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Biochemical Characterization of a PLA2 Btae TX-I Isolated from *Bothriopsis taeniata* Snake Venom: A Pharmacological and Morphological Study

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Abstract

In this research a preliminary identification and biochemical and biological characterization of a PLA_2 (Btae TX-I) from the venom of a viperid snake, *Bothriopsis taeniata* (Speckled forest pit viper) were obtained. Btae TX-I was purified by two chromatographic steps, molecular exclusion chromatography followed by analytical chromatography reverse phase HPLC. Molecular mass behaved as a homogeneous single chain protein on SDS–PAGE, confirmed by MALDI-TOF spectrometry, indicating a molecular mass of 13889.98 Da. Tryptic peptides were determined *in tandem* mass spectrometry and showed similarity with other myotoxic PLA_2s. Btae TX-I belongs to the Asp49 PLA_2 class, is enzymatically active in presence of a synthetic substrate and shows a minimum sigmoidal behavior, reaching its maximal activity at pH 8.0 and 35–45°C. PLA_2 activity in presence of Mn^{2+}, Mg^{2+}, Cd^{2+} and Zn^{2+} was reduced either in presence or absence of Ca^{2+}, suggesting that the arrangement of the catalytic site presents an exclusive structure for Ca^{2+}. Crotalic crotapotins from rattlesnake venom has significantly inhibited ($p<0.05$) the enzymatic activity of Btae TX-I. In ex vivo experiment, Btae TX-I caused partial blockade of the neuromuscular transmission in chick biventer cervicis preparations in a similar way to other *Bothrops* species. Btae TX-I also inhibited contractures in the upper concentration (50 µg) to exogenous KCl (20 mM). Histological analysis of the biventer cervicis incubated with Btae TX-I showed that just the highest Btae TX-I PLA_2 dose (50 µg) caused almost 27.4 ± 0.3% damaged fibers. The results give evidence that the main effect of type Asp49 Btae TX-I PLA_2 from *Bothriopsis taeniata* is at the post-synaptic site.

Keywords: Speckled forest pit viper; *Bothriopsis taeniata* venom; Phospholipase A_2; Histopathological analysis; Neurotoxic activity

Introduction

Phospholipase A_2s (PLA_2s, EC 3.1.1.4) represent a superfamily of lipolytic esterases that hydrolyze the *sn*-2 ester bond of phospholipids releasing free fatty acid and lysophospholipid. Initially, PLA_2 family members were included according with their biochemical characteristics and cellular origin in three main classes, secretory ($sPLA_2$), cytosolic ($cPLA_2$) and intracellular ($iPLA_2$) [1]. At present with the discovery of additional PLA_2s and based on amino acid sequences, 16 distinct groups of PLA_2s were now reported [2,3]. The $sPLA_2$ are the oldest class of PLA2, being also found in prokaryotes [4]. $sPLA_2$ molecular mass ranges from 13 to 19 kDa and typically requires Ca^{2+} at millimolar concentrations for their catalytic activity [5-7]. In eukaryotes, $sPLA_2$s are found in the secretion of glands such as salivary, lacrimal, seminal and exocrine pancreas, as well in glands of snakes, bees and wasps, among others, where they participate in pivotal physiological and pathological functions [8]. Despite sharing primary, secondary and tertiary structures and common catalytic properties, differences in the sequence of amino acids, confer to $sPLA_2$ a wide spectrum of pharmacological effects. The venom of snakes may contain myotoxins that provoke important muscle necrosis at the snakebite site. These myotoxins include small myotoxic peptides that affect Na^+ channels at the sarcolemma or sarcoplasmic reticulum, cardiotoxins or cytotoxic polypeptides present in the venom of elapid snakes and myotoxins with PLA_2 activity or myotoxic PLA_2 [9].

The PLA_2 myotoxins are natural components of a number of snake venoms including Bothropic venoms. They usually are small proteins and peptides that induce either local or systemic necrosis of muscle tissue (rhabdomyolysis), the latter leading to myoglobinuria and acute renal failure and death [10]. Myotoxic PLA_2 can be divided into two broad groups: Asp49 PLA_2 group, which possesses an aspartic acid residue at position 49, and Lys49 PLA_2 group, in which a lysine residue substitutes the aspartic acid at the same 49 position [11,12]. Both types disrupt the integrity of myofibers plasma membrane, by a catalytic-dependent or -independent mechanism, respectively [13].

The present study aimed to isolate and characterize a PLA_2 from the venom of the arboreal *Bothriopsis taeniata* Amazonian snake (*Viperidae*), popularly known as speckled forest pit-viper. In addition, it was sought to investigate if the isolated PLA_2 possesses neurotoxic activity as well to evaluate its myotoxicity on *in vitro* avian nerve-muscle preparation by using pharmacological and morphological approaches.

Herein, we showed for the first time an Asp-49 PLA_2 isolated from *B. taeniata* venom, called as Btae TX-I (*Botrhiopsis taeniata* Toxin-I), which looks like a post-synaptic-acting myotoxin whose myotoxic activity was significant just when 50 µg of toxin was used.

Materials and Methods

Venom, chemicals and drugs

Bothriopsis taeniata venom was a pool obtained by manual compression of the venom glands. All other chemicals and reagents used in this work were of analytical or sequencing grade, and were purchased from Sigma-Aldrich Corporation (St. Louis, MO. USA).

Animals

The Animal Services Unit of the State University of Campinas (UNICAMP) supplied male chicks (4–8 days old, HY-LINE W36 lineage). Animals were housed at 25°C on a 12 h light/dark cycle and had free access to food and water. All experiments described here were done with approved protocol number 1492-1, for the use of animals in research, conducted in accordance with guidelines established by the Ethics Committee of the Biology Institute in the Animals Use - UNICAMP (CEUA, COBEA).

Biochemical Studies

Isolation and purification of phospholipase A2: $sPLA_2$ was purified after two chromatographic steps. The first step was conducted using molecular exclusion chromatography followed by reverse phase HPLC (RP-HPLC) as described by Ponce-Soto et al. [14] and Bonfim et al. [15]. Approximately 50 mg of *Bothriopsis taeniata* venom was dissolved in 1 mL of 1 M $(NH_4)_2CO_3$ buffer, pH 8, homogenized in the sonic bath, clarified by centrifugation at 9,000 rpm for 5 min, and applied to a Kontex Flex column (78x2 cm) of Sephadex G-75, which had been pre-equilibrated with the same buffer (0.2 M). The proteins were eluted at a flow rate of 0.25 ml/min and the chromatographic profile was determined by measuring absorbance at A280 nm. The fraction corresponding to main peaks were pooled, lyophilized and stored at -20°C. Five milligrams of the fraction containing PLA_2 activity were dissolved in 120 µl of 0.1% TFA (buffer A) until complete dissolution. The supernatant was then applied to an analytical reverse phase HPLC column, Shim Pack CLC-ODS C18 (4.6 mmx25 cm). Protein elution was carried out using a linear gradient (0-100%) of buffer B (66.5% of acetonitrile in 0.1% TFA) at a constant flow rate of 1 ml/min. The chromatographic run was monitored at 280 nm, and the fractions obtained were then collected, lyophilized and stored at -20°C.

The degree of purity of the PLA_2 isoform was assessed by Tricine SDS–PAGE in a discontinuous gel and a buffer system was used to estimate the molecular mass of the proteins, under reducing and non-reducing conditions [16].

Protein determination by the Bradford method (Comassie blue): Protein content of the fractions was determined using bovine gamma globulin as a standard by the Coomassie blue method [17]. The absorbances reading were made in wavelength of 595 nm in reader ELISA Versa Max microplate reader (Molecular Devices, Sunnyvale, CA, USA). These calculated protein concentrations, performed in triplicate for each fraction sample, were the basis of other analyses.

Phospholipase A2 activity: PLA2 activity was measured using the assay described by Cho and Kézdy [18] and Holzer and Mackessy [19] modified for 96-well plates by Beghini et al. [20] using 20 µl of 4-nitro-3-octanoyloxy-benzoic acid as a substrate (Biomol, USA), 200 µl of buffer (10 mM Tris-HCl, 10 mM $CaCl_2$, 100 mM NaCl, pH 8.0), 20 µl of water and 20 µl of PLA_2 in a final volume of 260 µl. After the addition of PLA_2 (20 µg), the mixture was incubated for 40 min at 37°C, and the absorbance was checked with 10 min intervals. Enzyme activity, expressed as the initial velocity of the reaction (V_o), was calculated based on the absorbance increase after 20 min.

Btae TX-I obtained from RP-HPLC was determined by studying the kinetic parameters. The effect of substrate concentration (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 of incubation in 10 mM Tris-HCl buffer, pH 8.0, at 37°C. The optimum pH and temperature of the PLA2 were determined by incubating the enzyme in buffers (10 mM citrate, 10 mM phosphate, 10 mM Tris and 10 mM glycine) of different pH values (4 to 10) and in Tris-HCl buffer, pH 8.0, at different temperatures (25 to 45°C), respectively.

The inhibition of PLA_2 activity by crotapotins from *Crotalus durissus terrificus* (CdtF5 and CdtF7) and *C. d. colillineatus* (CdcolF3 and CdcolF4) were determined by incubating both proteins (Btae TX-I : crotapotins) for 30 min at 37°C prior assaying the residual enzyme activity at 425 nm.

Determination of the molecular mass of the purified protein by mass spectrometry: Purified lyophilized protein Btae TX-I PLA_2 from RP-HPLC was resuspended in 8 M urea containing 10 mM DTT at pH 8.0 and the disulfide bridges were then reduced by incubation at 37°C for 2 h. Iodoacetamide (IAA) was used to alkylate the free thiols of cysteine residues, based on previous experiments, a 30% molar excess of iodoacetamide relative to the total number of thiols was eventually chosen and the mixture was incubated for 1.5 h at 37°C in the dark. The reaction was stopped by injecting the mixture onto a RP-HPLC column followed by lyophilization of the collected peak.

The molecular mass of intact native and alkylated Btae TX-I PLA_2 were 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) and 1 µL of sample in 0.1% TFA was mixed with 2 µL of the matrix sinapinic acid (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: accelerate voltage of 25 kV, the laser fixed in 2890 µJ/com^2, delay 300 ns, and linear analysis mode [21].

Electrospray ionization mass spectrometry (ESI-CID-MS/MS) analyses were performed using a quadrupole-time of flight (Q-TOF) hybrid mass spectrometer Q-TOF Ultima from Micromass (Manchester, UK) equipped with a nano Zspray source operating in a positive ion mode. The ionization conditions of usage included a capillary voltage of 2.3 kV, a cone voltage and RF1 lens of 30 V and 100 V, respectively, and collision energy of 10 eV. The source temperature was 70°C and the cone gas was N2 at a flow of 80 l/h; nebulizing gas was not used to obtain the sprays. Argon was used for collisional cooling and for fragmentation of ions in the collision cell. External calibration with sodium iodide was made over a mass range from 50 to 3000 m/z. All spectra were acquired with the TOF analyzer in "Vmode" (TOF kV=9.1) and the MCP voltage set at 2150 V [22].

Identification of tryptic digests: The protein was reduced with 5 mM DTT and alkylated with 14 mM IAA prior to addition of trypsin (Promega-Sequence Grade Modified). After trypsin addition (20 ng/µl in 0.05 M Ambic), the sample was incubated for 16 h at 37°C. To stop the reaction, 0.4% formic acid was added and the sample centrifuged at 2,500 rpm for 10 min. The pellet was discarded and the supernatant dried in a speed vac. The resulting peptides were separated by C18

(100 µm x 100 mm) RP-UPLC (nanoAcquity UPLC, Waters) coupled with nano-electrospray tandem mass spectrometry on a Q-Tof Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nl/min. 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, in order to select the ion of interest. Subsequently, these ions were fragmented in the collision cell (TOF MS/MS mode).

Raw data files from LC-MS/MS runs were processed using MASSlynx 4.1 software package (Waters) and analyzed using the Mascot search engine version 2.3 (Matrix Science Ltd) against the NCBI-BLAST database, using the following parameters: peptide mass tolerance of ±0.1 Da, fragment mass tolerance of ± 0.1 Da, oxidation as variable modifications in methionine and trypsin as enzyme.

Pharmacological assay

Neurotoxic activity: Chick biventer cervicis muscle preparation (BC): Male young chicks (4–8 days old, HY-LINE W36 lineage, n=5) were anesthetized and killed by halothane inhalation and *biventer cervicis* muscles were removed and mounted [23] under a tension of 1 g in a 5 ml organ bath at 37°C (Automatic organ multiple-bath LE01 Letica Scientific Instruments, Barcelona, Spain), containing carbogen-aerated (95% O_2 and 5% CO_2) Krebs solution. Contractures to exogenously applied acetylcholine (73.3 µM ACh for 60 s) and KCl (20 mM for 180 s) were recorded in the absence of field stimulation, prior to the addition of different doses (5, 10, 20 and 50 µg/ml) of Btae TX-I PLA_2 and at the end of the experiment. A bipolar platinum ring electrode was placed around the tendon in which runs the nerve trunk supplying the muscle. Indirect stimulation (0.1 Hz, 0.2 ms, 5-6 V) was performed with a Grass S48 stimulator (Powerlab AD Instruments, Barcelona, Spain). Muscle contractions and contractures were isometrically recorded via a force-displacement transducer (Model MLT0201 Force transducer 5 mg-25 g Panlab s.l. AD Instruments Pty Ltd, Barcelona, Spain) connected to a Power Lab/4SP (OUAD Bridge AD Instruments, Barcelona, Spain).

Myotoxic Activity: Morphological and morphometric analyses: After 120 minutes of Btae TX-I incubation (10 and 50 µg), biventer cervicis muscles (n=5/concentration) were immersed for 24 h in 4% paraformaldehyde, washed three times with saline solution, dehydrated in increasing ethanol concentration series (70, 80, 95 and 100%, v/v), clarified in xylene and embedded in paraffin. Sections (5 µm thick) obtained using a Leica RM2035 microtome were stained with hematoxylin-eosin (HE) and analyzed with a Olympus BX51 microscope equipped with image analysis software (Image ProPlus 6.0, Media Cybernetics, Inc.). Control preparations were prepared from muscle incubated with Krebs solution. The extent of damage in control and treated muscles was assessed by counting the total number of normal and damaged fibers per histological section and then expressing the number of damaged fibers as a percentage of the total number of fibers counted. Normal fibers were defined as those with a polygonal appearance, peripheral nucleus and evenly distributed myofibrils.

Statistics

Results were reported as mean±SEM. Statistical comparisons were done using ANOVA followed by Tukey-Kramer test. Values of $p<0.05$ indicated significance.

Results

Purification and biochemical characterization of the Btae TX-I PLA_2

Fractionation of crude *B. taeniata* venom on Sephadex G-75 at pH 7.8 resulted in four main peaks (I-IV; Figure 1A). The whole venom, with fractions detected by molecular exclusion, was monitored for phospholipasic activity on specific chromogenic substrate and peak III showed PLA2 activities (12.075 ± 0.138 nmoles/min/mg). Subsequently, the peak III was pooled, dialyzed, lyophilized and fractionated in an analytical shim-pack CLC-ODS (C18) column (4.6 mm x 25 cm x 0.5 µm) by RP-HPLC and resulted in the purification of three well-defined peaks (III-1 to III-3), followed by several smaller peaks (Figure 1B).

Step	Volume (ml)	Protein (mg/ml)	Activity (U/ml)	Total Activity (U.T.)	Specific Activity (U/mg)	Recovery (%)	Purification
Whole Venom	12	0.1966	109.26	1311.0	555.85	100.00	1.00
Sephadex G-75 (peak III)	3.6	0.0144	284.39	1023.8	19746.97	78.08	35.53
HPLC-FR (Btae TX-I)	1	0.0151	772.30	772.3	51303.31	58.90	92.30

Table 1: Parameters of Btae TX-I purification.

The purification procedures of PLA_2 Btae TX-I are summarized in Table 1 and this purified protein was selected for further biochemical and pharmacological characterization. The crude venom had catalytic activity of 2.185 ± 0.078 nmol/min/mg, which increased to 5.668 ± 0.091 mol/min/mg for peak III (Sephadex G75), resulting in a yield of 78.08%, with a purification factor of 35.53. The second chromatographic step (RP-HPLC) yielded 58.90% in relation to the whole venom with purified factor of 92.30.

After SDS-PAGE analysis, the enzyme showed a single chain with molecular mass (Mr) around 14 kDa, under reducing (1M DTT) and no reducing conditions, suggesting that it is a monomeric protein (Figure 2). The Btae TX-I homogeneity was confirmed by MALDI-TOF mass spectrometry analysis, to be determined an intact molecular weight of 13,889.98 Da, as well as, an exact molecular mass in reduced and alkylated samples of 14,701.98 Da (Figure 3A).

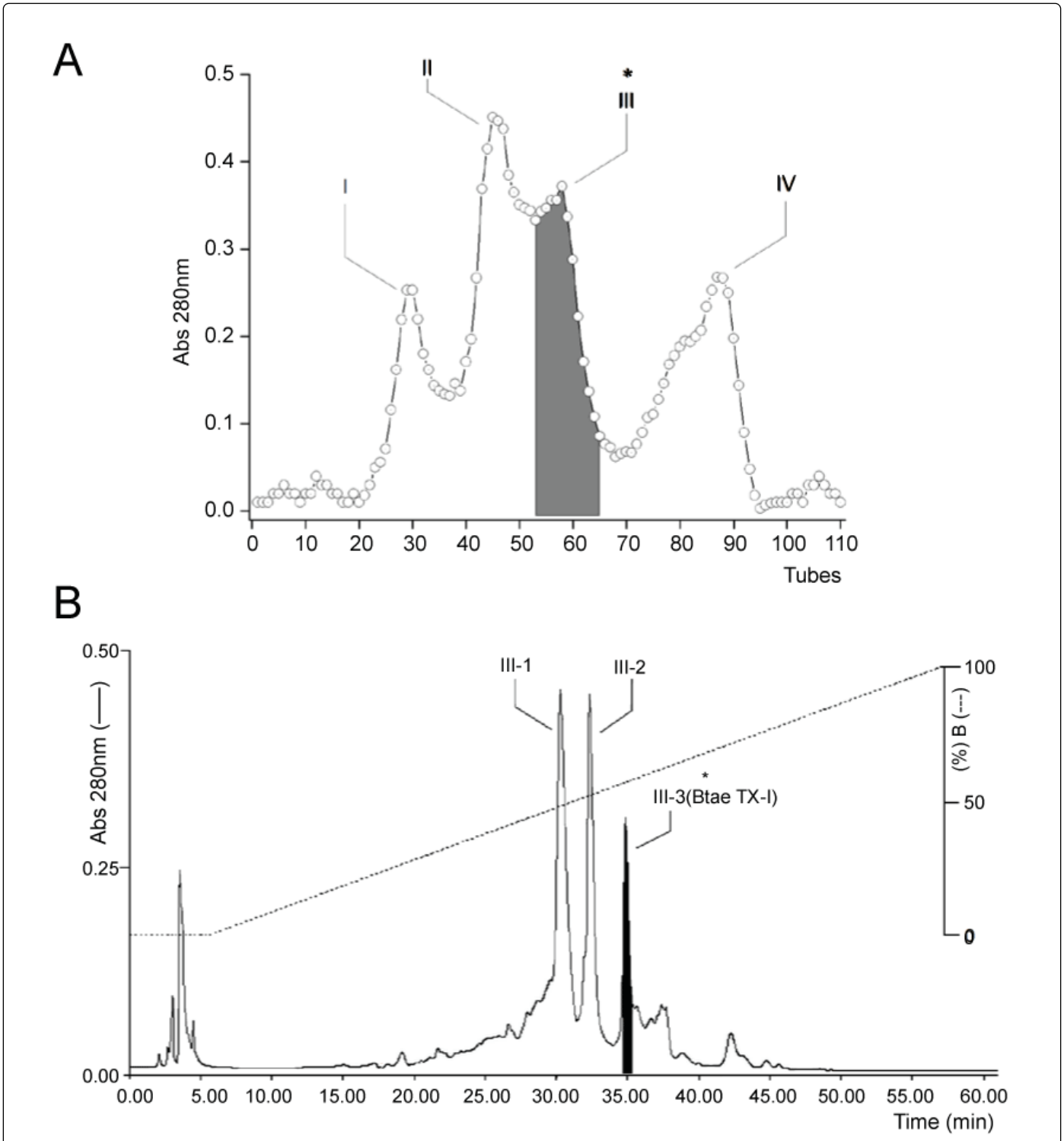

Figure 1: (A) Elution profile of *Bothriopsis taeniata* venom by molecular exclusion chromatography on a Sephadex G-75 column (Kontex Flex Column 78x2 cm). Fraction III contained PLA_2 activity. (B). Elution profile of peak III following RP-HPLC on a reversed-phase column, shim-pack CLC-ODS (C18), 4.6 mmx25 cmx0.5 µm. The peak corresponding to the phospholipase A_2 (Btae TX-I) from *Bothriopsis taeniata* venom is indicated (*).

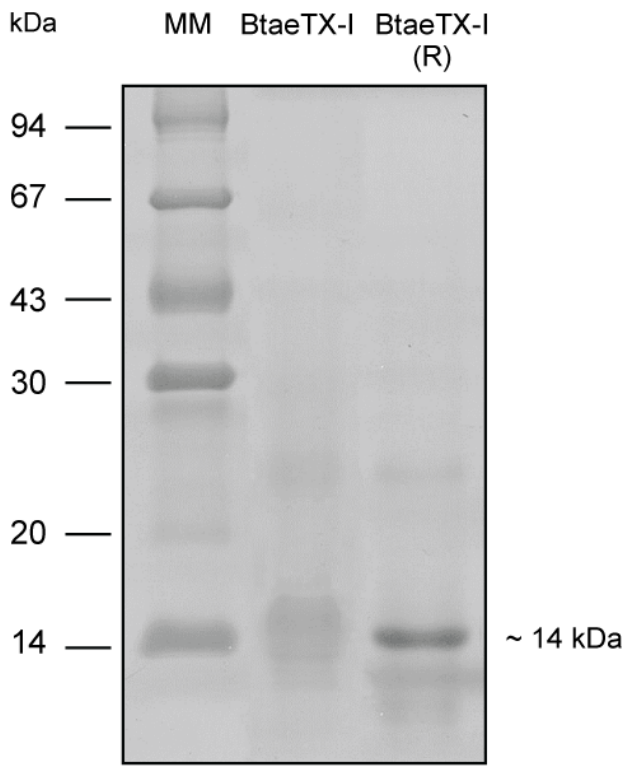

Figure 2: Electrophoretic profile of Btae TX-I protein (PLA2) by SDS-PAGE (12.5% gel). Lane 1. Molecular mass markers (MM); Lane 2. Btae TX-I not reduced (NR); Lane 3. Btae TX-I reduced with 1M DTT (R). Protein standards and their molecular weights are: phosphorylase b–94, albumin–67, ovalbumin–43, carbonic anhydrase–30, trypsin inhibitor–20.1, α-lactalbumin–14.4.

Additionally the amino acid composition determined was: N,D/11; Q,E/12; S/2; G/12; H/2; R/4; T/8; A/6; P/7; Y/10; V/4; M/2; C/14; I/5; L/6; F/4; K/13; W/not determined. This analysis revealed a high content of Lys, Tyr, Gly, Thr, and 14 half-Cys residues-typical of a basic PLA2 protein. The basic amino acids (His, Lys and Arg) represent 20.74% of the total amino acids of Btae TX-I. Also, this enzyme possesses 41.48% of hydrophobic amino acids.

Identification of tryptic peptides from Btae TX-I PLA_2 by ESI-MS/MS

Alkylated Btae TX-I PLA_2 was digested with trypsin and its tryptic peptides were fractionated by RP-HPLC. All tryptic digests submitted to the analysis in electrospray ionization-tandem mass spectrometry (ESI-MS/MS) were searched using the Mascot MS/MS Ion Search software (www.matrixscience.com). Table 2 shows some of these alkylated peptides with their deduced sequences and measured masses. Isoleucine and leucine residues were not discriminated in any of the sequences reported since they were indistinguishable in low energy CID spectra. Because of the external calibration applied to all spectra, it was also not possible to resolve the 0.036 Da difference between glutamine and lysine residues, except for the lysine that was deduced based on the cleavage and missed cleavage of the enzyme. Each of the peptides identified from Btae TX-I PLA_2 were submitted to NCBI database search, using the program BLAST-p with a search restricted to sequenced proteins from the basic phospholipase A_2 family.

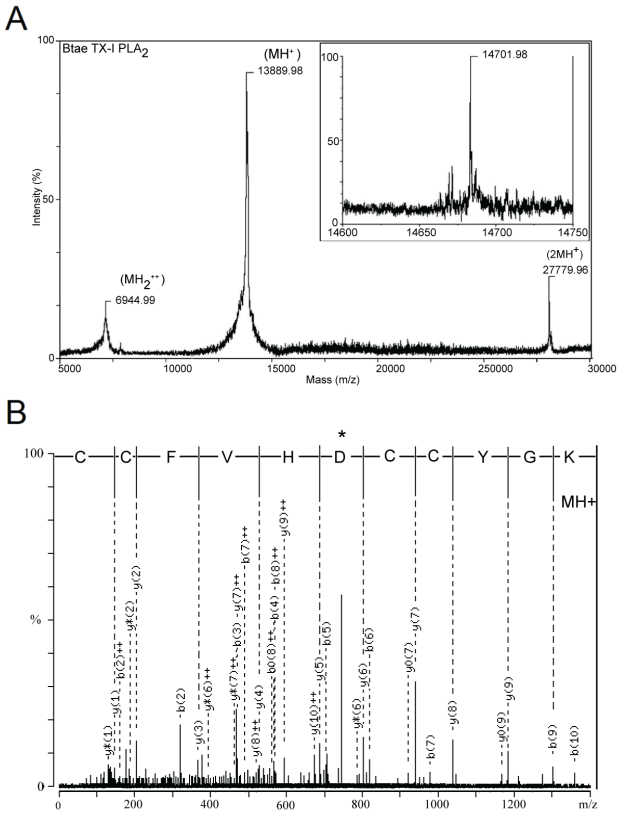

Figure 3: (A). Mass determination of the native Btae TX-I PLA_2 by MALDI-TOF mass spectrometry. The MH^+, MH^{2++} and 2MH+ species are shown in the mass spectrum. Insert MALDI-TOF mass spectrum, showing multiple alkylation channels of alkylated Btae TX-I. (B). ESI-QTOF-MS/MS spectrum of the tryptic peptides of 1504.5356 Da. Series of b and y ions, which are correspondent to the 11-residue-long tryptic peptide eluted in fraction 3 of the RP-HPLC of the alkylated Btae TX-I containing the aspartic acid residue at position 49 in the amino acid sequence.

The *tandem mass* spectra shown in Figure 3B, relative to the peptide 3 of the digest (sequence C C F V H D C C Y G K), allows classifying the protein as an Asp49 PLA_2. A comparison of the sequence of Btae TX-I deduced peptides with others myotoxic PLA_2s from *Bothrops* genus was shown. Sequences of the other peptides would indicate that they are part of regions highly conserved as well as also of variable regions. Such conserved residues are involved in the binding of Ca^{2+} (Tyr28, Gly30, Gly32 and Asp49) and the His48 residue, a key component of the active site, is also conserved in Btae TX-I (Figure 4). Furthermore, a majority of residues involved in the formation of a hydrophobic channel (Leu2, Phe5, and Ile9) are also conserved in Btae TX-I.

Btae TX-I (PLA_2)			
HPLC Fraction	Measured Mass (Da)	Amino acid sequence	Theorical Mass (Da)
1	1377.7115	DL/IWQ/KFGQ/KMI/LL/IK/Q	1377.6687
2	2599.1301	L/ IPFPYYTTYGCYCGWGGQ/KGQ/KPK/Q	2599.1233
3	1504.5356	CCFVHDCCYGK/Q	1504.4954
4	1661.7389	ENGVI/LI/LCGEGTPCEK/Q	1661.6716
5	951.3790	Q/KI/LCECDK/Q	951.3886
6	793.3905	AAAVCFR	793.3890
7	1414.6737	RYMAYPDVL/ICK/Q	1414.6905
8	975.4549	I/LDSYTYSK/Q	975.4623

Table 2: Measured molecular masses and deduced amino acid sequences obtained by ESI-MS/MS based on the alkylated tryptic peptides of Btae TX-I. The peptides were separated by RP-HPLC and sequenced by mass spectrometry. C = alkylated cysteine, lysine residues shown in bold were deduced on the cleavage and missed cleavage by trypsin. All molecular masses are reported as monoisotopic.

Enzymatic characterization of Btae TX-I

The activity of Btae TX-I was examined using the synthetic substrate 4-nitro-3-(octanoyloxy) benzoic acid. Btae TX-I showed to be a typical PLA_2, since it hydrolyzes synthetic substrates at position 2. Under the conditions used, this enzyme showed classic Michaelis–Menten kinetics with a discrete sigmoidal behavior, mainly at low substrate concentrations (Figure 5A).

Kinetic analysis of PLA_2 activity at different concentrations of 4N3OBA showed apparent Michaelis-Menten constant (*Km*) value of 0.168 mM and apparent maximum velocity (*Vmax*) value of 0.115 nmol/min (Figure 5B), and optimum pH set at 8.0 (Figure 5C). Maximum enzyme activity occurred between 35°C and 45°C, temperature. At 25°C, the enzyme showed decreased activity relative to first ones (Figure 5D). The *in vitro* phospholipase activity of Btae TX-I at equimolar ratios with each of crotalic crotapotins from *C. d. terrificus* and *C. d. collilineatus* was inhibited around 65-70% (Figure 5E). The PLA2 activity required Ca^{2+}, at 1 and 10 mM concentrations (Figure 5F). The substitution of Ca^{2+} by other cations (10 mM) alone or in presence of Ca^{2+} 1 mM reduced the enzymatic activity. However, when Mg^{2+} (10 mM) was added in presence of Ca^{2+} (1 mM) it did not reduce significantly the activity of Btae TX-I PLA_2 (Figure 5F).

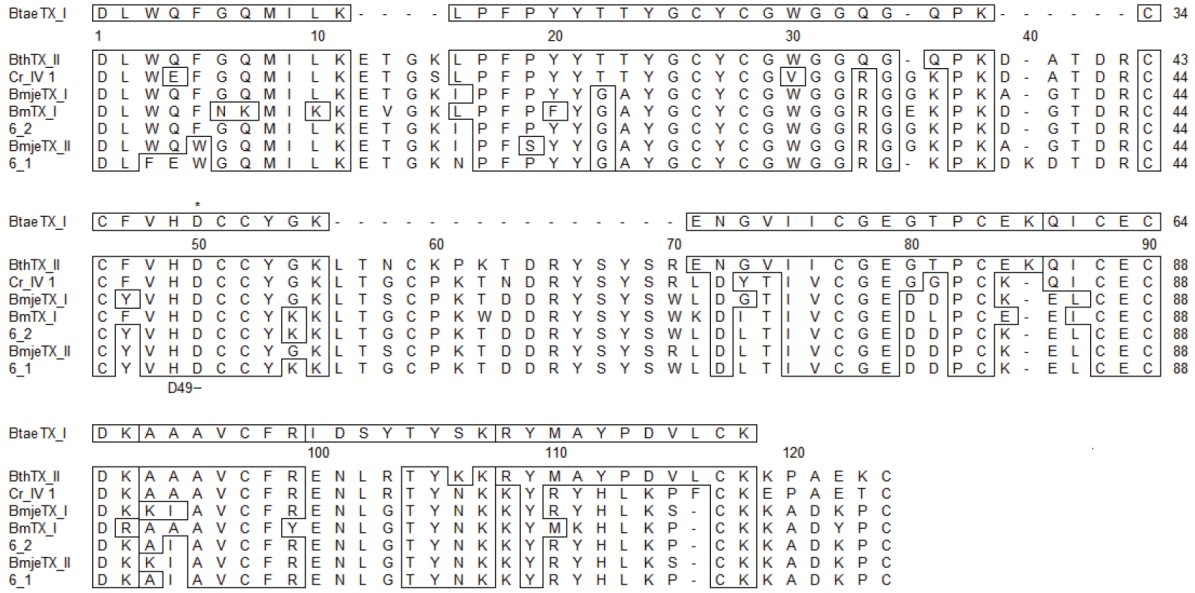

Figure 4: Comparison of the obtained amino acid sequence from tryptic peptides of the Btae TX-I with others Asp49 PLA_2. BthTX-II of bothropstoxin II from *Bothrops jararacussu* [50], Cr-IV-1 from *Calloselasma rodhostoma* [51], PLA_2 isoforms (6-1 and 6-2) of the fraction BthTX-II from *Bothrops jararacuçu* [21], PLA_2 isoforms (BmjeTX-I and BmjeTX-II) from *Bothrops marajoensis* [34] and PLA_2 (BmTX-I) from *Bothrops moojeni* [31]. The spaces are inserted in the sequences to reach the maxim homology.

Pharmacological characterization of the Btae TX-I PLA2

Neurotoxic *in vitro* activity was tested in isolated chick *biventer cervicis* preparation indirectly stimulated. Btae TX-I concentrations of 5, 10, 20 and 50 µg/mL produced a slow and discreet decrease in the twitch muscle responses (Figure 6A) and induced partial neuromuscular blockade at 120 min incubation even using the highest concentration. The time required for Btae TX-I to cause 50% blockade was 103.6 ± 2 min and 85.5 ± 2.5 min at 10-20 µg/mL and 50 µg/mL concentrations, respectively. Five µg/mL of toxin concentration was

ineffective in inducing 50% blockade within 120 min incubation (data not shown).

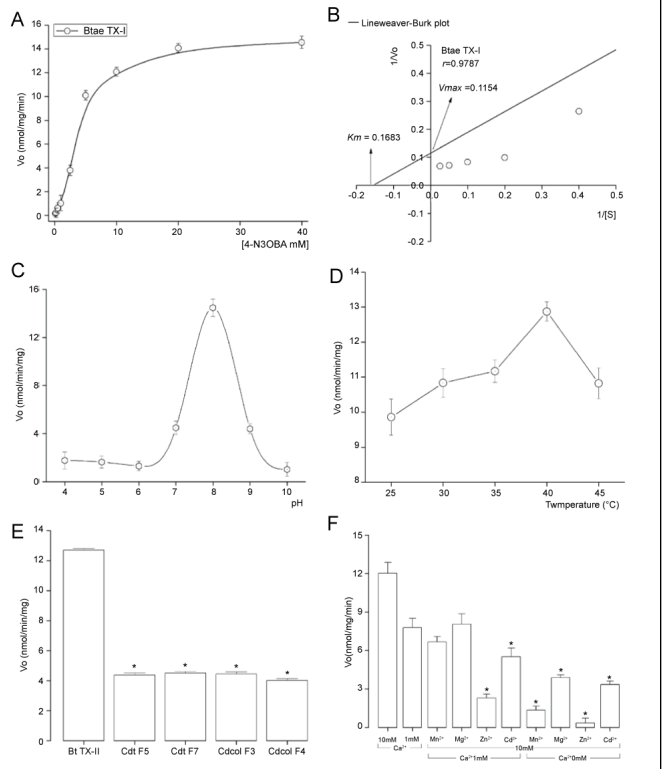

Figure 5: (A) Effect of substrate concentration on the kinetics of Btae TX-I PLA_2 activity. The graph showed a curvilinear shape at low substrate concentration. (B) Line weaver Burk's plot of Btae TX-I. (C) Effect of pH on Btae TX-I activity. (D) Effect of temperature on the PLA_2 activity of Btae TX-I. (E) Inhibition of Btae TX-I activity by crotapotin (CdtF5, CdtF7, CdcolF3 and CdcolF4) isolated from *C. durissus terrificus* and *C. durissus collilineatus* venom. (F) Influence of ions (10 mM each) on PLA_2 activity in the absence or presence of 1 mM Ca^{2+} compared to Btae TX-I activity in the presence of 10 mM Ca^{2+}. The results of all experiments are the mean ± S.E. of three determinations ($p<0.05$).

The toxin showed a discreet action on the nicotinic receptors since the contractures induced by ACh were reduced in just 1 ± 6.1% and 13 ± 4.7% with 10 µg/mL and 50 µg/mL concentrations, respectively after 120 min (n=5/concentration; $p<0.05$).

Myotoxicity: KCl and histopathological analysis

The criteria used to determine the myotoxicity was through KCl-induced contractures and by counting the number of degenerating muscle fibers against the number of normal-looking fibers. The KCl-induced contractures were reduced in 37.8 ± 3% and 59.7 ± 5.8% at 10 µg/mL and 50 µg/mL Btae TX-I concentrations, respectively. Control preparations showed contracture induced by ACh and KCl stable after the experiments (Figure 6B).

Morphologically, fibers considered normal were those presenting polygonal cross-sectional profile, peripheral nuclei, homogeneous myofibrils distribution inside the sarcoplasm and continuous sarcolemma. Biventer cervicis muscle incubated with Krebs solution showed 1 ± 0.6% of altered fibers. BC incubated with 10 µg/mL Btae TX-I PLA2 showed 6±3.4% of altered fibers which showed to be statistically equal to the number found in controls. However, Btae TX-I at 50 µg/mL concentration caused a 27.4±0.3% of damaged fibers, which was significantly different from both Krebs solution- and 10 µg/mL toxin-incubated BC. Muscles treated with 50 µg/mL Btae TX-I presented fibers with different pathologic states, the more frequent of which being ghost fibers (when myofibrils and sarcolemma were lysed and only the basement membrane persisted) or damaged fibers (when part of the fibers were lysed) (Figures 7,8).

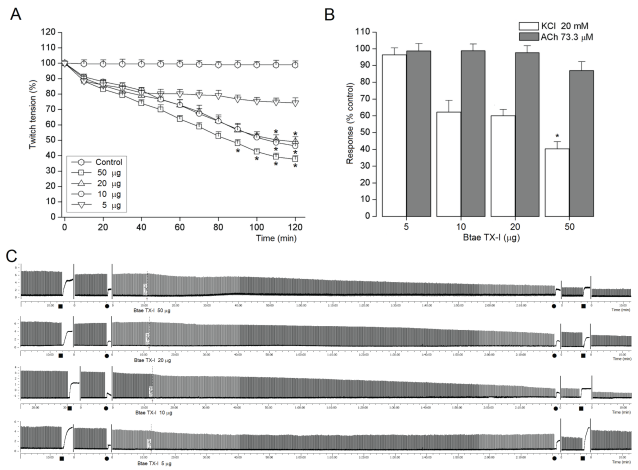

Figure 6: (A) Twitch-tension responses of indirectly stimulated chick *biventer cervicis* preparation. The preparations were incubated with Btae TX-I PLA2 (5–50 µg) active fraction, for 120 min at 37°C. (B) Representatives myographic profiles showing the muscle response to indirectly stimulated (0.1 Hz, 0.2 ms) muscle preparation and contractures in response to exogenous ACh (●) and KCl (▪). Btae TX-I in different concentrations were added at t=0 and left in contact with the tissue for the duration of the experiment. Each point represents the average from five experiments ± SEM; $p < 0.05$ compared with control.

Discussion

The characterization of snake venom containing-$sPLA_2$ is of importance because the enzyme is the major responsible for local and systemic degeneration and inflammation of skeletal muscle tissue [24]. Apart from that, the biochemical characterization of the molecule is important not only for evolutionary purposes but also in relation to structure-function studies. Although significant progress has been made to characterize the structural basis related with the diverse PLA_2 toxic activities, further researches are required. In the present study, we report for the first time a biochemical and pharmacological characterization of a PLA_2 isolated from the venom of *B. taeniata*, named as Btae TX-I. The enzyme was isolated through two chromatographic steps: in column of molecular exclusion and hydrophobic column coupled to a system of reverse phase HPLC. This methodological combination was efficient to preserve the selectivity, resolution capacity, high degree of molecular homogeneity and biological activity [25,26].

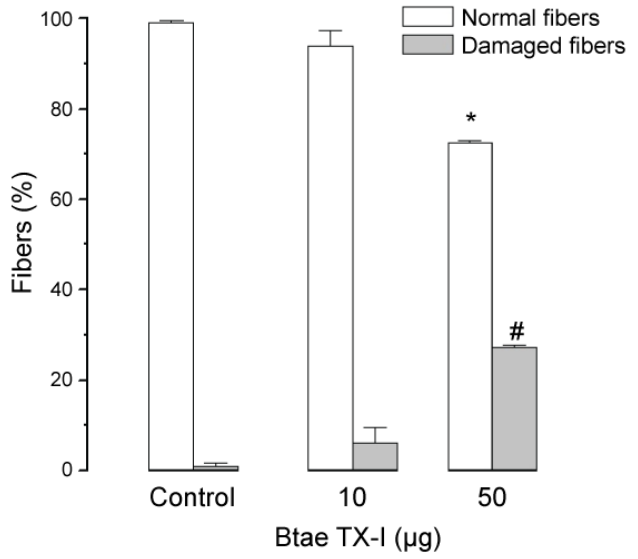

Figure 7: Percentage values of damaged and normal fibers in the whole area of the transverse section of *biventer cervicis* muscle control and treated with the concentrations of 10 µg and 50 µg Btae TX-I; *: $p < 0.05$ in relation to control; #: $p < 0.05$ in relation to 10 µg.

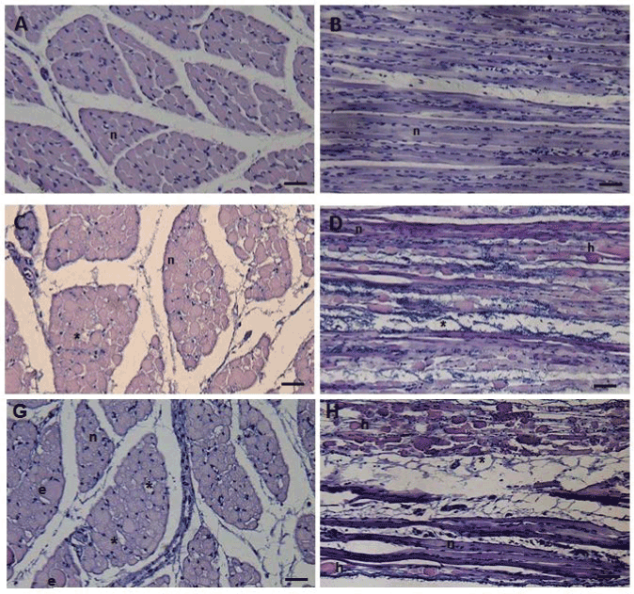

Figure 8: Transverse (A) and longitudinal section (B) of biventer cervicis muscle treated with Krebs solution (Control) showing normal (n) morphology of the muscular fibers. Transverse (C) and longitudinal section (D) of biventer cervicis muscle treated with 10 µg of Btae TX-I and 50 µg Btae TX-I (E) and (F), respectively. Observe normal fibers (n) between edematous (e), discontinuous or hipercontracted (h) and ghost fibers (*). Bar= 50 µm.

A variety of PLA2s isolated from *Bothrops sp* venoms are oligomers constituted by two or more subunits [27]. Nevertheless, SDS-PAGE under non-reducing conditions showed Btae TX-I running as a monomer and possessing a single polypeptidic chain of ~14 kDa (13,889.89 Da) after reduction, what was confirmed by MALDI-TOF mass spectrometry. Such configuration of Btae TX-I is similar to other basic Asp49 PLA_2s that also have monomeric structure like the PhTX-I from *Porthidium hyoprora* [28], blD-PLA_2 from *Bothrops leucurus* [29] and LmTX-I from *Lachesis muta muta* venoms [22]. The amino acid composition of the toxin revealed a high content of basic and hydrophobic residues, with 14 half-Cys, in agreement with the reported composition and primary structure of PLA_2 myotoxins isolated from *Bothrops* venoms [21,30,31]. In effect, the amino acid composition of Btae TX-I PLA_2 revealed the presence of 14 half-Cys residues so providing the basis for a common structural feature of PLA_2 in the formation of its seven disulfide bridges [30,32-34].

The high stability of snake venom PLA_2s, including Btae TX-I, is probably due to the relatively small molecular size of these proteins (121 amino acid residues), the presence of disulfide bridges and a high content of basic and hydrophobic residues. Such structural characteristics make this protein a compact and highly denaturation resistant molecule. PLA_2 remains active through a large temperature range as demonstrated by its highest activity at temperatures as high as 37°C and with optimum activity at pH 8.0. Temperature is another kinetic parameter utilized to characterize the Asp49 PLA_2. It has been shown that PLA_2 from *Naja naja naja* is very stable in extreme temperatures such as 100°C [35].

Together, these characteristics constitute the basis for the successful interaction between basic PLA_2 and negatively-charged phospholipids of cell membranes [36]. Such interaction is important to explain the hydrolyzing effect of these enzymes on different cell membrane types [37].

The effect of bivalent cations (Mn^{2+}, Mg^{2+}, Zn^{2+}, and Cd^{2+}) on the enzymatic activity of Btae TX-I revealed that the Ca^{2+} is an obligatory co-factor for PLA_2 catalysis role since its replacement prevents substrate binding to the enzyme. Studies have shown that is the presence of the Ca^{2+} which determines the electrophilic behavior of the catalytic site, as well as stabilizes the otherwise flexible Ca^{2+}-binding loop and appears to optimize the interaction enzyme-substrate [38-40].

Crotapotins (CA), the non-toxic subunit of *Crotalus* venom, are pharmacologically inactive and non-enzymatic acid protein; the toxin forms a non-covalent association with PLA_2 crotoxin complex (CB, toxic subunit). Crotapotins bind specifically to PLA_2 as a natural inhibitor of catalytic activity [41]. We suggest that crotapotin might interact in a less stable way, thus partially avoiding the substrate access to the catalytic site and hiding several key amino acid residues involved in the interfacial binding surface of PLA_2s. Crotapotin isoforms from *Crotalus d. collilineatus* (Cdcol F3 and Cdcol F4) and *Crotalus d. terrificus* (CdtF5 and Cdt F7) significantly inhibit the Btae TX-I activity by approximately 60%. Our results are in agreement with findings of Landucci and co-workers [42], who reported that highly purified crotapotin can inhibit pancreatic, bee, and other snake venom PLA2, and Bonfim et al. [15] and Calgarotto et al. [31], who reported that crotapotins from *C. d. terrificus* (F7), *C. d. cascavella* (F3 and F4) and *C. d. collilineatus* (F3 and F4) decreased by 50% the catalytic activity of BJ IV PLA_2 from *B. jararacussu* and BmTX-I PLA_2 from *B. moojeni* snake venoms. Together, these results suggest that crotapotin

may bind to bothropic PLA_2 in a manner similar to that from crotalic PLA_2.

Although accidents caused by *Bothrops venoms* show non visible clinical signs of neurotoxicity, experimentally a number of *in vivo* or *in vitro* studies have shown that motor nerve fibers, nerve terminals or nicotinic receptor can be affected by Bothropic venoms [43]. Venom of diverse Bothrops snakes abolishes contractions caused by direct and indirect electrical stimulation of skeletal muscle in mouse [44-46]. Herein, we found that Btae TX-I PLA_2 causes discreet and slow concentration-dependent decrease of elicited twitch muscle responses in the chick *biventer cervicis* preparation. Btae TX-I PLA_2 low concentration did not interfere with the muscle contractures to exogenous ACh. Also, only a slight decrease was observed with 10 µg/mL toxin concentration, indicating that the toxin had a minor action on nicotinic receptors. In fact, the blockade of the contracture induced by ACh is compatible with the neuromuscular blocking effect produced by the toxin at the concentrations used. However, 10 µg/mL Btae TX-I inhibited the contracture responses to KCl suggesting that Btae TX-I PLA_2 presents specially a post-synaptic action. A similar finding was obtained in BC with BmjeTX-I and II PLA_2s from *B. marajoensis* venom [34] and PhTX-I PLA_2 from *Porthidium hyoprora* [28].

Myotoxicity induced by *Bothrops* venoms, may result from the direct action of myotoxins on the plasma membrane of muscle cells, or indirectly, as consequence of blood circulation failure and resulting ischemia caused by hemorrhagins or metalloproteases [13,30, 47-48]. Our present results suggest that Btae TX-I PLA_2 showed a moderate, although significant local myotoxicity in avian muscle preparation *in vitro*, as observed by histopathological analysis and morphometric data. The data are in accordance to other venoms such as from *B. atrox, B. jararaca and B. alternatus*, which show a low to moderate myotoxicity [49]. This study is the first experimental evidence of a PLA_2 myotoxin isolated from *Bothriopsis taeniata* snake venom. The toxin exhibited a moderate myotoxicity and neurotoxic activity in avian biventer cervicis preparation; the findings suggest that the sarcolemma is a target for this BTae-TX-1 Asp49 PLA_2.

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Conflicts of interest

There are no conflicts of interest to disclose.

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