Research Article
Biochemical Characterization and Pharmacological Properties of New Basic PLA₂ BrTX-I Isolated from Bothrops roedingeri (Roedinger’s Lancehead) Mertens, 1942, Snake Venom

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BrTX-I, a PLA₂, was purified from Bothrops roedingeri venom after only one chromatographic step using reverse-phase HPLC on μ-Bondapak C-18 column. A molecular mass of 14358.69 Da was determined by MALDI-TOF mass spectrometry. Amino acid analysis showed a high content of hydrophobic and basic amino acids as well as 14 half-cysteine residues. The total amino acid sequence was obtained using SwissProt database and showed high amino acid sequence identity with other PLA₂ from snake venom. The amino acid composition showed that BrTX-I has a high content of Lys, Tyr, Gly, Pro, and 14 half-Cys residues, typical of a basic PLA₂. BrTX-I presented PLA₂ activity and showed a minimum sigmoidal behavior, reaching its maximal activity at pH 8.0, 35–45°C, and required Ca²⁺. In vitro, the whole venom and BrTX-I caused a neuromuscular blockade in biventer cervicis preparations in a similar way to other Bothrops species. BrTX-I induced myonecrosis and oedema-forming activity analyzed through injection of the purified BrTX-I in mice. Since BrTX-I exerts a strong proinflammatory effect, the enzymatic phospholipid hydrolysis might be relevant for these phenomena; incrementing levels of IL-1, IL-6, and TNFα were observed at 15 min, 30 min, one, two, and six hours postinjection, respectively.

1. Introduction

PLA₂s (phosphatide 2-acylhdrolase, EC 3.1.14) represent a superfamily of lipolytic enzymes which specifically catalyze the hydrolysis of the ester bond at the sn-2 position of glycerophospholipids resulting in the generation of fatty acid (arachidonate) and lysophospholipids. The PLA₂ superfamily consists of about 15 groups which are further subdivided into several subgroups, all of which display differences in terms of their structural and functional specificities. However, the four main types or classes of PLA₂s are the secreted, the cytosolic, the Ca²⁺-independent and the lipoprotein-associated PLA₂ [1]. PLA₂ structure/function, mechanism, and signaling [2].

Snake venom PLA₂s display a variety of activities, such as neurotoxicity, myotoxicity, cardiotoxicity, and hemolysis that may be modulated by specific receptors located on target cells [3–6]. Indeed, PLA₂ receptors classified as kinds M and N [7] have been identified in various kinds of cells, including vascular smooth muscle cells, platelets, neutrophils, chondrocytes, fibroblasts, hepatocytes, and mesangial cells, as well as in brain, lung, and skeletal muscle [8, 9]. Snake venom PLA₂ can bind to M receptors, which are the most common kind found in human macrophages and muscle cells, and these may mediate some of the deleterious actions of venom PLA₂s, although that was not conclusively demonstrated [5, 6].

Peru has a rich and diverse herpetofauna that includes venomous snake species of the families Elapidae (16 species
of Micrurus and the pelagic sea snake Pelamis platurus) and Viperidae (15 species) [10]. Snakebite envenomations represent a public health problem in this country. The vast majority of snakebites in Peru are inflicted by species of the genus Bothrops (family Viperidae) [11]. Bothrops atrox, Bothrops brazili, and Bothrops bilineatus are distributed in the tropical rainforests located in the eastern part of the country, whereas Bothrops barnetti and Bothrops roedingeri are found in the western dry coastal regions [10–12].

This variety of pharmacological roles derives from an accelerated microevolutionary process through which a high rate of amino acid substitutions have occurred in molecular regions located mainly at the surface of these molecules [13–15]. The purpose of this paper is to isolate, biochemically and pharmacologically characterize a basic PLA2 from Bothrops roedingeri venom, BrTX-I.

2. Materials and Methods

2.1. Venom and Reagents. The venom was obtained from the adult specimens of Bothrops roedingeri captured in the vicinity of Arequipa-Peru. Swiss mice (18–20 g) were supplied by the Animal Services Unit of the State University of Campinas (UNICAMP). All experiments were conducted in accordance with guidelines of the Committee for Ethics in Animal Research, UNICAMP No. 2006-1 (Campinas-Brazil). The reagents used in this work were of analytical or sequencing grade.

2.2. PLA2 Activity. PLA2 activity was measured using the assay described in [16, 17], modified for 96-well plates [18]. The standard assay mixture contained 200 µL of buffer (10 mM Tris-HCl, 10 mM CaCl2, 100 mM NaCl, pH 8.0), 20 µL of substrate (4-nitro-3-octanoyloxybenzoic acid), 20 µL of water, and 20 µL of PLA2 in a final volume of 260 µL. After the addition of PLA2 (20 µg), the mixture was incubated for up to 40 min at 37°C, with the absorbance being read at 10 min intervals. The enzyme activity, expressed as the initial velocity of the reaction (Vo), was calculated based on the increase in absorbance after 20 min.

All assays were done three times and the absorbances at 425 nm were measured using a VersaMax 190 multwell plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.3. Reversed-Phase HPLC (RP-HPLC). Five milligrams of the venom was dissolved in 200 µL solvent A (TFA 0.1%, pH 3.5). The resulting solution was clarified by centrifugation and the supernatant was applied to a µ-Bondapak C18 column (0.78 × 30 cm; Waters 991-PDA system). Fractions were eluted using a linear gradient (0–100%, v/v) of acetonitrile (solvent B) at a constant flow rate of 1.0 mL/min over 40 min. The elution profile was monitored at 280 nm, and the collected fractions were lyophilized and conserved at −20°C.

2.4. Electrophoresis SDS-PAGE. The relative molecular mass of the protein was determined by SDS-PAGE [19]. The molecular mass markers were (in kDa): phospholipase B—94, albumin—67, ovalbumin—43, carbonic anhydrase—30, soybean trypsin inhibitor—20, and lysozyme—14.

2.5. Amino Acid Analysis. Amino acid analysis was done on a Pico-Tag amino acid analyzer (Waters Corporation, Massachusetts, USA) as described by [20]. The purified protein (30 µg) was hydrolyzed at 105°C for 24 h in 6 M HCl acid (Pierce sequencing grade) containing 1% phenol (w/v). The hydrolyzates were reacted with 20 µL of derivatization solution (ethanol: triethylamine: water: phenylisothiocyanate, 7:1:1:1, v/v) for 1 h at room temperature after the phenylthiohydantoin (PTC)-amino acids were identified and quantified by HPLC by the comparison of their retention times and peak areas with those of a standard amino acid mixture.

2.6. Reduction and Alkylation. Purified lyophilized protein 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. Since the number of cysteine residues in the protein was initially unknown, the optimum concentration of iodoacetamide for alkylating the free thiols was derived empirically, based on results obtained from incubations using various concentrations of iodoacetamide and different amounts of protein, with each mixture being analyzed by mass spectrometry [21]. Based on these preliminary 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 ceased by injecting the mixture onto a RP-HPLC column followed by lyophilization of the collected peak.

2.7. Enzymatic Hydrolysis. The purified proteins were hydrolyzed with sequencing grade bovine pancreatic trypsin in 0.4% ammonium bicarbonate, pH 8.5, for 4 h at 37°C, at an enzyme:substrate ratio of 1:100 (w/w). The reaction was ceased by lyophilization.

2.8. Mass Spectrometry. All mass spectra were acquired 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 a collision energy of 10 V. 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.

2.9. Analysis of Native and Alkylated Protein. Lyophilised RP-HPLC fractions of intact native and alkylated protein were dissolved in 10% acetonitrile in 0.1% TFA and was introduced...
into the mass spectrometer source with a syringe pump at a flow rate of 500 nL/min. Mass spectra were acquired over the mass range of 1000–2800 m/z for the native protein and over the range of 800–2000 m/z for the alkylated protein, both at a scan speed of 1 s/scan. The masses were analyzed by the MassLynx-MaxEnt 1 deconvolution algorithm. The data obtained were processed using the Mascot MS/MS Ion Search software http://www.matrixscience.com/.

2.10. De Novo Sequencing of Tryptic Peptides. Alkylated tryptic peptides fractionated by RP-HPLC were lyophilized and re-suspended in 20% acetonitrile in 0.1% TFA prior to injection into the mass spectrometer source at a flow rate of 500 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). Different collision energies were used, depending on the mass and charge state of the ions. The resulting ion spectra was acquired in the TOF analyser and deconvoluted using the MassLynx-MaxEnt 3 algorithm. Singly charged spectra were processed manually using the PepSeq application included in MassLynx.

2.11. Pharmacological Activity

2.11.1. Young Chicken Biventer Cervicis Preparation. Male chicks (4–8-days-old) were killed with isoflurane and the biventer cervicis muscles were removed [22]. The biventer cervicis muscles were mounted under a tension of 0.5 g, in a 5 mL organ bath (Automatic organ multiple-bath LE01 Letica cervicis muscles were mounted under a tension of 0.5 g, in a biventer cervicis muscle was removed [22]. The biventer cervicis muscles were removed under a tension of 0.5 g, in a 5 mL organ bath (Automatic organ multiple-bath LE01 Letica Scientific Instruments. Barcelona, Spain) at 37°C containing aerated (95% O₂ - 5% CO₂) Krebs solution (pH 7.5) of the following composition (mM): NaCl 118.7, KCl 4.7, CaCl₂ 1.88, KH₂PO₄ 1.17, MgSO₄ 1.17, NaHCO₃ 25.0 and glucose 11.65. Contracture to exogenously applied acetylcholine (ACh; 55 and 110 μM for 60 s) and KCl (20.1 mM for 130 s) was obtained in the absence of field stimulation, prior to the addition of a single dose of BrTX-I (50 μg/mL). A bipolar platinum ring electrode was placed around the tendon, which runs the nerve trunk supplying the muscle. Indirect stimulation was performed with a (MAIN BOX LE 12404 Panlab s.l. Powerlab AD Instruments Barcelona, Spain) stimulator (0.1 Hz, 0.2 ms, 3–4 V). Muscle contractions and contractures were isometrically recorded by force-displacement transducers (Model MLT0201 Force transducer 5 mg–25 g Panlab s.l. AD Instruments Pty Ltd. Spain) connected to a PowerLab/4SP (OUAD Bridge AD Instruments, Barcelona, Spain).

2.11.2. Myotoxic Activity. Groups of four Swiss mice (18–20 g) received an intramuscular (i.m.) or an intravenous (i.v.) injection of variable amounts of BrTX-I, in 100 μL of PBS, in the gastrocnemius. A control group received 100 μL of PBS. At different intervals of time (2, 4, 6, 9, and 24 h) blood was collected from the tail into heparinized capillary tubes, and the plasma creatine kinase (CK; EC 2.7.3.2) activity was determined by a kinetic assay (Sigma 47-UV). Activity was expressed in U/L, one unit defined as the phosphorylation of 1 μmol of creatine/min at 25°C.

2.11.3. Edema-Forming Activity. The ability of BrTX-I to induce edema was studied in groups of five Swiss mice (18–20 g) according Ponce-Soto et al. [6, 23, 24]. Twenty microliters of phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2) with BrTX-I (1, 5, 10 and 20 μg/paw) were injected in the subplantar region of the right footpad. The control group received an equal volume of PBS alone. The swelling of the paw was measured at 0.5; 1; 3; 6, and 24 h after administration. Edema was expressed as the percentage increased in the volume of the treated group to that of the control group at each time interval.

2.11.4. Cytokines. The percentage of cytotoxicity was of IL-1, IL-6, and TNF-α in the plasma were collected and measured at 30, 60, 180, and 360 min after i.p. injection of the BrTX-I PLÁ (1.0 mg/kg) (20 μg/100 μL) or sterile saline. After centrifugation, the supernatants were used for determination of IL-1 and IL-6 levels by a specific EIA. The levels of cytokines II-1, II-6, and TNF-α in the serum from BALB/c mice were assayed by two-site sandwich enzyme-like immunosorbent assay (ELISA). In brief, ELISA plates were coated with 100 μL (1 μg/mL) of the monoclonal antibodies anti-II-1, in 0.1 M sodium carbonate buffer (pH 8.2) and incubated for 6 hours at room temperature. The wells were then washed with 0.1% phosphate-buffered saline (PBS/Tween-20) and blocked with 100 μL of 10% fetal calf serum (FCS) in PBS for 2 hours at room temperature. After washing, duplicate sera samples of 50 μL were added to each well. After 18 hours of incubation at 4°C, the wells were washed and incubated with 100 μL (2 μg/mL) of the biotinylated monoclonal antibodies anti-II-1, anti-II-6, as second antibodies for 45 minutes at room temperature. After a final wash, the reaction was developed by the addition of orthophenyldiamine (OPD) to each well. Optical densities were measured at 405 nm in a microplate reader were measured using a VersaMax 190 multwell plate reader (Molecular Devices, Sunnyvale, CA, USA).

The cytokine content of each sample was read from a standard curve established with the appropriate recombinant cytokines (expressed in picograms per millilitre). The minimum levels of each cytokine detectable in the conditions of the assays were 10 pg/mL for IL-1, IL-6.

2.12. Statistical Analysis. The results are reported as the means ± SEM. The significance of differences among the means was assessed by ANOVA followed by Dunnett’s test when various experimental groups were compared to the control group. A value of P < 0.05 indicated significance.

3. Results

The elution profile of Bothrops roendiigeri venom following RP-HPLC performed on a C18 column showed fifteen fractions (1–15) (Figure 1). The fifteen eluted peaks were screened for PLÁ activity. Only the fraction labeled in figure
peak 8 presented PLA₂ activity, which was eluted with 58% of buffer B.

To confirm the level of purity, peak 8 was re-purified in a μ-Bondapack C18 column in HPLC of the reverse phase, showing a high level of molecular homogeneity (95%), for the presence of a single peak for the peak 8 (BrTX-I), with a very small retention time difference (37.19 ± 0.34 min) (Figure 1 insert). SDS-PAGE show of PLA₂ BrTX-I only band with molecular masses of ~14 kDa (Figure 1 insert) confirmed by MALDI-TOF mass spectrometry in 14,358.69 Da (Figure 2).

The amino acid composition determined was: N, D/10; Q, E/7; S/6; G/6; H/3; R/9; T/6; A/5; P/7; Y/8; V/5; M/1; C/14; I/5; L/7; F/3; K/18; W/Not determined (Figure 5(f)).

Samples of the native with mass 14,358.69 Da (Figure 2) and alkylated 15,170.35 Da (Figure 2 inserted) BrTX-I were digested with trypsin and the digests were analyzed by RP-HPLC. Table 1 shows the masses of the tryptic peptides obtained for from the BrTX-I. It is possible to see that these proteins presented five common peptides to the other Bothrops snake venoms. The data obtained were processed using the Mascot MS/MS Ion Search software (http://www.matrixscience.com/).

To obtain detailed structural information, the native protein was alkylated and then digested to be analyzed through ESI-MS/MS. The alkylated protein digest was fractionated by RP-HPLC and each chromatographic peak marked in the chromatogram was manually collected and lyophilized. De novo sequencing by ESI-MS/MS was obtained from the alkylated BrTX-I (Table 1). Ile and Leu residues were not discriminated in any of the sequences, since they were indistinguishable in low-energy collision-induced dissociation spectra. Due to the external calibration applied to all the spectra, it was also not possible to distinguish between Gln and Lys residues based on the 0.035 Da that separates these amino acids, except for Lys, marked in bold in Table 1, which was deduced by analysis of the cleavage and missed cleavage sites of the enzyme.

Each de novo sequenced peptide of the BrTX-I was submitted separately to the NCBI database, using the protein search program BLAST-p with the search being restricted to the sequenced proteins from the PLA₂ from snake venom family. In order to determine the presence and number of cysteine residues, BrTX-I was reduced and alkylated as described in Section 2.6.

The protein mass registered in peak 1–4 after alkylation was 15170.35 Da; the mass increase of 812Da indicated the presence of 14 Cys modified residues. The primary structure of the BrTX-I was determined by sequence tryptic digested and deduction of the SwissProt database http://br.expasy.org/. BrTX-I presented a sequence of 54 amino acid residues sequenced, being BrTX-I: DLWQWNKMIK --------- ---YGCYCGW GGR--

From BrTX-I, five peptides, with molecular masses of 1,360.65 Da (peak 1), 1,404.67 Da (peak 2), 1,791.07 Da
Table 1: Measured molecular masses and deduced amino acid sequences obtained by ESI-MS/MS based on the alkylated tryptic peptides of BrTX-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.

<table>
<thead>
<tr>
<th>BrTX-I HPLC fraction</th>
<th>Measured mass (Da)</th>
<th>Amino acid Sequence</th>
<th>Theoretical mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1360.65</td>
<td>DLI/WQ/KWKNK/QMI/LK/Q</td>
<td>1360.61</td>
</tr>
<tr>
<td>2</td>
<td>1404.67</td>
<td>DI/LTI/LVCGEDL/IPCK/Q</td>
<td>1404.64</td>
</tr>
<tr>
<td>3</td>
<td>1791.07</td>
<td>AAAVCFYENL/GTYNK/QK/Q</td>
<td>1791.03</td>
</tr>
<tr>
<td>4</td>
<td>1120.28</td>
<td>YGCYGWGGR</td>
<td>1020.25</td>
</tr>
<tr>
<td>5</td>
<td>616.79</td>
<td>L/ITGCPK/Q</td>
<td>616.75</td>
</tr>
</tbody>
</table>

The measured masses and deduced amino acid sequences obtained by ESI-MS/MS based on the alkylated tryptic peptides of BrTX-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.

**Figure 3**: MS/MS spectrum of the peptide tryptic ion of m/z 1120.310. Ion of the major sequence-specific peptide of the complementing ions YGCYGWGGR, from which the sequence of BrTX-I tag was deduced.

(peak 3), 1,120.28 Da (peak 4), and 616.79 Da (Peak 5). After the determination of these molecular masses and with the utilization of iodoacetamide, the cysteines presented in the peptides were alkylated (Table 1).

The peptide eluted in fraction 4 of BrTX-I, having the sequence YGCYGWGGR (tandem MS spectra shown in Figure 3) and the sequence of the BrTX-I protein was deduced and returns high homology with the others PLA2 proteins from the B. russelli species registered in the database Blast-p and showed high sequence homology with other PLA2 in the region associated with the catalytic site (Figure 4).

The PLA2 activity was examined in the Bothrops roedingeri venom and in BrTX-I using the synthetic substrate 4-nitro-3(octanoyloxy) benzoic acid [25]. The PLA2 activity was higher in BrTX-I (Figure 5(a)). Under the conditions used, BrTX-I showed a discrete sigmoidal behavior (Figure 5(b)) insert, mainly at low substrate concentrations. Maximum enzyme activity occurred at 35–40°C (Figure 5(c)) and the pH optimum was 8.0 (Figure 5(d)). PLA2s require Ca2+ for full activity, being only 1 mM of Ca2+ needed for BrTX-I to present phospholipase A2 activity. The addition of Zn2+, Mg2+, Mn2+, and Cd2+ (10 mM) in the presence of low Ca2+ concentration (1 mM) decreases the enzyme activity. The substitution of Ca2+ by Mg2+, Cd2+ and Mn2+ also reduced the activity to levels similar to those in the absence of Ca2+ (Figure 5(e)).

In the neuromuscular activity in chick nerve-muscle preparation, the whole venom concentrations of the 50 μg/mL were tested as well as the concentrations of 5, 20, 50, and 100 μg/mL of BrTX-I. The tested concentration, in both venom and BrTX-I, caused an irreversible dose-dependent blockade of the neuromuscular transmission (P < 0.05). The time required for the venom to achieve 50% twitch tension blockade, through an indirect stimulation, was: 22.60 ± 0.61 min (50 μg/mL) (Figure 6). The time required for BrTX-I to achieve 50% twitch tension blockade, also through indirect stimulation only doses of 50 (31.51 ± 0.52 min) and 100 μg/mL (25.29 ± 0.28 min) (Figure 6(a)). The twitch tension records of the control preparation remain stable at 98% to the venom and 97% to the BrTX-I (5 μg) along the 120 min of incubation with Krebs solution.

Regarding the venom, the concentration of 50 μg/mL altered significantly the ACh (110 μM) and KCl (20 mM) induced contractures when compared to the control values. In the concentration of the 50 μg/mL, the complete blockade was not accompanied by significantly inhibition of the response to ACh and KCl (Figure 6(b)). In the control preparations, the contracture to ACh and KCl was kept stable after a 120 min indirect stimulation.

In vivo, BrTX-I induced a conspicuous local myotoxic effect when injected by the i.m. route, only doses 10 and 20 μg (Figure 6(c)), but no increase in plasma CK levels occurred after their i.v. injection even in the same dose of 20 μg. Time-course analysis showed a maximum increase in plasma CK 1 h after i.m. injection, returning to normal by 24 h (Figure 6(d)). Compared to PBS-injected animals, those which received subplantar injections of the BrTX-I (1, 5, 10 and 20 μg/paw) presented marked paw edema all doses (Figure 7(a)). Maximal activity was attained 2 h to BrTX-I after injection and receded to normal levels after 24 h. The level of edema induction by 20 μg of BrTX-I PLA2 was similar to the other doses tested.
To further analyze and compare the mechanisms of the inflammatory events induced by BrTX-I PLA₂, the concentrations of the IL-1, IL-6, and TNF-α in the serum were measured. BrTX-I caused a marked increase in the TNF-α concentrations only at 1h (Figure 7(b)). In both the case of IL-1, the maximum peak was recorded at 6h, on the other hand for IL-6 level the peak was at 3h (Figures 7(c) and 7(d)).

4. Discussion

The purification procedure for basic PLA₂s developed by Ponce-Soto et al. [6, 15, 23, 24] showed to be also efficient for the obtainment of the Bothropsroedingeritoxin I PLA₂ (BrTX-I) from Bothropsroedingeri snake venom. Fractionation protocol of this crude venom using a single pass chromatographic in a column µ-Bondapack C-18 coupled to a system of reverse phase HPLC (0.78cm–30cm; Waters 991-PDAsystem) gaverisulto15fractionsat280nm, the eight last being the basic PLA₂ named BrTX-I (Figure 1).

SDS-PAGE showed (Figure 1 insert) the isolated toxin, BrTX-I have Mr of ∼14kDa similarly to basic PLA₂ isolated from other myotoxins from Bothrops snake venoms.

The molecular masses obtained by MALDI TOF mass spectrometry showed to be similar to that of other snake venom PLA₂s (14358.69Da) (Figure 2). Sequence homology studies had showed that there are extremely conserved positions in the PLA₂s. In positions 1 and 2, there is a predominance of the amino acids sequence (DL), in position 4 (Q). One of the highly conserved regions in the amino acid sequences of PLA₂ is the Ca²⁺-binding loop, segment from...YGCGCGG... and HD(49)CC (Figure 3). Residues forming the Ca²⁺-binding loop and the catalytic network of BrTX-I PLA₂ show a high conservation grade, reflecting the nondecreased catalytic activity.

The primary structure of BrTX-I determined by deduced sequencing (SwissProt database http://br.expasy.org/) method is aligned with the sequences of some other homologous snake venom PLA₂ from snake of the crotalidae family. It was very similar to that of other PLA₂ (Figure 4).

The PLA₂ activity showed to be higher in BrTX-I (16.87 ± 0.643 nmol/min/mg) when compared with the whole venom (2.59 ± 0.617 nmol/min/mg) (Figure 5(a)).

The PLA₂ from Crotalus durissus terrificus venom is a typical PLA₂, since it hydrolyzes synthetic substrates at position 2 and preferentially attacks substrates in their micellar state [28]. They can hydrolyze phospholipids in monomeric, micellar or lipid bilayer phases. PLA₂ enzymes exhibit a large and abrupt increase (up to 10,000 times) in their catalytic activity when monomeric phospholipids aggregate forms micelles at their critical micellar concentration [29]. This is due to the higher efficiency of interfacial catalysis, which depends on the absorption of the enzyme onto the lipid-water interface, strongly promoted by the presence of anionic amphipatic molecules within the membrane [30].

With synthetic substrate, BrTX-I behaved allosterically, especially at low substrate concentrations, which is in agreement with the results obtained by Beghini et al. [31], Bonfim et al. [32, 33], Ponce-Soto et al. [18], Calgarotto et al. [26], Huancahuire-Vega et al. [27] and for other PLA₂ using the same nonmicellar substrate also observed that the dependence of activity on substrate concentration was markedly sigmoidal (Figure 5(b)).

The PLA₂s from snake are highly stable and resistant to heat, acid, and urea, but catalytic activity is inactivated at high...
Figure 5: (a) PLA$_2$ activity of Bothrops roedingeri venom and peak 4 (BrTX-I); (b) effect of substrate concentration on the kinetics of BrTX-I (PLA$_2$) activity. (c) effect of temperature on the PLA$_2$ activity of BrTX-I; (d) effect of pH on BrTX-I activity; (e) influence of ions (10 mM each) on PLA$_2$ activity in the absence or presence of 1 mM Ca$^{2+}$. The results of all experiments are the mean ± SE, of three determinations ($P < 0.05$) and (f) amino acid composition of BrTX-I from Bothrops roedingeri snake venom.
pH. When micellar substrates are used, maximum catalytic activity occurs at pH 7-8 and 30–55°C [17, 28, 34–36] (Figure 5(c)). BrTX-I showed maximum enzyme activity at 35–45°C and greatest activity at around pH 8.0 (Figure 5(d)).

A strict requirement for Ca²⁺ is characteristic of some PLA₂ [18, 31, 35, 37]. BrTX-I showed typical Ca²⁺-dependent PLA₂ activity similar to other PLA₂ and this activity was lower in the presence of other divalent cations. Beghini et al. [31] observed the same for PLA₂ from Crotalus durissus casavella venom and Ponce-Soto et al. [18] for PLA₂ from Crotalus durissus collilineatus (Figure 5(e)).

The amino acid composition of the BrTX-I PLA₂ toxin revealed a high content of basic and hydrophobic residues, with 14 half-Cys, in agreement with the reported compositions and primary structures of PLA₂ toxins isolated from Bothrops venoms (Figure 5(f)). [6, 15, 38, 39]. The pharmacological activities investigated for BrTX-I PLA₂ includes neurotoxicity ex vivo in preparation BCP, in vivo inducing rapid damaging action to skeletal muscle tissue, paw oedema and increase of IL-1, IL-6 and TNF-α in the mice serum.

Some authors [3, 4, 6, 15, 40–44] have proposed several models to explain PLA₂-catalytic and pharmacological activities. In these models PLA₂ has two separated places; one is responsible for catalitic activity and other for biological activity expression. In accordance to them, the pharmacological place would be located in the surface of PLA₂ molecules.

The BrTX-I caused an irreversible concentration-dependent blockade of the indirectly elicited twitch responses of the chick biventer cervicis muscle preparation (BCP). Only doses 20, 50, and 100 µg/mL caused an irreversible dose-dependent blockade of the neuromuscular transmission (Figure 6(a)). The complete blockade of the muscle contraction all of the doses, was not accompanied by any significant inhibition of the responses to ACh. Inhibition response to KCl was progressive in terms of increasing the dose, suggesting a myotoxic effects due to destabilization of the membrane (Figure 6(b)).

Thus, the neuromuscular blockade produced by BrTX-I may be attributed to presynaptic activity, either by blocking axonal conduction or by affecting transmitter release at the motor nerve-terminal. The fact that the BrTx-I from Bothrops moojeni did not significantly affect the response to ACh and KCl, except when high doses were used, suggests that the venom presents a primordial presynaptic nature. Such neuromuscular blockade characteristics have been attributed to presynaptic-acting PLA₂ from snake [45, 46] as those of Crotalus durissus terrificus [47], Micruurus species [48, 49], and other Bothrops, Bothrops insularis [50], Bothrops pauloensis [47, 51], and Bothriopsis bilineata smaragdina [52], which did not show any detectable effect on the nicotinic receptor and, in some cases, showed only a mild muscle alteration.

In accordance to the model proposed by [42], the anticoagulant place would be located in a region between the 53 and 76 residues, considering this region charged positively in the PLA₂ with high anti-coagulant activity. In PLA₂ with moderate or low anti-coagulant activity, there is a predominance of negative charges. This region is placed in a distinct local and separated of foreseen regions by neurotoxicity and myotoxicity.

Local and systemic skeletal muscle degeneration is a common consequence of envenomations due to snakebites and mass bee attacks. PLA₂ is an important myotoxic component in these venoms, inducing a similar pattern of degenerative events in muscle cells. The bothrops PLA₂ myotoxins generally present low systemic toxicity, in contrast to myotoxic PLA₂ that are also strongly neurotoxic [5, 53].

Our studies on local and systemic myotoxicity in vivo revealed the BrTX-I is non-systemic myotoxin with local action due to decrease of the plasmatic CK levels (Figures 6(a) and 6(b)). This fact reinforces the hypothesis of differentiated action of local and systemic myotoxicity proposed by Gutiérrez and Ownby [5] and also the unspecificity and specificity proposed by Kini [3], Ponce-Soto et al. [6] and Gutiérrez et al. [54].

PLA₂s from snake venoms exert a large number of pharmacological activities [35, 54] due to a process of accelerated micro-evolution through which a high mutational rate in the coding regions of their genes has allowed the development of new functions, mainly associated with the exposed regions of the molecules [13]. The integral analysis of the inflammation elicited by BrTX-I from Bothrops roedingeri venom in the mouse serum performed in the present study allowed a parallel evaluation of the increase in microvascular permeability, by paw oedema and the production of various inflammatory mediators.

The PLA₂s from snake induced an increase in vascular permeability in peritoneal cavity of mice. This is in agreement with previous observations on the edema forming activity of similar molecules in the rodent footpad model [55, 56]. The increase of vascular permeability was detected after BrTX-I injection and developed rapidly, indicating that the observed plasma extravasation is primarily due to formation of endothelial gaps in vessels of microcirculation (Figure 7(a)). Previous studies have documented polymorphonuclear and mononuclear cellular infiltrate after injection of myotoxic PLA₂s from the venoms of Bothrops asper [57], Bothrops nummifer [58], and Bothrops jararacussu [59] in mouse skeletal muscle, and after intraperitoneal administration of similar myotoxins from Bothrops jararacussu and Bothrops pirajai venoms [60]. The mediators involved in this effect of BrTX-I was not addressed in this study. However, the immediate plasma extravasation in response to BrTX-I, strongly suggests the involvement of vasoactive mediators derived from mast cell granules. Previously, the ability of venom PLA₂ to degranulate mast cells has been shown [55].

TNF-α is also likely to be involved in inflammation induced by BrTX-I, since the PLA₂ caused a significant increase of TNF-α levels in the serum. TNF-α is also likely to be involved in leukocyte infiltration induced by BrTX-I, since the PLA₂ caused a significant increase of TNF-α levels in the serum. TNF-α induces the expression of E-selectin, CD11b/CD18 and ICAM-1 and triggers the release of several cytokines such as IL-1 and IL-6 (Figure 7(b)). Thus, our results suggest that TNF-α may have a role in the expression of CD18 and the release of other cytokines.
following BrTX-I injection, thereby being relevant for neutrophil influx and for increase of vascular permeability on the paw edema.

Cytokines, such as IL-1, IL-6, and TNF-α, are also relevant mediators for leukocyte migration and participate in several inflammatory conditions. Our results showed that BrTX-I induce increase in IL-1 and IL-6 in the serum, exerting a stronger effect (Figures 7(c) and 7(d)). IL-1 induced the expression of adhesion molecules by endothelial cells and stimulates the release of both IL-6 and TNF-α [61]. Thus,
our results suggest that IL-1 may contribute for the leukocyte migration.

All these biological effects induced by the BrTX-I occur in the presence of a measurable PLA\textsubscript{2} activity. Although the catalytic activity of PLA\textsubscript{2} contributes to pharmacological effects, it is not a prerequisite [55, 56, 62–64]. However, further studies are necessary to identify the structural determinants involved in these pharmacological activities.

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References


