza, and S. Marangoni, "Structural and functional characterization of Brazilian toxins II and III (BbTX-II and -III), two myotoxins from the venom of *Bothrops brazili* snake," *Toxicon*, vol. 54, no. 6, pp. 818–827, 2009.
- [41] D. G. Beghini, M. H. Toyama, S. Hyslop, L. C. Sodek, and S. Marangoni, "Enzymatic characterization of a novel phospholipase A₂ from *Crotalus durissus cascavella* rattlesnake (maracambóia) venom," *Journal of Protein Chemistry*, vol. 19, no. 8, pp. 679–684, 2000.
- [42] D. C. S. Damico, S. Lilla, G. de Nucci et al., "Biochemical and enzymatic characterization of two basic Asp₄₉ phospholipase A₂ isoforms from *Lachesis muta muta* (Surucucu) venom," *Biochimica et Biophysica Acta (BBA)—General Subjects*, vol. 1726, no. 1, pp. 75–86, 2005.
- [43] E. Habermann and H. Breithaupt, "The crotoxin complex—an example of biochemical and pharmacological protein complementation," *Toxicon*, vol. 16, no. 1, pp. 19–30, 1978.
- [44] J. J. Daniele, I. D. Bianco, C. Delgado, D. B. Carrillo, and G. D. Fidelio, "A new phospholipase A₂ isoform isolated from

- Bothrops neuwiedii* (Yararáchica) venom with novel kinetic and chromatographic properties,” *Toxicon*, vol. 35, no. 8, pp. 1205–1215, 1997.
- [45] W. A. Pieterse, J. J. Volwerk, and G. H. de Haas, “Interaction of phospholipase A₂ and its zymogen with divalent metal ions,” *Biochemistry*, vol. 13, no. 7, pp. 1439–1445, 1974.
- [46] R. K. Arni, R. J. Ward, J. M. Gutierrez, and A. Tulinsky, “Structure of a calcium-independent phospholipase-like myotoxic protein from *Bothrops asper* venom,” *Acta Crystallographica—Section D Biological Crystallography*, vol. 51, no. 3, pp. 311–317, 1995.
- [47] R. Miledi, “Strontium as a substitute for calcium in the process of transmitter release at the neuromuscular junction,” *Nature*, vol. 212, no. 5067, pp. 1233–1234, 1966.
- [48] U. Meiri and R. Rahamimoff, “Activation of transmitter release by strontium and calcium ions at the neuromuscular junction,” *Journal of Physiology*, vol. 215, no. 3, pp. 709–726, 1971.
- [49] H. Rasgado-Flores and M. P. Blaustein, “ATP-dependent regulation of cytoplasmic free calcium in nerve terminals,” *American Journal of Physiology: Cell Physiology*, vol. 252, no. 6, part 1, pp. C588–C594, 1987.
- [50] A. Ghassemi, D. S. Dhillon, and P. Rosenberg, “ β -Bungarotoxin-induced phospholipid hydrolysis in rat brain synaptosomes: effect of replacement of calcium by strontium,” *Toxicon*, vol. 26, no. 5, pp. 509–514, 1988.
- [51] L. Rodrigues Simioni, N. Borgese, and B. Ceccarelli, “The effects of *Bothrops jararacussu* venom and its components on frog nerve-muscle preparation,” *Neuroscience*, vol. 10, no. 2, pp. 475–489, 1983.
- [52] M. I. Homsi-Brandenburg, L. S. Queiroz, H. Santo-Neto, L. Rodrigues-Simioni, and J. R. Giglio, “Fractionation of *Bothrops jararacussu* snake venom: partial chemical characterization and biological activity of bothropstoxin,” *Toxicon*, vol. 26, no. 7, pp. 615–627, 1988.
- [53] S. R. Zamunér, M. A. Da Cruz-Höfling, A. P. Corrado, S. Hyslop, and L. Rodrigues-Simioni, “Comparison of the neurotoxic and myotoxic effects of Brazilian *Bothrops* venoms and their neutralization by commercial antivenom,” *Toxicon*, vol. 44, no. 3, pp. 259–271, 2004.
- [54] C. R. Borja-Oliveira, A. M. Durigon, A. C. C. Vallin et al., “The pharmacological effect of *Bothrops neuwiedii pauloensis* (jararaca-pintada) snake venom on avian neuromuscular transmission,” *Brazilian Journal of Medical and Biological Research*, vol. 36, no. 5, pp. 617–624, 2003.
- [55] M. H. Toyama, P. D. Costa, J. C. Novello et al., “Purification and amino acid sequence of MP-III 4R D49 phospholipase A₂ from *Bothrops pirajai* snake venom, a toxin with moderate PLA₂ and anticoagulant activities and high myotoxic activity,” *Journal of Protein Chemistry*, vol. 18, no. 3, pp. 371–378, 1999.
- [56] M. F. Pereira, J. C. Novello, A. C. Cintra et al., “The amino acid sequence of bothropstoxin-II, an Asp-49 myotoxin from *Bothrops jararacussu* (Jararacucu) venom with low phospholipase A₂ activity,” *Journal of Protein Chemistry*, vol. 17, no. 4, pp. 381–386, 1998.