

Research Article

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

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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: \sim 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 $\mu\text{J}/\text{cm}^2$, 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

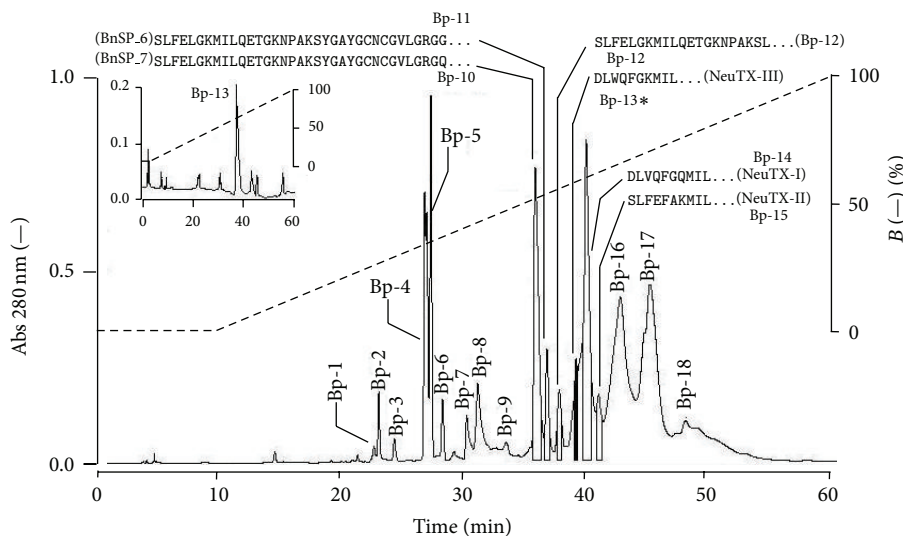


FIGURE 1: RP-HPLC chromatography of *Bothrops pauloensis* venom on μ -Bondapak C 18 column (0.78 cm \times 30 cm; Waters 991-PDA system Waters). A sample of 20 mg from venom was eluted with solvent B (acetonitrile, 0–66%) at 25°C. The elution profile was monitored at 280 nm. The main fractions obtained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

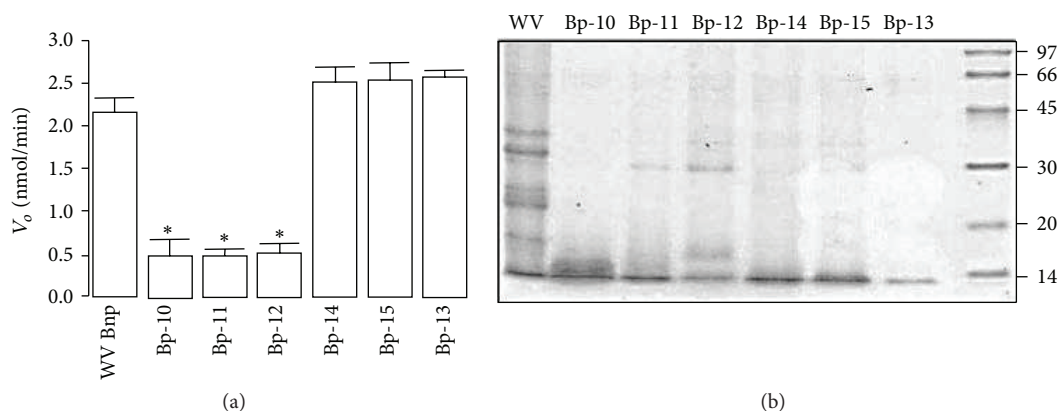


FIGURE 2: (a) PLA₂ activity of *Bothrops pauloensis* whole venom (WV), Bp-13 PLA₂. ANOVA and followed by Mann-Whitney test for comparison between two groups. Data are represented as mean \pm SEM. (b) SDS-PAGE profile of Bp-13 PLA₂, Mm, molecular mass markers ($\times 10^{-3}$).

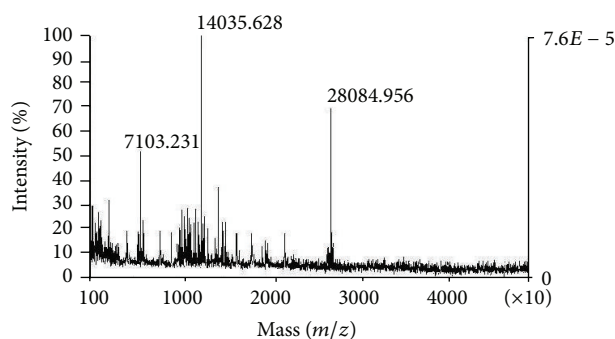


FIGURE 3: Mass determination of *Bothrops pauloensis* Bp-13 by MALDI-TOF mass spectrometry.

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 KENGLKSPFSF YGAYGCGYGGW GRRRKPDKT 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 \pm 1 min, 28 \pm 3 min, and 120 \pm 4 min, respectively ($P < 0.05$); Bp13 at 0.71 μ M concentration induced a 25% paralysis only after 120 \pm 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

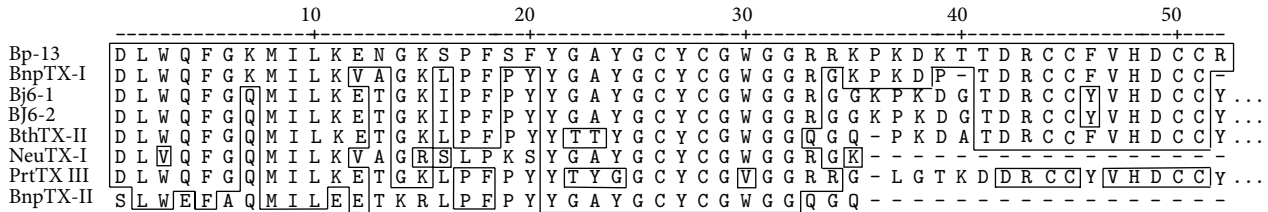


FIGURE 4: The amino acid sequence N-terminal, alignment of Bp-13 with selected PLA₂ sequences obtained from the BLAST protein data bank (PubMed-Medline). PrtTX III from *Bothrops pirajai* [55], PLA₂Bj p from *Bothrops jararacussu* [30], PLA₂ 6-1 and PLA₂ 6-2 [22] and BthTx-II, bothroptoxin II, from *Bothrops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

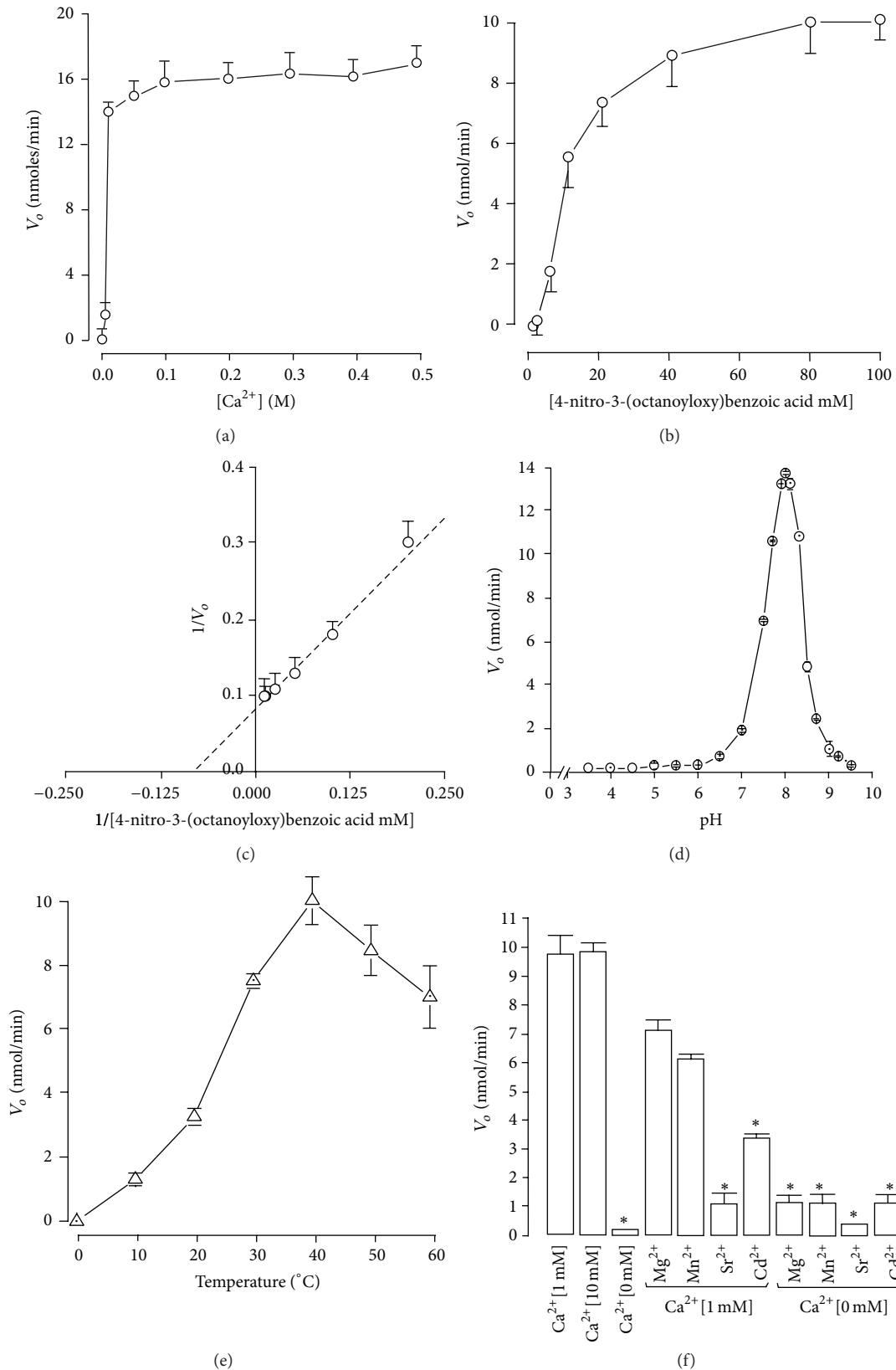


FIGURE 5: Kinetic analysis of Bp-13 PLA₂ activity. (a) Influence of calcium ion on PLA₂ activity; (b) effect of substrate concentration on the kinetics of Bp-13 PLA₂; (c) Lineweaver-Burk (double-reciprocal) plot of Bp-13 PLA₂; (d) optimal pH for PLA₂ activity; (e) optimal temperature for PLA₂ activity; (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.

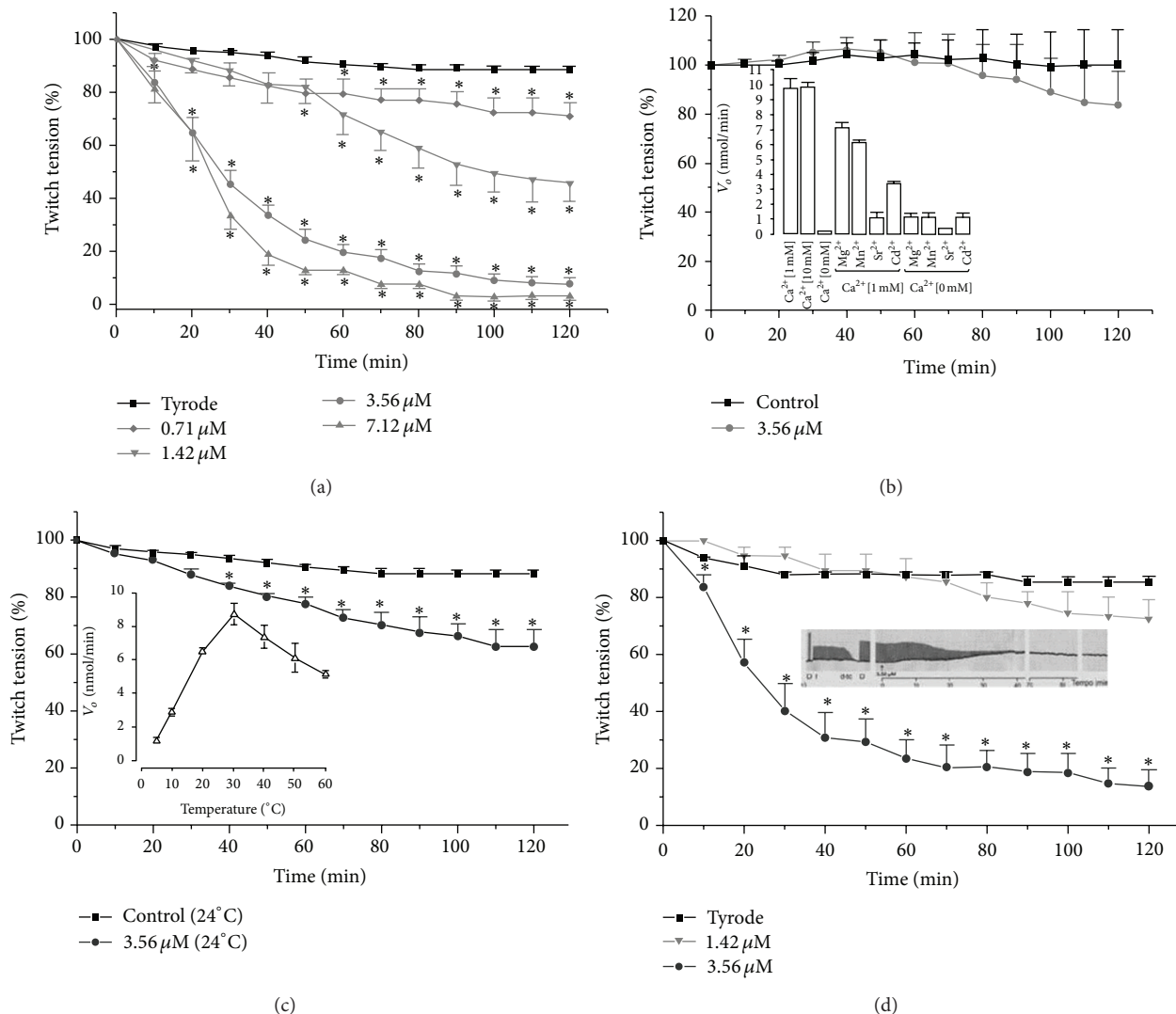


FIGURE 6: Twitch-tension response of direct and indirect stimulated PND preparations for 120 min. (a) The preparations were incubated with Tyrode (control) or Bp-13 PLA₂ (0.71–7.12 μM) at 37°C ($n = 3-6$ experiments); (b) twitch-tension response of PND preparations incubated with Bp-13 PLA₂ in Tyrode 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 μM) 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 μM) 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

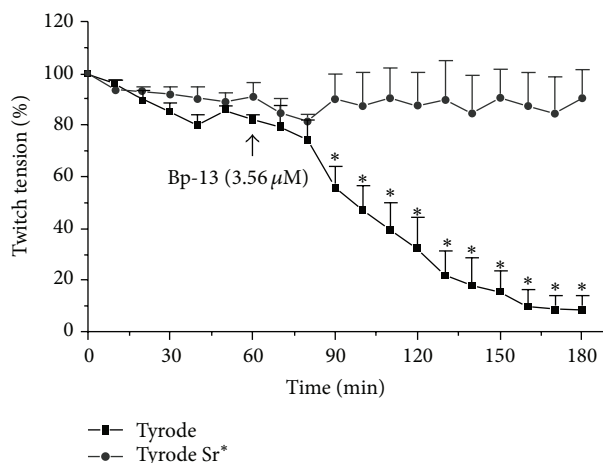


FIGURE 7: Twitch-tension response of indirectly stimulated EDL preparations incubated with $3.56 \mu\text{M}$ of Bp-13 in Tyrode and Tyrode Sr^{2+} . Each point represents the mean \pm SEM of 3–6 experiments; * $P < 0.05$ compared to control values. ANOVA and followed by Mann-Whitney test for comparison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

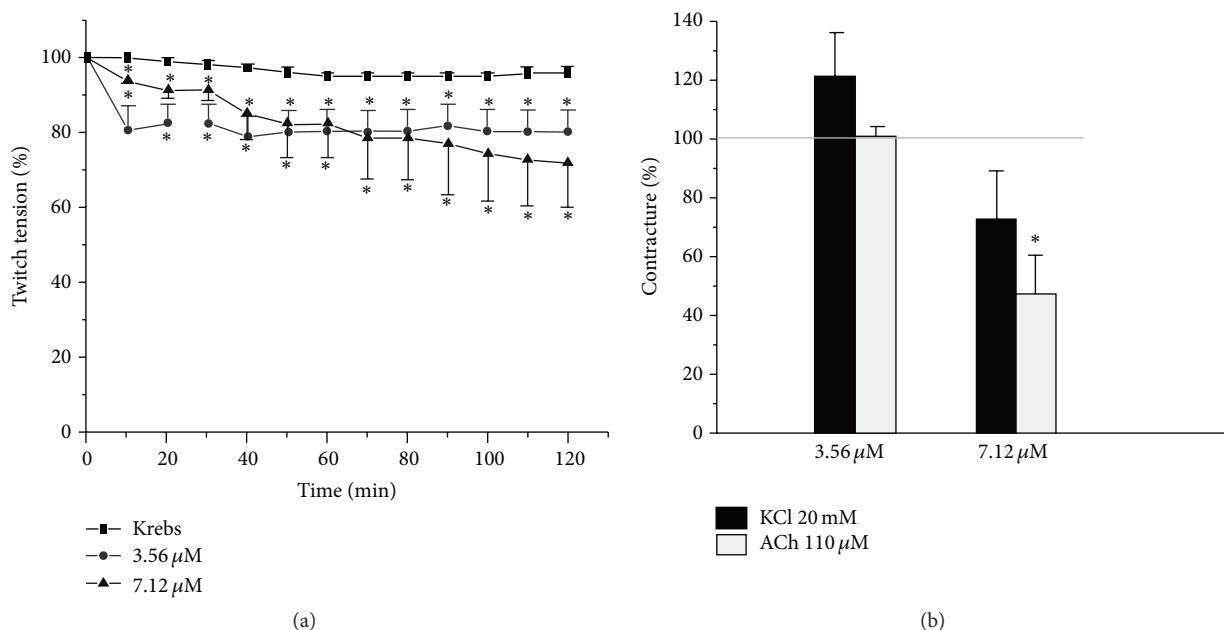


FIGURE 8: Twitch-tension response curve of indirectly stimulated BC preparations incubated with 3.56 and $7.12 \mu\text{M}$ (50 and $100 \mu\text{g}/\text{mL}$, resp.) at 37°C . (a) Each point represents the mean \pm SEM of 3–6 experiments. (b) Percentage of ACh ($110 \mu\text{M}$) and KCl (20mM) evoked 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- PLA_2 , 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 PLA_2 was resistant to heat and acid like PLA_2 s from *Crotalus d. cascavella* [38, 41], *C. d. collilineatus*, 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 PLA_2 from *C. mitchelli pyrhrus* [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 PLA_2 , the activity of Bp-13 PLA_2 was shown to be completely Ca^{2+} -dependent [45]. The coinubation of Bp-13 PLA_2 with other divalent ions (Mg^{2+} , Mn^{2+} , Sr^{2+} , and Cd^{2+}) in the presence of 1mM 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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