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# Biological and biochemical characterization of new basic phospholipase $A_2$ BmTX-I isolated from *Bothrops moojeni* snake venom ☆

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ABSTRACT

BmTX-I, an Asp49 phospholipase $A_2$, was purified from *Bothrops moojeni* venom after only one chromatographic step using reverse-phase HPLC on μ-Bondapak C-18 column. A molecular mass of 14238.71 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 BmTX-I $PLA_2$ had a sequence of 121 residues of amino acids: DLWQFNKMIK KEVGKLPFPF YGAYGCYCGW GGRGEKPKDG TDRCCFVHDC CYKKLTGCPK WDDRYSYSWK DITIVCGEDL PCEEICECDR AAAVCFYENL GTYNKKYMKH LKPCKKADYP C and p*I* value 7.84, and showed a high degree of homology with basic Asp49 $PLA_2$ myotoxins from other *Bothrops* venoms.

BmTX-I presented $PLA_2$ activity in the presence of a synthetic substrate and showed a minimum sigmoidal behavior, reaching its maximal activity at pH 8.0 and 35–45 °C. Maximum $PLA_2$ activity required $Ca^{2+}$ and in the presence of $Mg^{2+}$, $Cd^{2+}$ and $Mn^{2+}$ it was reduced in presence or absence of $Ca^{2+}$. Crotapotin from *Crotalus durissus colillineatus* rattlesnake venom has significantly inhibited ($P<0.05$) the enzymatic activity of BmTX-I. *In vitro*, the whole venom and BmTX-I caused a blockade of the neuromuscular transmission in young chick biventer cervicis preparations in a similar way to other *bothrops* species.

In mice, BmTX-I and the whole venom-induced myonecrosis and a systemic interleukin-6 response upon intramuscular injection. Edema-forming activity was also analyzed through injection of the venom and the purified BmTX-I into the subplantar region of the right footpad. Since BmTX-I exert a strong proinflammatory effect; the enzymatic phospholipids hydrolysis might be relevant for these phenomena.



## 1. Introduction

Most of venomous species in Latin America belong to the Crotalinae subfamily, and in Brazil, the genus *Bothrops* is responsible for approximately 90% of envenomation cases, followed by *Crotalus* and *Lachesis* (Rosenfeld, 1971).

*Bothrops moojeni* is commonly known as "caiçaca" and is found in warm and dry regions of several Brazilian

☆ *Ethical statement*: All procedures were done in accordance with the general guidelines proposed by the Brazilian Council for Animal Experimentation (COBEA), protocol 1040-1.

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States. The importance of the study with this species lies on its geographic distribution as well as on the clinic characteristics that its venom may cause (Kouyoumdjian et al., 1990).

Snake venoms comprise a complex pool of proteins (more than 90% of the dry weight), organic compounds with low molecular mass and inorganic compounds. Among the toxic compounds are included non-enzymatic proteins such as desintegrins and bradykinin-potentiating peptides and enzymes such as metalloproteases, serinoproteases and phospholipases, for example. Among these bioactive proteins, phospholipases $A_2$ ($PLA_2$ E.C 3.1.1.4) and $PLA_2$-like myotoxins are the main components (Serrano et al., 2005).

$PLA_2$s are calcium-dependent enzymes and catalyze the hydrolysis of the sn-2 ester bond of 1,2-diacyl-3-phosphoglycerides to produce lysophosphatidylcholine and free acids, including the arachidonic acid (Six and Dennis, 2000). Phospholipases $A_2$ have been classified as groups I, II and III on the basis of their primary structure and disulfide bridge pattern (Dennis, 1994; Balsinde et al., 1999). Group I $PLA_2$s are composed of both mammalian pancreas and snake venoms belonging to the Elapidae and Hydrophidae families. Group II secreted $PLA_2$s enzymes include those from *Viperidae* snake venom. Group III $PLA_2$s have mainly been isolated from bee (*Apis mellifera*) and lizard venoms (Serrano et al., 2005).

Independently of its primary catalytic function, PLA2s from snake venoms can induce important toxic/pharmacological effects including myonecrosis, neurotoxicity, cardiotoxicity, hemolytic, hemorrhagic, hypotensive, anticoagulant, platelet aggregation inhibition and edema-inducing activities (Gutiérrez and Lomonte, 1997; Valentin and Lambeau, 2000).

Two basic Lys49 myotoxins of approximately 13.5 kDa, myotoxins-I (MjTX-I) and -II (MjTX-II), were isolated from *B. moojeni* venom (Lomonte et al., 1990; Soares et al., 1998, 2000a). The purpose of this paper is the isolate, biochemically and pharmacologically characterize a basic Asp-49 phospholipase $A_2$ from *B. moojeni* venom, BmTX-I.

## 2. Materials and methods

### 2.1. Venom and reagents

The venom and solvents (HPLC grade), 4-nitro-3 (octanoyloxy) benzoic acid, all chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

### 2.2. Reversed-phase HPLC (RP-HPLC)

Five milligrams of the venom was dissolved in 200 μl ammonium bicarbonate 0.2 M pH 8.0. 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.

### 2.3. $PLA_2$ activity

$PLA_2$ activity was measured using the assay described by Holzer and Mackessy, (1996) modified for 96-well plates (Ponce-Soto et al., 2002). The standard assay mixture contained 200 μl of buffer (10 mM Tris–HCl, 10 mM $CaCl_2$ and 100 mM NaCl, pH 8.0), 20 μl of substrate (3 mM), 20 μl of water and 20 μl of $PLA_2$ (1 mg/ml) in a final volume of 260 μl. After adding $PLA_2$ (20 μg), the mixture was incubated for up to 40 min at 37 °C, with the reading of absorbance 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 inhibition of $PLA_2$ activity by crotapotins from *Crotalus durissus colillineatus* was determined by incubating the protein (BmTX-I and crotapotin) for 30 min at 37 °C prior to assaying the residual enzyme activity. The pH and optimal temperature of the $PLA_2$ were determined by incubating the enzyme in four buffers of different pH values (4–10) and at different temperatures, respectively. The effect of substrate concentration (10, 5, 2.5, 1.25, 0.625 and 0.312 mM) on enzyme activity was determined by measuring the increase of absorbance after 20 min. All assays were done in triplicate and the absorbances at 425 nm were measured with a VersaMax 190 multiwell plate reader (Molecular Devices, S., CA).

### 2.4. Electrophoresis

Tricine SDS-PAGE in a discontinuous gel and buffer system was used to estimate the molecular mass of the proteins, under reducing and non-reducing conditions (Schagger and Von Jagow, 1987).

### 2.5. MALDI-TOF mass spectrometric analysis (MS)

The molecular mass of isolated BmTX-I was analyzed by MALDI-TOF mass spectrometry using a Voyager-DE PRO MALDI-TOF apparatus (Applied Biosystems, Foster City, CA, USA). One microliter 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 25 kV, the laser fixed in 2890 μJ/com2, delay 300 ns, and linear analysis mode (Ponce-Soto et al., 2006).

### 2.6. Amino acid analysis

Amino acid analysis was performed on a Pico-Tag analyzer (Waters Systems) as described by Heinrikson and Meredith (1984). The purified BmTX-I sample (30 μg) was hydrolyzed at 105 °C for 24 h, in 6 M HCl (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 which the 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.

### 2.7. Sequencing procedure

Two milligrams of the purified protein were dissolved in 200 μl of a 6 mol/l guanidine chloride solution (Merck, Darmstadt, Germany) containing 0.4 mol/l of Tris–HCl and 2 mMol/l EDTA (pH 8.15). Nitrogen was blown over the top of the protein solution for 15 min; it was then reduced with DTT (6 M, 200 μl) and carboxymethylated with 14C-iodoacetic acid and icy iodoacetic acid. Nitrogen was again blown over the surface of the solution and the reaction tube was sealed. This solution was incubated in the dark at 37 °C for 1 h and desalted using a Sephadex G25 column (0.7 × 12 cm) with 1 mol/l acetic acid buffer.

The reduced carboxymethylated $PLA_2$ (RC-$PLA_2$) protein was digested with *Staphylococcus aureus* protease SV8 for 16 h at 37 °C; using a 1:30 enzyme to substrate molar ratio and the reaction was stopped by lyophilization. The RC-$PLA_2$ was also digested with clostripain for 8 h at 37 °C and then lyophilized again. The digested products of these treatments were fractionated by reverse-phase HPLC using a Waters PDA 991 system and a C18 μ-Bondapack column. The elution of peptide peaks was made using a linear gradient consisting of 0–100% of acetonitrile in 0.1% trifluoroacetic acid (v/v).

The sequencing of the N-terminal was conducted for the RC-$PLA_2$ protein, using a Procise f automatic sequencer. The phenylthyoidantoin (PTH) amino acids were identified by comparing their retention times to the 20 PTH amino acid standards. Peptides containing 14C-CM-Cys were monitored by detecting the radioactivity label using a liquid scintillation counter (Beckman model L- 8 250). The primary structure of the $PLA_2$ D49 BmTX-I, was build mainly based on the purified peptides from the protein digested, protease SV8 and clostripain (Ponce-Soto et al., 2007c).

### 2.8. Chick biventer cervicis muscle preparation (BCp)

Male young chickens (4–8 days old, HY-LINE W36 lineage) were supplied by Granja Ito S/A (Campinas, SP, Brazil). Animals had free access to food and water. All procedures were done in accordance with the general guidelines proposed by the Brazilian Council for Animal Experimentation (COBEA), protocol 1040-1.

Animals were anesthetized with chloral hydrate (3 mg/kg) and sacrificed by exsanguination. The biventer cervicis muscles were removed and mounted under a tension of 0.5 g, in a 5 ml organ bath at 37 °C (Automatic organ multiple-bath LE01 Letica Scientific Instruments. Barcelona, Spain), containing aerated (95% $O_2$–5% $CO_2$) Krebs solution (pH 7.5) of the following composition (mM): NaCl 118.7, KCl 4.7, $CaCl_2$ 1.88, $KH_2PO_4$ 1.17, $MgSO_4$ 1.17, $NaHCO_3$ 25.0 and glucose 11.65. Contracture to exogenously applied acetylcholine ACh (110 μM for 60 s) and KCl (20 mM for 130 s) was obtained in the absence of field stimulation, prior to the addition of a single dose (100, 50 and 10 μg/ml) of whole venom, and (50 and 10 μg/ml) of BmTX-I. A bipolar platinum ring electrode was placed around the tendon with 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 via a force–displacement transducer (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). Experiments were carried out in triplicate.

### 2.9. 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 the whole venom and $PLA_2$ BmTX-I. Samples (20 μl) containing 10 or 20 μg in the gastrocnemius. A control group received 50 μl of PBS. At different intervals, 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.

### 2.10. Edema-forming activity

The ability of *B. moojeni* venom and BmTX-I to induce edema was studied in groups of five Swiss mice (18–20 g). Twenty microliters of phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2) with venom (10 μg/paw) and BmTX-I (5 and 10 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. Quantification of interleukin-6

Groups of five Swiss mice (18–20 g) received an (i.m.) injection of 10 and 20 μg of whole venom and BmTX-I in 20 μl at the tibial muscle. Blood samples were collected into heparinized capillaries in 1, 3 and 8 h after injection. Briefly, 96-well plates were coated with 100 μl of the first capture monoclonal antibody anti-IL-6 and incubated for 2 h at 37 °C. Abs at 450 nm were recorded and concentrations of IL-6 were estimated from standard curves prepared with recombinant IL-6.

## 3. Results

The elution profile of *B. moojeni* venom following RP-HPLC performed on a C18 column showed 13 fractions (I–XIII) (Fig. 1A). The 13 eluted peaks were screened for $PLA_2$ activity. Only the fraction labeled in Fig. 1a presented

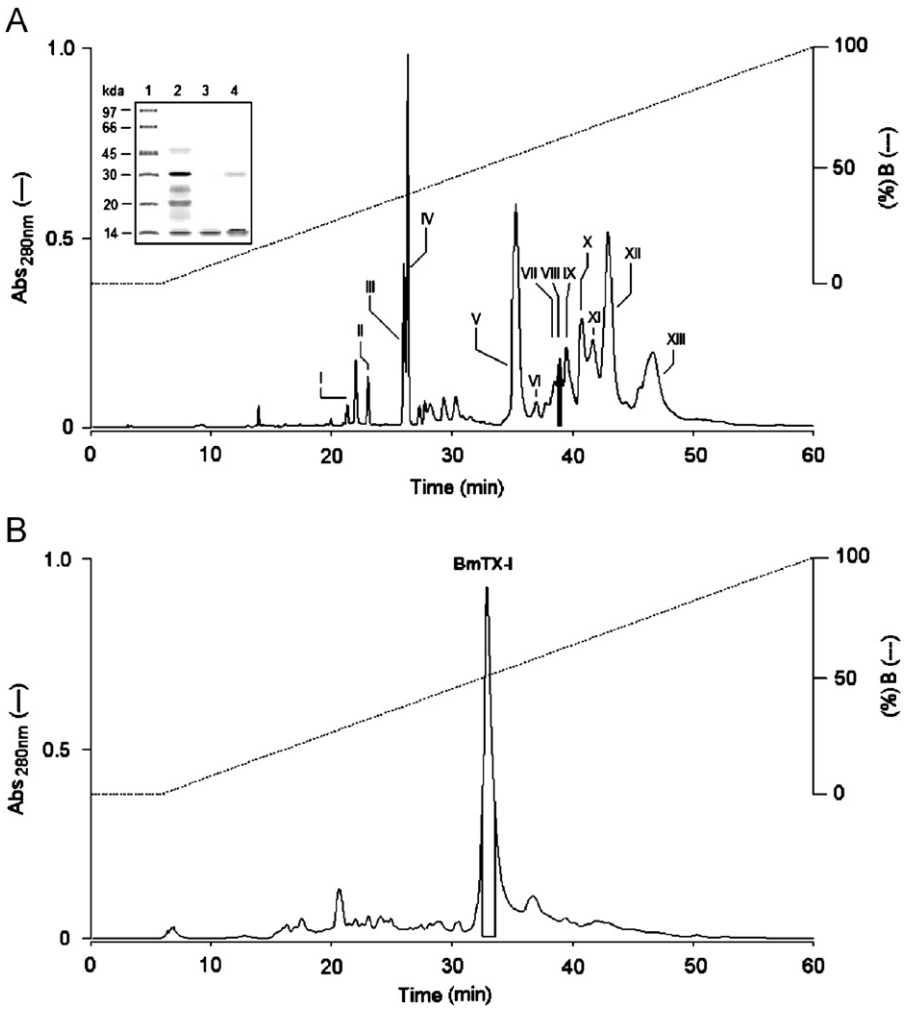


**Fig. 1.** (A) Elution profile of *Bothrops moojeni* venom by RP-HPLC on a μ-Bondapack C18 column. Fraction VIII contained $PLA_2$ activity. Insert: Electrophoretic profile in SDS-PAGE (12.5% gel). (1), molecular mass markers; (2) *Bothrops moojeni* venom; (3) BmTX-I-$PLA_2$ from *B. moojeni*, and (4) BmTX-I-$PLA_2$ from *B. moojeni* reduced. (B) Elution profile of FVIII following RP-HPLC on a μ-Bondapack C18 column. The peak corresponding to active fraction (BmTX-I) is indicated.

$PLA_2$ activity, which was eluted with 58% of buffer B. This peak was further purified by the same chromatography system used in the first fractionation step (RP-HPLC). The result of the repurification showed the presence of only one peak, named BmTX-I (Fig. 1B).

The electrophoretic profile (insert Fig. 1a) showed the *B. moojeni* venom, the fraction BmTX-I in the absence and presence of DTT (1 M), respectively. BmTX-I in the absence of DTT presented two electrophoretic bands with of ~14 and 28 kDa. In the presence of DTT, BmTX-I showed only one electrophoretic band with of ~14 kDa confirmed by MALDI-TOF mass spectrometry in 14238.71 Da.

The amino acid composition determined was: N,D/13; Q,E/11; S/3; G/10; H/2; R/6; T/6; A/6; P/7; Y/3; V/5; M/1; C/14; I/4; L/9; F/6; K/22; W/not determined.

The primary sequence of BmTX-I was determined by treating the purified enzyme with clostripain and bovine trypsin followed by sequencing of the resulting peptides. Purified enzyme was used as a control for enzymatic digestion and appeared as a single major peak. RP-HPLC of the peptide mixture obtained by digestion of BmTX-I $PLA_2$ with clostripain revealed the presence of 9 peptides and the digestion with the protease trypsin resulted in 7 peptides for BmTX-I $PLA_2$ (data not shown). The primary structure of BmTX-I $PLA_2$ was determined comparing the sequences of the overlapping peptides purified from the above digests. Although various peptides have been purified and sequenced, only three (CLT-3, CLT-5 CLT-6, T-1, T-2, T3 and T-6) were important for determining the primary structure of BmTX- I $PLA_2$ (Fig. 2).

The BmTX-I $PLA_2$ is built by 121 amino acid residues and showed high-sequence homology with other $PLA_2$ and 83% with 6-2 $PLA_2$ (Ponce-Soto et al., 2006) in the region associated with the catalytic site (Fig. 2).

The $PLA_2$ activity was examined in the *B. moojeni* venom and in BmTX-I using the synthetic substrate 4-nitro-3 (octanoyloxy) benzoic acid (Holzer and Mackessy, 1996).

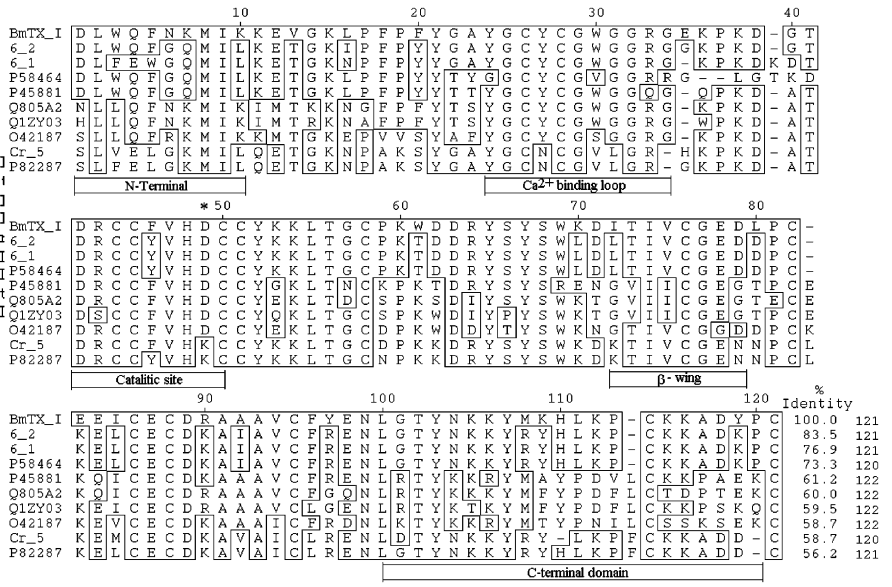


**Fig. 2.** Amino acid alignment of *Bothrops moojeni* BmTX-I with Asp49 $PLA_2$ homologs. 6_2 and 6_1: phospholise $A_2$ from *Bothrops jararacussu* (Ponce-Soto et al., 2006); P58464: piratoxin III from *Bothrops pirajai* (Toyama et al., 1999); P45881: phospholipase $A_2$ from *Bothrops jararacussu* (Moura-da-Silva et al., 1995); Q805A2: phospholipase $A_2$ from *Trimeresurus flavoviridis* (Chijiwa et al., 2003); Q1ZY03: phospholipase $A_2$ from *Deinagkistrodon acutus* (Wang et al., 1996); O42187: phospholipse $A_2$ from *Agkistrodon halys Pallas* (Pan et al., 1996); Cr_5: phospholipase $A_2$ from *Calloselasma rhodostoma* (Bonfim et al., 2006); P82287: piratoxin II from *Bothrops pirajai* (Lee et al., 2001).

The $PLA_2$ activity was higher in BmTX-I (6.5 nmols/mim/mg) when compared with the whole venom (0.39 nmols/min/mg) (Fig. 3A). Under the conditions used, BmTX-I showed a discrete sigmoidal behavior (Fig. 3B), mainly at low substrate concentrations. Maximum enzyme activity occurred at 35–40 °C (Fig. 3C) and the pH optimum was 8.0 (Fig. 3D). $PLA_2$s require $Ca^{+2}$ for full activity, being only 1 mM of $Ca^{+2}$ needed for BmTX-I to present phospholipase activity. The addition of $Mg^{2+}$, $Cd^{2+}$ and $Mn^{2+}$ (10 mM) in the presence of low $Ca^{2+}$ concentration (1 mM) decreases the enzyme activity. The substitution of $Ca^{2+}$ by $Mg^{2+}$, $Cd^{2+}$ and $Mn^{2+}$ also reduced the activity to levels similar to those in the absence of $Ca^{2+}$ (Fig. 3E).

The crotapotins are pharmacologically inactive and non-enzymatic acid protein, binds specifically of the $PLA_2$ inhibited the activity. Crotapotins isoforms from *Crotalus durissus collilineatus* (F3 and F4) significantly inhibit the BmTX-I activity by approximately 50% (Fig. 3F).

In the neuromuscular activity in chick nerve–muscle preparation, the whole venom concentrations of the 100, 50 and 10 μg/ml were tested as well as the concentrations of 50 and 10 μg/ml of BmTX-I. All the tested concentrations, in both venom and BmTX-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: 50 ± 2.30 min (100 μg/ml), 60 ± 3.1 min (50 μg/ml) and 70 ± 4.3 min (10 μg/ml) (Fig. 4A). The time required for BmTX-I to achieve 50% twitch tension blockade, also through indirect stimulation, was: 40 ± 3.18 min (50 μg/ml) and 50 ± 2.15 min (10 μg/ml) (Fig. 4B). The twitch tension records of the control preparation remain stable at 98% to the venom and 97% to the BmTX-I all along the 120 min of incubation with Krebs solution.

Regarding the venom, the concentration of 100 and 50 μg/ml altered significantly the ACh- (110 μM) and KCl (20 mM)-induced contractures when compared with the control values. In the concentration of the 10 μg/ml, the complete blockade was not accompanied by significantly inhibition of the response to ACh and KCl (Fig. 4C). To BmTX-I, no concentration (50 and 10 μg/ml) has significantly altered induced contractures when compared with control values (Fig. 4D).

In the control preparations, the contracture to ACh and KCl was kept stable after a 120 min indirect stimulation (data not shown).

Compared with PBS-injected animals, those which received subplantar injections of the whole venom (10 μg/paw) and BmTX-I (5 and 10 μg/paw) presented marked paw edema (Fig. 4E). Maximal activity was attained 1 h to the whole venom and BmTX-I after injection and receded to normal levels after 24 h. The level of edema induction by 10 μg of $PLA_2$ and venom, 1 h after administration, was similar: 159 ± 10.4% and 172 ± 4.4%, respectively, showing a dose-dependent activity.

As shown in Fig. 5, intramuscular injections of either *B. moojeni* whole venom (Fig. 5A) or purified BmTX-I (Fig. 5B) produced myotoxic effects characterized by increased plasma CK activity in tested animals. BmTX-I (10 and 20 μg) produced an increase in plasma CK activity similar to that seen for the whole venom (10 and 20 μg) when compared with the control (PBS-treated). The highest level was observed 1 h after injection, decreasing

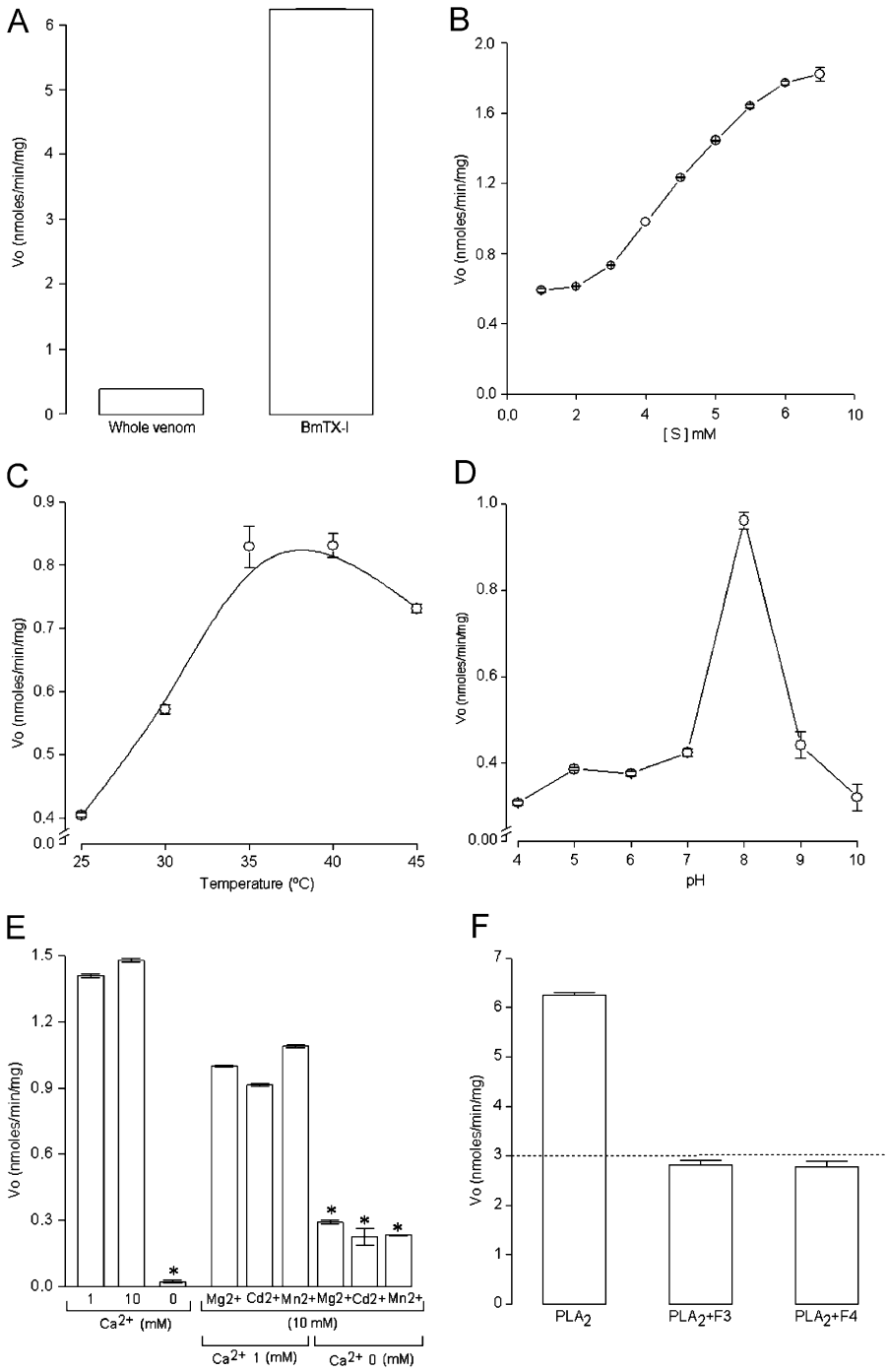


**Fig. 3.** (A) $PLA_2$ activity of *Bothrops moojeni* venom and BmTX-I; (B) effect of substrate concentration on the kinetics of BmTX-I ($PLA_2$) activity; (C) effect of temperature on the $PLA_2$ activity of BmTX-I; (D) effect of pH on BmTX-I activity; (E) influence of ions (10 mM each) on $PLA_2$ activity in the absence or presence of 1 mM $Ca^{2+}$; (F) inhibition of BmTX-I activity by crotapotins (F3 and F4) isolated from *Cdcoll* venom. The results of all experiments are the mean ± S.E. of three determinations ($P < 0.05$).

afterwards. The CK levels after 8 h were not significantly different from the control value.

To further analyze and compare the mechanism of the inflammatory events induced by *B. moojeni* venom (Fig. 5C) and purified BmTX-I $PLA_2$ (Fig. 5D), the concentrations of IL-6 in the plasma were measured. BmTX-I (20 μg) caused a marked increased in IL-6 levels, reaching maximum increase 3 h after the injection when compared with the control; 8 h after the injection, there was a decrease of the IL-6 levels.

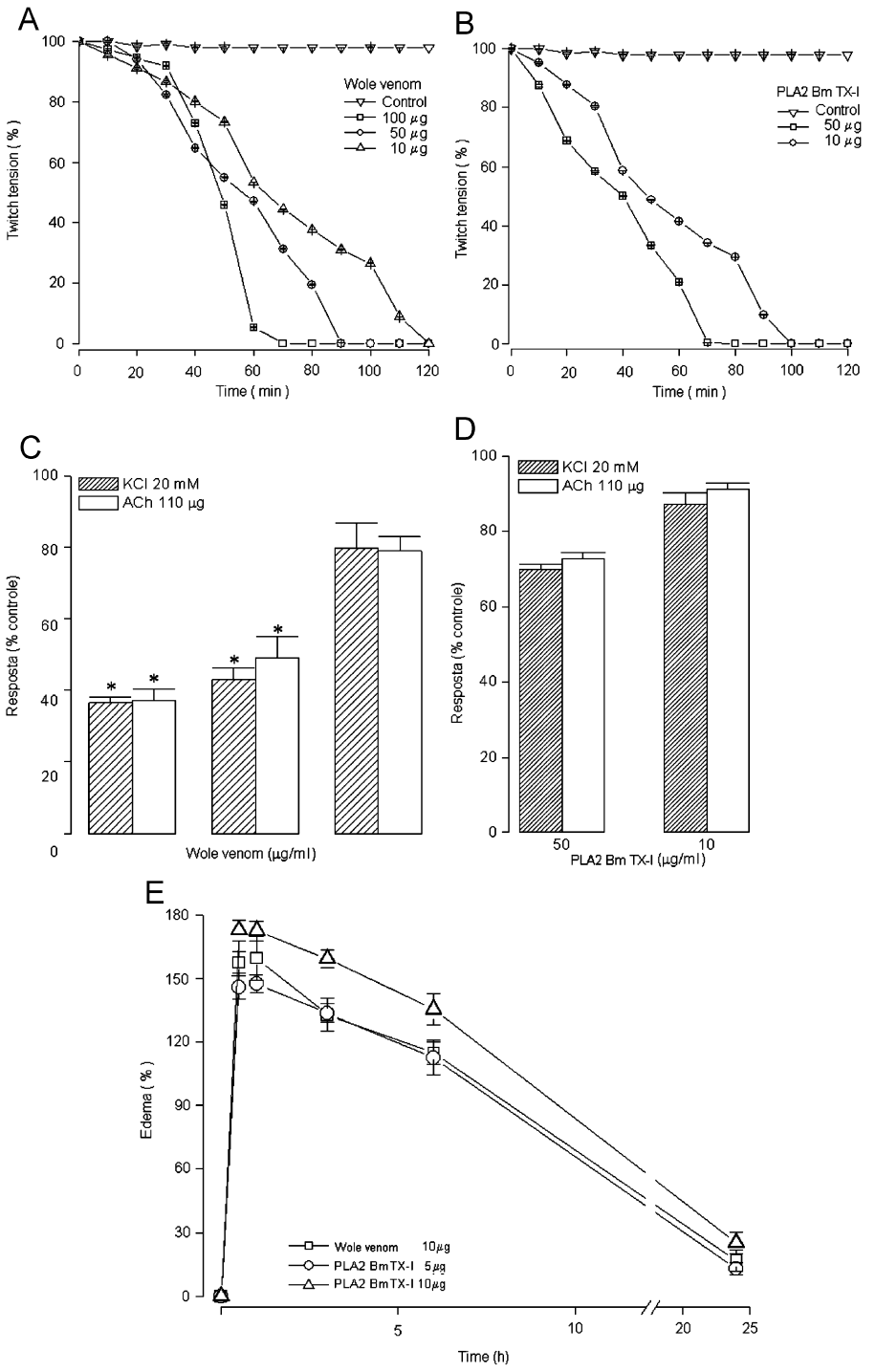


**Fig. 4.** (A) Neuromuscular blockade induced by the whole venom from *Bothrops moojeni*, (B) the $PLA_2$ (BmTX-I) on the chick biventer cervicis muscle preparation. Each point represents the average from five experiments ± SEM. $P < 0.05$ compared with control; (C) effect of the whole venom from *Bothrops moojeni* and the $PLA_2$ BmTX-I (D) on the response of the chick biventer cervicis preparation to the acetylcholine (ACh) and potassium (KCl). Each point represents the average from five experiments ± SEM $P < 0.05$. Compared with control and (D) mice paw edema induced by selected doses of *Bothrops moojeni* venom (10 μg) and $PLA_2$ BmTX-I (5 and 10 μg). The edema, which was expressed as the percentage increased in the volume of the treated group to that of the control group at each time interval, was maximal after 0.5 h and decreased thereafter. The points are the mean ± SEM of five mice.

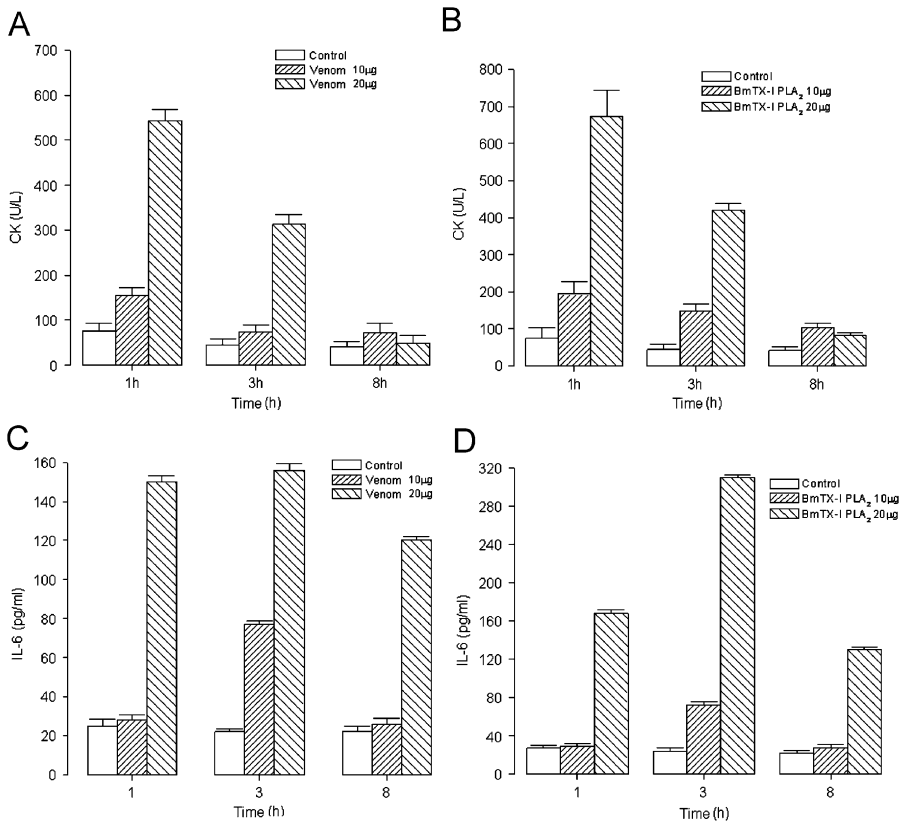


**Fig. 5.** (A) Myotoxic activity induced by *Bothrops moojeni* venom (10 and 20 μg) and (B) by $PLA_2$ BmTX-I (10 and 20 μg) as measured by creatina kinase (CK) release. The whole venom was injected into the tibial muscle of mice. Controls were injected with 20 μl of PBS. At different times, blood was collected, and serum CK levels were measure. Values are means ± SEM of five mice at each time point; (C) systemic interleukin-6 response induced by i.m. injection of *Bothrops moojeni* venom (10 and 20 μg) and (D) $PLA_2$ BmTX-I (10 and 20 μg). Plasma levels of IL-6 were determined at the indicated time points, by enzyme immunoassay. Each point represents ± SEM of five mice.

## 4. Discussion

The Myotoxin-I and -II homologs purified from *B. moojeni* (MjTX-I and -II) are Lys49-$PLA_2$ and have already been isolated and characterized (Lomonte et al., 1990; Soares et al., 1998, 2000a, b).

Our results using a simple and rapid procedure based on RP-HPLC showed that the *B. moojeni* venom can be separated in XIII fractions. The fraction VIII presents $PLA_2$ activity and was denominated BmTX-I. To confirm the homogeneity, the fraction VIII was further purified using the same chromatography system.

SDS-PAGE under non-reducing conditions showed that BmTX-I occurs as a dimmer, which dissociates into a single chain polypeptide of ~14 kDa after reduction, confirmed by MALDI-TOF mass spectrometry (14,238.71 Da). 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 compositions and primary structures of myotoxins $PLA_2$ isolated from *Bothrops* venoms (Gutierrez and Lomonte, 1995; Ponce-Soto et al., 2006; Damico et al., 2006; Gutierrez et al., 2007).

The $PLA_2$ activity, although it is moderated when compared with other $PLA_2$ isolated from crotalic snakes (data not shown). The primary structure revealed a high level of sequence homology between BmTX-I and Asp49 $PLA_2$s such as the $PLA_2$ isoforms 6-1 and 6-2 from *Bothrops jararacussu* (Ponce-Soto et al., 2006).

The conserved residues Y28, G30, G32, D49, H48 and Y52 are directly or indirectly linked in the catalyses of the BmTX-I. On the other hand, BmTX-I presents some important mutations: T13−>V13, F20−>Y20, F46−>Y46, L80−>D80, E83−>K83 and R90−>90 K that are strategically positioned to the expression of catalytic activity. Despite these substitutions, no decrease was detected in the catalytic or in the pharmacologic activity of BmTX-I.

Sequence homology studies had showed that there are extremely conserved positions in the $PLA_2$s. In positions 1 and 2, there is a predominance of the amino acids sequence (SL), in position 4 (Q), in position 7–10 (KMIL), in position 12 and 13 (ET), in position 21 (Y), in position 25–26 and 28–29 (GC and CG). In the Asp49 $PLA_2$s, there are many conserved residues that also have important functions in the activity expression of the $PLA_2$ activity: W/YCG-G to be essentials for the formation of the channel calcium binding (Pereira et al., 1998; Breithaupt, 1976; Arni and Ward, 1996; Ponce-Soto et al., 2007b).

The $PLA_2$ activity showed to be higher in BmTX-I ($6.25 \pm 0.01$ nmoles/min/mg) when compared with the whole venom ($0.39 \pm 0.005$ nmoles/min/mg). $PLA_2$ enzyme from snake venom shows classic Michaelis–Menten behavior against micellar substrates (Breithaupt, 1976). With a synthetic substrate, BmTX-I behaved allosterically, especially at low concentrations, what is in agreement with the results obtained by Bonfim et al. (2001) for the $PLA_2$ of *B. jararacussu* venom and Damico et al. (2005a) for the $PLA_2$ isoform purified from *Lachesis muta muta* venom. Using the same synthetic non-micellar substrate, it was also possible to observe that the dependence of activity on substrate concentration was markedly sigmoidal for the PrTX-III from *Bothrops pirajai* (Rigden et al., 2003).

$PLA_2$s from crotalic venoms have showed a similar behavior to the one presented by bothropic $PLA_2$s with the same substrate used in the kinetic studies to BmTX-I (Beghini et al., 2000; Ponce-Soto et al., 2002). Despite the structural and functional differences among bothropic and crotalic $PLA_2$s, both show allosteric behavior in the presence of the same substrate.

The $PLA_2$ activity could be verified with different pH levels; the optimum pH to basic $PLA_2$s is around 7.0 and 8.5 (Kini, 1997; Breithaupt, 1976). BmTX-I can be considered basic since its highest activity is evidenced at pH 8.0. Temperature is another kinetic parameter utilized to characterize the $PLA_2$ (Asp49). It has been shown that $PLA_2$ from *Naja naja naja* is very stable in extreme temperatures such as 100 °C (Kini, 1997). The optimum temperature to BmTX-I was around 37 °C, but at 40–45 °C, the BmTX-I activity did not present a huge decrease.

A strict requirement for $Ca^{2+}$ is characteristic of some $PLA_2$ (Dennis, 1994; Arni and Ward, 1996). BmTX-I showed typical $Ca^{2+}$-dependent $PLA_2$ activity similar to other Asp49 $PLA_2$, and this activity was lower in the presence of other cations. Beghini et al. (2000) observed the same for $PLA_2$ from *Crotalus durissus cascavella* venom.

The crotapotin isoforms from *C. durrissus collilineatus* (F3 and F4) venom inhibit significantly the $PLA_2$ activity of BmTX-I by approximately 50%. Our results are in agreement with the finding by Landucci et al. (2000), who reported that highly purified crotapotin can inhibit pancreatic, bee, and other snake venom $PLA_2$, and Bonfim et al. (2001), who reported that crotapotins from *Crotalus durissus terrificus* (F7), *C. durissus collilineatus* (F3 and F4) and *C. durissus cascavella* (F3 and F4) decreased the catalytic activity of BJ IV ($PLA_2$ from *B. jararacussu*) by 50%. Together, these results suggest that crotapotin may bind to bothropic $PLA_2$ in a manner similar to that from crotalic $PLA_2$.

The whole venom and BmTX-I caused an irreversible concentration-dependent blockade of the indirectly elicited twitch responses of the chick biventer cervicis muscle preparation (BCp) at all concentrations (100, 50 and 10 μg/ml of the venom; 50 and 10 μg/ml of BmTX-I). In the concentrations of 10 μg/ml of the venom and of 50 and 10 μg/ml of BmTX-I, the complete blockade of the muscle contraction was not accompanied by any significant inhibition of the responses to KCl and to ACh.

However, using the highest concentration of the venom (100 and 50 μg/ml), a muscle contracture, concomitant with inhibition of the response to ACh and KCl, was observed, pointing though to a postsynaptic effect. The fact that the whole venom from *B. 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. In this sense, *B. moojeni* venom behaves as *Bothrops insularis* venom, which does not inhibit the response to KCl and ACh, presenting a preponderant presynaptic action (Cogo et al., 1993).

The *B. moojeni* venom and the $PLA_2$ BmTX-I showed an *in vitro* neuromuscular pre-synaptically blocking activity in chick nerve–muscle preparations, in a similar way to those of other *Bothrops* species.

The pharmacological activities investigated for *B. moojeni* venom and purified BmTX-I $PLA_2$ includes local, rapid damaging action to skeletal muscle tissue, the induction of paw edema and increase of IL-6 in the plasma. Accumulating evidences have strongly shown that venom $PLA_2$ are among the major mediators of myonecrosis (Mebs and Ownby, 1990; Gutierrez and Lomonte, 1995), hemolysis (Condrea et al., 1981), mast cell degranulation (Landucci et al., 1998) and edema formation (Lomonte et al., 1993).

The main edema formation occurred 1 h after the injection of BmTX-I and crude venom with constant decrease. *B. moojeni* venom and BmTX-I caused paw edema in mice with a time course similar to that reported for other *Bothrops* venoms in mice and rats, i.e. a fairly rapid onset (generally $\leqslant 3$ h to peak) followed by a gradual decline over the following 24 h (Chacur et al., 2001; Faria et al., 2001; Carneiro et al., 2002; Kanashiro et al., 2002).

$PLA_2$ isolated from *Bothrops* venoms are frequently myotoxic (Gutierrez and Lomonte, 1995) and can cause edema in rats and mice (Gutiérrez et al., 1986; Landucci et al., 1998, 2000; Soares et al., 1998; Andrião-Escarso et al., 2000; Kanashiro et al., 2002). These results suggest that, for some PLA2s, catalytic activity plays a role in the oedematogenic effect.

There was an increase of CK and IL-6 levels by the injection of BmTX-I and the crude venom from *B. moojeni*. Myonecrosis might be the trigger for the systemic release of the proinflammatory cytokine IL-6 observed, in agreement with earlier findings with *Bothrops asper* myotoxin II (Lomonte et al., 1993).

All these biological effects induced by the toxin occur in the presence of a measurable $PLA_2$ activity. Although the catalytic activity of $PLA_2$ contributes to pharmacological effects, it is not a prerequisite (Díaz et al., 1992; Chaves et al., 1998; Landucci et al., 1998, 2000; Andrião-Escarso et al., 2000; Kanashiro et al., 2002). However, further studies are necessary to identify the structural determinants involved in these pharmacological activities.

Some authors (Kini, 2003; Gutiérrez and Ownby, 2003; Ponce-Soto et al., 2006; Gutiérrez et al., 2008), have proposed several models to explain $PLA_2$ catalytic and pharmacological activities. In these models $PLA_2$ has two separated places; one is responsible for catalitic activity and other for biological activitiy expression. In according to them, the pharmacological place would be located in the surface of $PLA_2$ molecules. In according to the model

proposed by Kini and Evans (1987), the anti-coagulant place would be located in a region between the 53 and 76 residues, considering this region charged positively in the $PLA_2$ with high anti-coagulant activity. In $PLA_2$ with moderate or low anti-coagulant activity, there is a predominancy of negative chargings. This region is placed in a distinct local and separated of foreseen regions by neurotoxicity and myotoxicity.

Hence, $PLA_2$ are important tools for the development of anti-inflammatory drugs since several lines of evidence (Nevalainen et al., 2000; Da Silva et al., 2008) indicate that human $PLA_2$s, particularly the pancreatic group IB $PLA_2$ ($PLA_2$-IB) and synovial-type group IIA $PLA_2$ ($PLA_2$-IIA), have a pivotal role in inflammatory diseases.

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