Toxicon 127 (2017) 22–36

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Exploring and understanding the functional role, and biochemical and structural characteristics of an acidic phospholipase A_2, AplTx-I, purified from *Agkistrodon piscivorus leucostoma* snake venom

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ARTICLE INFO

Article history:
Received 4 September 2016
Received in revised form
28 November 2016
Accepted 3 January 2017
Available online 4 January 2017

Keywords:
Acidic phospholipase A_2
Snake venom
Agkistrodon piscivorus leucostoma
Pharmacological activity

ABSTRACT

Phospholipases A_2 (PLA_2s) constitute a class of extensively studied toxins, isolated from snake venoms. Basic PLA_2 isoforms mediate various toxicological effects, while the acidic isoforms generally have higher enzymatic activities, but do not promote evident toxic effects. The functions of these acidic isoforms in snake venoms are still not completely understood and more studies are needed to characterize the biological functions and diversification of acidic toxins in order to justify their abundant presence in these secretions. Recently, Lomonte and collaborators demonstrated, in a proteomic and toxicological study, high concentrations of PLA_2s in the venom of *Agkistrodon piscivorus leucostoma.* We have, herein, purified and characterized an acidic PLA_2 from this snake venom, denominated AplTx-I, in order to better understand its biochemical and structural characteristics, as well as its biological effects. AplTx-I was purified using two chromatographic steps, in association with enzymatic and biological assays. The acidic toxin was found to be one of the most abundant proteins in the venom of *A. p. leucostoma;* the protein was monomeric with a molecular mass of 13,885.8 Da, as identified by mass spectrometry ESI-TOF and electrophoresis. The toxin has similar primary and tridimensional structures to those of other acidic PLA_2s, a theoretical and experimental isoelectric point of ≈5.12, and a calcium-dependent enzyme activity of 25.8985 nM/min/mg, with maximum values at 37 °C and pH 8.0. Despite its high enzymatic activity on synthetic substrate, AplTx-I did not induce high or significant myotoxic, coagulant, anticoagulant, edema, neuromuscular toxicity in mouse phrenic nerve-diaphragm preparations or antibacterial activities. Interestingly, AplTx-I triggered a high and selective neuromuscular toxicity in chick *biventer cervicis* preparations. These findings are relevant to provide a deeper understanding of the pharmacology, role and diversification of acidic phospholipase A_2 isoforms in snake venoms.

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1. Introduction

The *Agkistrodon piscivorus leucostoma*, also known locally as the Western Cottonmouth is a venomous pit viper subspecies that is geographically found from southern Illinois to Alabama (Jia et al., 2008). Little information exists in the medical literature regarding the clinical features of envenomation by this subspecies and only some proteomic and transcriptomic studies on its venom have been published (Lomonte et al., 2014). Furthermore, until now, none of the proteins or peptides have been directly purified from this snake venom for functional and structural characterization, although some studies have reported the characterization of some proteins

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http://dx.doi.org/10.1016/j.toxicon.2017.01.002
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from this subspecies using the venom gland and molecular cloning techniques (Jia et al., 2009).

In a previous study of the composition of the *A. p. leucostoma* snake venom and the expression of transcripts at a molecular level, genes encoding phospholipases A_2 (PLA_2s) were the most expressed and accounted for 35% of gene expression (Jia et al., 2008). In line with this transcriptomic analysis, suggesting a predominance of PLA_2s, Lomonte et al. (2014) recently compared *Agkistrodon* venom proteomes and demonstrated that the venom of the Western Cottonmouth pitviper is composed mainly of PLA_2s (33.6%), metalloproteases (33.1%) and serinoproteases (13.2%) and presents low myotoxicity activity.

The PLA_2s (EC 3.1.1.4) constitute one of the most extensively studied toxins of snake venoms and are known to induce a variety of pharmacological activities, such as platelet aggregation, pro-inflammatory, anticoagulant, hypotensive, hemolytic, bactericidal, myotoxicity, neurotoxicity, among others effects (Schaloske and Dennis, 2006; Carvalho et al., 2013). Owing to this range of effects, the PLA_2s are commonly referred to as multifunctional toxins (Gutierrez and Lomonte, 2013; Almeida et al., 2016a). The majority of these multifunctional proteins are aggressive and toxic, being frequently associated with important roles in the detention and death of the prey (Ghazaryan et al., 2015; Corrêa et al., 2016).

Snake venoms PLA_2s may exhibit different isoelectric point (pI) values, ranging from acidic to highly basic values (Vargas et al., 2012). Different proportions of each isoform can be found and diversified in the venom of a single snake species (Saikia et al., 2011). The subtle features and biochemical properties displayed by these toxins, such as cationic or anionic nature, may result in different pharmacological activities that may, consequently, modulate the pathophysiological effects mediated by a snakebite accident (Vija et al., 2009).

All acidic PLA_2s that have been characterized from the venoms of snakes belonging to the Viperidae family present an Asp residue at position 49 and often exert a higher enzymatic activity than the basic PLA_2s isoforms upon their substrates (Vargas et al., 2012). Curiously, despite their higher catalytic activities, acidic toxins do not induce significant toxic effects and some authors have suggested that they play a digestive role (Fernandez et al., 2010). When the effects induced by the acidic PLA_2s are analyzed, they are usually non-lethal, non-toxic and only a few of these acidic PLA_2s mediate pharmacological effects, such as inhibition of platelet aggregation (Vija et al., 2009), myotoxic (Santos-Filho et al., 2008), antiplatelet (Teixeira et al., 2011), bactericidal (Vargas et al., 2012) and antitumoral effects (Roberto et al., 2004).

As most toxic PLA_2 are basic proteins with lower enzymatic activities than the acidic PLA_2s, a greater understanding of the diversification, biological roles and structure-function/enzymatic-pharmacological relationships of acidic toxins is needed, making it essential to isolate and characterize new variants of these isoforms (Jimenez-Charris et al., 2016). Given the above, we herein isolated, and structurally and biologically characterized an acidic phospholipase A_2 from the venom of *A. p. leucostoma,* in order to better understand its structural, biochemical and pharmacological features.

2. Material and methods

2.1. Venom and reagents

The venom of *Agkistrodon piscivorus leucostoma* was provided by the National Natural Toxins Research Center (NNTRC) of the University Kingsville, Texas (USA). All reagents used were of sequencing or analytical grade.

2.2. Animals

The animals used were purchased from the Multidisciplinary Center for Biological Research in Science Area with Laboratory Animals (CEMIB) of the State University of Campinas. The following animals were used; Swiss male mice (18–20 g), male Wistar rats (300–400 g) and HY-Line W36 chicks (4–8 days old) maintained at 24-28 °C with food and water *ad libitum*. All biological tests were carried out with the authorization of the Committee for Ethics in Animal Experimentation of the Institute of Biology of Campinas, Protocol 3951-1.

2.3. Purification of AplTx-I from Agkistrodon piscivorus leucostoma venom

To isolate the acidic PLA_2 Asp49, AplTx-I, from *A. p. leucostoma* venom, two chromatographic steps were used. Initially, a gel filtration system with two Superdex-75 columns coupled in series was employed. Twenty mg of crude venom were dissolved in 200 μL of 50 mM ammonium bicarbonate buffer at pH 8.0, before vortexing for 2 min and centrifuging at 9000 rpm for 3 min. The pellet was discarded and the supernatant applied to the chromatographic system, previously equilibrated with 50 mM ammonium bicarbonate. The fractions were eluted with 0.2 M ammonium bicarbonate and the run was monitored at 280 nm under a flow of 0.8 mL/min at 60 min. All fractions were collected and lyophilized. This experimental procedure was repeated forty times. In the second purification step, the fraction that showed higher phospholipase activity, called Apl-V, was subjected to reversed-phase high-performance liquid chromatography (RP-HPLC). The system used was a HPLC- PDA 991 (Waters), equipped with two Waters model 510/B pumps, an U6K automatic injector sample with a "loop" of 200 μL and a Column Discovery Bio Wide Pore C18 Supelco (4.6 mm × 25 cm, 10 μm column), previously equilibrated with 0.1% trifluoroacetic acid (TFA), pH 3.5 (buffer A). Five mg of the Apl-V fraction were dissolved in 120 μL 0.1% trifluoroacetic acid (TFA) and 80 μL of 1M ammonium bicarbonate buffer. The solution was vortexed for 2 min and clarified at 8000 rpm for 3 min. The supernatant was applied to the chromatographic system and the elution was monitored at 280 nm under a constant flow of 1 mL/min using a gradient (0–100%) of buffer B (66% acetonitrile in buffer A). The molar absorption coefficient of the purified enzyme was determined by the method of Edelhoch (1967).

2.4. Biochemical and structural characterization

2.4.1. Mono-dimensional electrophoresis

The homogeneity of AplTx-I was evaluated by SDS-PAGE under reducing and non-reducing conditions following the methodology described by Laemmli (1970). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out discontinuously using a gel concentration of 5% and a running gel of 12.5%. Briefly, the purified reduced and non-reduced acidic PLA_2, as well as the molecular weight markers were dissolved in sample buffer (0.075 M Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 0.001% bromophenol blue) and heated. The electrophoretic run was performed at 30 mA and the gel was stained; the dye excess was removed using 7% acetic acid.

2.4.2. Two-dimensional electrophoresis (isoelectric focusing)

The theoretical pI of the purified toxin was calculated using a protein analysis tool (Compute pI/Mw tool), available on the Expasy server, and the experimental pI was determined following the methodology used and described by Corrêa et al. (2016). Briefly,

AplTx-I was solubilized and applied on a polyacrylamide strip (7 cm) with a pH gradient from 3.0 to 10.0, immobilized linearly (Immobiline DryStrip®). Isoelectric focusing was performed in an IPGphord III System (GE Healthcare Life Sciences). After this step, the strip was placed in the superior part of a 12.5% SDS-PAGE (m/v) to perform the second dimension (15 mA, 90 min). Posteriorly, the gel was fixed and stained with Coomassie Blue G 250.

2.4.3. Enzymatic activity

Determination of PLA_2 activity was assayed using the synthetic chromogenic substrate, 4-nitro-3-octanoyloxybenzoic acid (NOBA). The colorimetric method described by Holzer and Mackessy (1996) was employed and adapted for use in a 96-well microplate, according to Calgarotto et al. (2008). Briefly, 3.1 mg substrate was dissolved in 1 mL of 1 M acetonitrile and subsequently added to 9 mL buffer (10 mM Tris-HCl, 1 M $CaCl_2$, 10 mM NaCl, pH 8.0). After this, 100 μg of each fraction obtained or the purified toxin were dissolved in 100 μL 0.15 M NaCl. Two-hundred-and-twenty μL of substrate solution and 20 μL test sample were added to each microplate well. The reaction product was colorimetrically quantified using a VERSA max microplate reader (Molecular Devices, Sunnyvale, CA) and calculated according to the change in absorbance, monitored at 425 nm for 40 min at 37 °C. The PLA_2 activity was expressed as the initial rate of reaction (V_o) based on the increase in absorbance after 20 min and performed in triplicate.

To evaluate optimal pH, the following buffers were used: sodium citrate (pH 4, 5 and 6), Tris-HCl (pH 7 and 8) and glycine (pH 9 and 10). To determine the optimal temperature for reaction, the assays were carried out at different temperatures (10, 15, 20, 25, 30, 35, 37, 40, 45, 50, 55 and 60 °C). The influence of substrate concentration on the PLA_2 activity of AplTx-I was assayed using different substrate concentrations; 0.1, 0.2, 0.5, 1, 2.5, 5, 10 and 20 mM. Furthermore, enzymatic activity was tested in the presence of the divalent ions: Ca^{2+}, Zn^{2+}, Cd^{2+}, Mg^{2+} and Mn^{2+}. For the preparation of buffers containing these cations, the following metal salts were used, respectively: $CaCl_2$, $ZnCl_2$, $CdCl_2$, $MgCl_2$, $MnCl_2$.

2.4.4. Mass spectrometry analysis

2.4.4.1. Determination of AplTx-I mass. The isotope-averaged molecular mass of AplTx-I was determined using a NanoAcquity Ultra Performance LC, coupled with a nanoelectrospray source, using a Waters Q-TOF Ultima mass spectrometer. An aliquot of purified protein (4.5 μL) was injected into a C18 reverse phase column UPLC (nanoACQUITY UPLC Waters) coupled with a mass spectrometry system. A gradient of 0–50% acetonitrile in 0.1% formic acid was used for 45 min at a flow rate of 600 nL/min. The instrument was operated in continuous mode and MS data acquisition was carried out at 100–3000 m/z, with a speed of 1 s and delay of 0.1 s. The processing parameters were; output 6000–20,000 Da mass range with a resolution of 0.1 Da/channel.

2.4.4.2. Determination of primary structure of AplTx-I

2.4.4.2.1. N-terminal sequencing. The N-terminal sequence of 50 μg ApLTx-I was carried out in an automatic microsequencer PPSQ-33A (Shimadzu) according to the chemical process of sequencing by N-terminal cleavage derivative method described by Edman (1950).

2.4.4.2.2. AplTx-I digestion and analysis of digested proteins. For AplTx-I digestion, initially, 50 μL of protein and 10 μL of 50 mM ammonium bicarbonate, at pH 8.5, were added to a microtube. The sample was subjected to a rapid centrifugation, followed by the addition of 5 μL dithiothreitol (DTT), vortexing and incubation at 56 °C for 25 min. Subsequently, 150 μL 5 mM iodoacetamide were added and the tube incubated for 30 min at room temperature protected from light. Five μL of DTT 5 mM were then added and incubated for 15 min at room temperature, also protected from light. Afterwards, a solution of 1 mM calcium chloride and 10 μL proteolytic enzyme (trypsin) was added, followed by incubation for 16 h at 37 °C. In order to stop the restriction digestion of the purified toxin, 100 μL of 0.4% trifluoroacetic acid (TFA) were added, vortexed and centrifuged at 2500 × g for 10 min. The pellet was discarded and the supernatant was transferred to a vial with 5 μL 1 N ammonium hydroxide. The same experimental protocol was performed using the endoproteinases, GluC and trypsin/CNBr (chemical reagent).

The analysis of peptides obtained from the digestion of AplTx-I with proteolytic enzymes was performed at the National Biosciences Laboratory (LNBio) CNPEM-ABTLuS, Campinas, in a nanoACQUITY Ultra LC Performance coupled with a nanoelectrospray source and Waters quadrupole-time-of-flight analyzer (Q-TOF) mass spectrometer Ultima (Milford, Massachusetts, USA). A study of overlapping peptides obtained from the enzymatic digestion was performed. All fragments obtained from spectrometry were interpreted manually to derive de novo amino acid sequences. The alignment of the peptide fragments and the identification of structural homology with other PLA_2s were carried out using available snake venom databases, including NCBI-BLAST.

2.5. Tridimensional structure modeling

2.5.1. Template selection for homology modeling

The first step in the three-dimensional determination of AplTx-I was the identification of the protein-template, which was selected from the Protein Data Bank (PDB) after bioinformatic and homology studies. The protein template selected for determination of the 3D model of AplTx-I was characterized and published by Zhao et al. (1998) and Xu et al. (2003); this protein is an acidic and monomeric PLA_2, isolated from the *Agkistrodon halys pallas* snake venom (PDB access: 1M8R). The alignment of the sequences of these two proteins (AplTx-I and 1M8R) demonstrated 82% identity.

2.5.2. Molecular dynamic studies and model validation

The homology model of AplTx-I were built and optimized using the Prime software from Schrödinger suite. After constructing the 3D model of AplTx-I, molecular dynamic (MD) simulations were conducted to enhance it and to verify the stability of the obtained structure. Counter-ions were added, aiming to neutralize the protein for the definition of the protonation states of the charged groups at a pH of 7. MD was performed using the OPLS-AA force field within the Desmond package v2.0 contained in Maestro 9.2 suite. The simulation was set in 20 ns and number of atoms constant using the Nosé-Hoover method with a relaxation time of 1 ps applying the MTK algorithm. The SHAKE algorithm was employed for every hydrogen atom and the cutoff for van der Waals forces was set at 9 Å and the long-range electrostatic forces were modeled using the particle mesh Ewald method. A restriction was applied on the backbone atoms with a constant force of 0.5 kcal × mol^{-1} × $Å^{-2}$. Data were collected every 4 ps during the MD. The model constructed was validated and analyzed using Root Mean Squared Deviation and PROCHECK.

2.6. Pharmacological characterization

2.6.1. Local and systemic myotoxic activity

The ability of AplTx-I to induce the local myotoxic and systemic activity was evaluated in groups of five mice (18–20 g), according to methodology also used by Gutiérrez et al. (2008). The animals received intramuscular (local myotoxicity) or intravenous injections (systemic myotoxicity) of different amounts of acidic PLA_2 (5, 10, and 20 μg) dissolved in 50 μL of PBS buffer. As a negative

control, the mice received an intramuscular or intravenous injections of 50 μL of PBS alone. Following toxin administration, tail blood samples were collected into heparinized capillaries from the mice tail at various time intervals (0, 1, 3, 6, 9, 12 and 24 h). The blood samples collected were immediately centrifuged and the plasma used for plasma creatine kinase (CK) quantification using a colorimetric assay, CK-NAC UV UniTest kit (Wiener lab). For this kinetic assay, 200 μL of substrate were pre-incubated for 4 min at 37 °C, followed by the addition of 5 μL of plasma. The reaction production was monitored at 340 nm for 6 min, according to the manufacturer's instructions. Myotoxic activity was expressed in U/L, which is defined as the phosphorylation of 1 mmol of creatine/min at 25 °C. The toxin, ColTx-I (5 μg), characterized and provided by Almeida et al. (2016b) was used as a positive control in both local and systemic assays.

2.6.2. Inflammatory activity (paw edema)

To evaluate the edema-forming activity caused by AplTx-I, groups of mice (18–20 g) received subcutaneous injections of different concentrations of toxin (5, 10, and 20 μg) dissolved in 50 μL of PBS in the right footpad. As a negative control, the left footpad received an identical injection of PBS (50 μL) alone. Footpad thickness was determined at different time intervals (0, 0.5, 1, 3, 6, 9, 12 and 24 h) after the injection of toxins and control solution, using a low-pressure spring caliper. Edema-forming activity was calculated as described by Lomonte et al. (1993); based on the percentage of increase in volume produced when the volume of the left and right footpads are compared. The toxin, ColTx-I (5 μg), characterized and provided by Almeida et al. (2016b) was used as a positive control.

2.6.3. Effect on coagulation

2.6.3.1. Coagulant activity. Blood samples (10 mL) from male Wistar rats (300–400 g), obtained from the descending abdominal aorta, were added to 1 mL of 3.8% sodium citrate and centrifuged at 2500 × g for 15 min (4 °C) to obtain platelet-rich plasma (PRP). Triplicate assays were carried out of the positive controls (20U thrombin or 40 μg *B. jararaca* snake venom), negative control (saline) or with AplTx-I toxin (20–160 μg). Coagulant activity was estimated from the ability of the purified toxin to accelerate the coagulation of normal rat plasma and determined by the clotting time. If the sample did not induce plasma clotting within 600 s (10 min), it was considered to present no plasma coagulant activity.

2.6.3.2. In vitro anticoagulant activity. In vitro anticoagulant activity was determined according to the methodology used by Clement et al. (2012) with some adaptations. Briefly, different amounts of AplTx-I toxin (20–160 μg) in 0.15 M NaCl in a final volume of 100 μl were added to 0.4 ml of rat plasma with sodium citrate at 0.4%, which were incubated for 1 min at 37 °C. Subsequently, 100 μl of 50 mM $CaCl_2$ was added and the clotting time was determined. Plasma samples treated in the same manner, but without addition of acidic toxin were used as control.

2.6.4. Antibacterial activity

The antibacterial potential of AplTx-I was assayed using the standardized single disk method in agar, as described by Bauer et al. (1966). For the assay, five bacterial strains were used: *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* 31NM, *E. coli* ATCC 25922, *Staphyloccocus aureus* BEC9393 and *Staphyloccocus aureus* Rib1. The isolated acidic PLA_2 was tested in increasing concentrations up to 3 mg/mL. Commercially available antibiotics, Ofloxacin and Imipinen, were used as positive controls.

2.6.5. Neurotoxic activity assay

The neurotoxic activity of AplTx-I was evaluated using two distinct nerve preparations (animal models): the chick *biventer cervicis* (BC) nerve-muscle preparations and the mouse phrenic nerve-diaphragm (PND) preparations.

The chick *biventer cervicis* (BC) nerve-muscle preparation was isolated and assembled, following the protocol described Ginsborg and Warriner (1960). According to this method, the chicks were anaesthetized with halothane and, after isolation, the muscle was suspended in a 5 mL bath (Automatic organ multiple-bath LE01, Letica Scientific Instruments Barcelona, Spain) containing Krebs nutrient solution with the following composition in mM: NaCl, 118.7; KCl, 4.69; $CaCl_2$, 1.88; KH_2PO_4, 1.17; $MgSO_4$, 1.17; $NaHCO_3$ 25.0 and $C_6H_{12}O_6$ 11.65. The solution was constantly aerated with carbogen (95% O_2 and 5% CO_2 mixture) and maintained at 37 °C. The preparation was subjected to a constant tension of 1 g and stimulated through bipolar electrodes (field stimulation). Supramaximal pulses of 0.1 Hz frequency and 0.2 ms were applied (MAIN BOX 12404 LE Panlab s.l. Powerlab AD Instruments Barcelona, Spain). The maximum muscle contractions caused by electrical stimuli and contraction in response to the addition of KCl (20 mM) and ACh (110 mM) were recorded using isometric transducers (Model MLT0201 Force transducer 5 mg - 25 g Panlab sl, AD Instruments Pty Ltd. Spain) connected to a PowerLab/4SP (Ouad Bridge AD Instruments, Barcelona, Spain). Records of contractions for KCl and ACh were performed with the absence of electrical stimulation at the beginning (before addition of toxin) and at the end of the experiment (after 120 min incubation with different amount of AplTx-I).

The diaphragm and its phrenic nerve were dissected from mice euthanized with isoflurane. The preparations were mounted under a resting tension of 1 g in a 5 mL organ bath containing aerated (95% O_2 and 5% CO_2) and Tyrode solution (composition: 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.42 mM NaH_2PO4, 11.9 mM $NaHCO_3$ and 11.1 mM glucose) at 37 °C, as described elsewhere by (Bülbring, 1997). Supramaximal stimuli (0.1 Hz and 0.2 ms for indirect stimulation) were delivered from a Grass S88 stimulator (Grass Instrument Co., Quincy, MA, USA). After stabilization for 20 min, the preparations were exposed to AplTx-I (different concentrations) for 120 min. Twitch responses were recorded and compared to those recorded from control preparations (Tyrode solution).

2.7. Statistical analysis

Each experiment was repeated at least 3 times and the results are reported as the means ± standard deviations (SD). The statistical significance of differences between two groups was evaluated by Student's t-test, while the comparison of more than two experimental groups was determined by ANOVA tests, followed by Tukey's Post Hoc test using the Origin Pro 8 Software (OriginLab Corporation). Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Purification of AplTx-I from A. p. leucostoma

The acidic Asp49 PLA_2, AplTx-I, was isolated from the whole venom of *A. p. leucostoma* using two chromatographic methods; molecular exclusion and reverse-phase chromatography. The fractionation profile of the *A. p. leucostoma* snake venom on a gel filtration system, employing two Superdex-75 columns coupled in series, for the first purification steps is shown in Fig. 1A. The chromatogram demonstrates twelve major fractions, which were

numbered and denominated according to their elution, from Apl-I to Apl-XII (Fig. 1A). Due to its high PLA_2 activity, the Apl-V fraction was selected for separation by RP-HPLC (shown in Fig. 1B). The peaks obtained in this second chromatography step were screened for PLA_2 activity and the peak with the highest enzymatic activity highlighted in the chromatography was denominated AplTx-I and selected for structural and functional characterization. Probably, AplTx-I is one of the most abundant proteins of this snake venom. From 800 mg snake venom used, 15.8 mg of pure enzyme was obtained.

The course of toxin purification was accompanied by an increase in PLA_2 activity. AplTx-I showed a higher PLA_2 activity, when compared with Apl-V and crude venom (Fig. 1C). The homogeneity and purity of the fraction selected for our study was assayed by electrophoretic analysis in SDS-PAGE and by RP-HPLC. When analyzed by RP-HPLC, a single well-resolved sharp peak was observed, demonstrating the purity of protein (Fig. 1D). In the electrophoretic analyzes by SDS-PAGE, the purified protein migrated as a single band of ~14 kDa under non-reducing and reducing conditions, confirming the high level of purity and its monomeric nature (Fig. 1D - insert). The purified enzyme presented a molar absorption coefficient of 29,370 $g^{-1}M^{-1}$, determined according to the methodology described by Edelhoch (1967).

3.2. Biochemical and structural characterization of AplTx-I PLA_2

The electrophoretic mobility and relative molecular mass of AplTx-I, assayed by SDS-PAGE, is consistent with its isotope-averaged molecular mass of 13,885.79 Da, as determined by mass spectrometry (Fig. 2).

The primary structure of AplTx-I was obtained by N-terminal sequencing and digesting the toxin with different proteolytic enzymes and chemical reagent, together with analysis by tandem mass spectrometry and manual interpretation of mass spectrometry to derive de novo amino acid sequences. Seventeen peptides were identified (Table 1) from the digested protein, which allowed the classification of the protein as an Asp49 PLA_2. AplTx-I is composed of 122 amino acids residues, containing the conserved His48/Asp49 dyad (in the common numbering system). These data are in agreement with the enzymatic assay results, which demonstrated the capacity of AplTx-I to cleave a synthetic substrate.

Comparison of the primary structure of AplTx-I with those of similar proteins from snake venoms in a database revealed that the toxin shares functional motifs with group IIA PLA_2s. The highest similarity was obtained with the acidic toxins that have been purified and characterized from Viperidae venoms (Fig. 3A). AplTx-I shows a theoretical pI of 5.12. Two-dimensional electrophoresis confirmed the acidic nature of the purified molecule and showed an approximately experimental pI of 5.0 (Fig. 3B), which is in line with its theoretical value, as determined by bioinformatic tools.

The three-dimensional model of AplTx-I, constructed by homology modeling using the crystallographic structure of *Agkistrodon halys pallas* acidic PLA_2 (PDB access: 1M8R) as a template, demonstrated that AplTx-I presents an expected molecular architecture that is similar to that of other acidic PLA_2s. AplTx-I presented secondary structures typically found in this group of proteins (three parallel α-helixes and a β-wing - one double-stranded anti-parallel β-sheet) (Fig. 4A). The evaluation of the quality of the tridimensional structure by Ramachandran plot analysis showed that the model obtained is energetically and stereochemistry favorable (Fig. 4B).

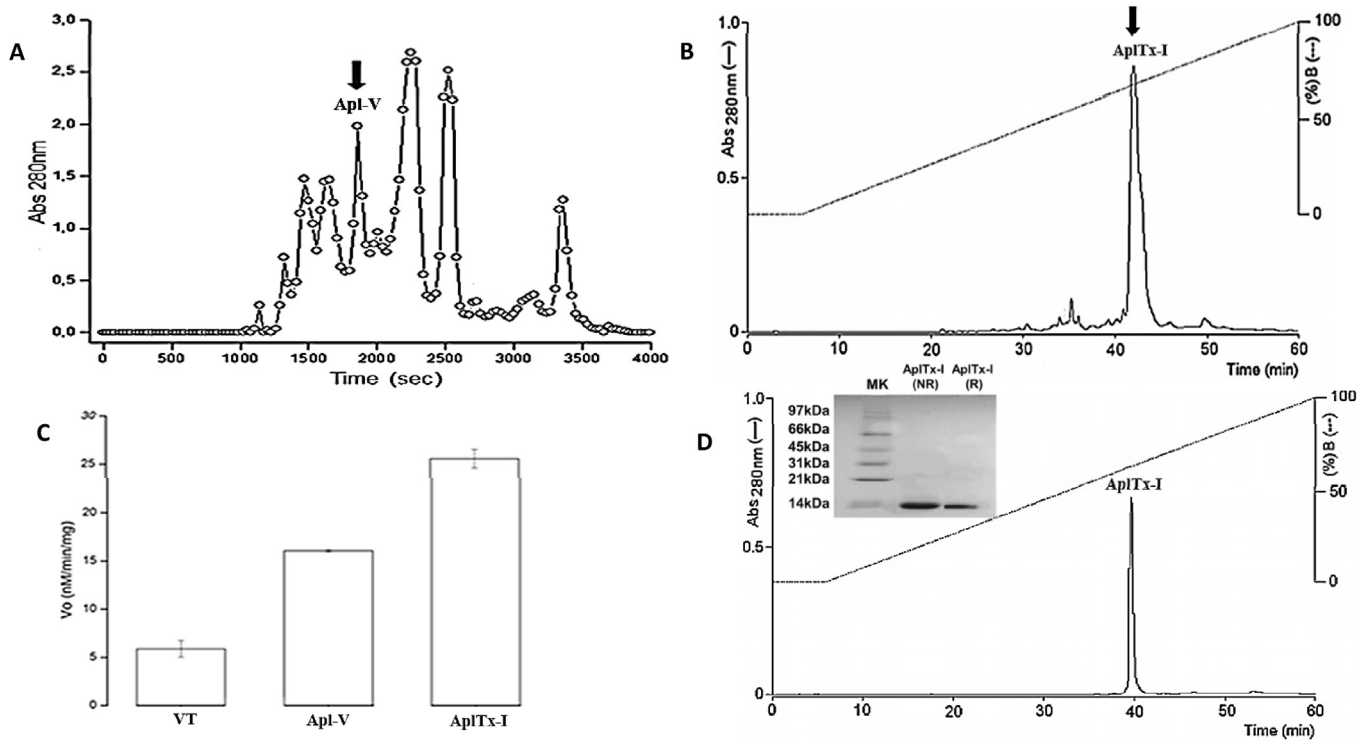

Fig. 1. Sequential purification of the acidic Aps49 PLA_2, AplTx-I, from *A. p. leucostoma*. **(A)** Fractionation of crude *A. p. leucostoma* snake venom on a gel filtration system with two Superdex-75 columns coupled in series eluted with 0.2 M ammonium bicarbonate. **(B)** Profile elution of Apl-V by RP-HPLC on an analytical Discovery Bio Wide Pore C18 Supelco Column (4.6 mm × 25 cm; 10 μm). The peak corresponding to AplTx-I is highlighted. **(C)** The PLA_2 activity was monitored during the purification procedure. PLA_2 activity of crude venom (VT), Apl-V (fractions selected from size exclusion) and AplTx-I (RP-HPLC). **(D)** Rechromatography of AplTx-I, obtained from the first purification step, on a C18 column. Insert: SDS-PAGE (12.5%) analysis of AplTx-I under reducing (R) and non-reducing conditions (NR). MK: low molecular weight marked, as indicated on the left, in kDa.

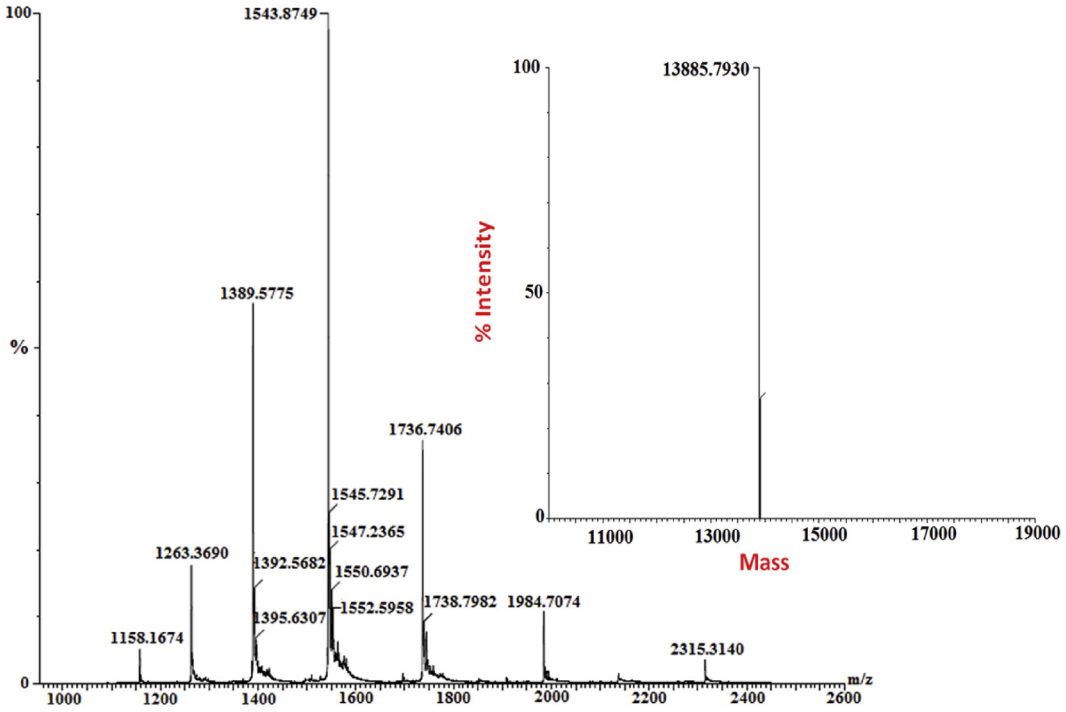

Fig. 2. Mass determination of the isolated acidic PLA_2 by mass spectrometry. The figure exhibits the convoluted spectrum of purified toxin. The isotope-averaged molecular mass of AplTx-I was determined using a NanoAcquity Ultra Performance LC coupled with nanoelectrospray source on a Q-TOF Ultima mass spectrometer. The insert shows the deconvoluted spectrum, indicating the exact mass of AplTx-I of 13885.7930 Da, confirming the relative molecular mass determined by SDS-PAGE.

Table 1
Peptide fragments from the digestion of AplTx-I with proteolytic enzymes and chemical reagent (CNBr), as identified by LC-MS/MS sequencing. The eluted peptides from proteolytic and chemical digestion were dried in a vacuum centrifuge and resuspended in 1% formic acid for LC-MS/MS analysis. All fragmentation spectra obtained were interpreted manually to derive de novo amino acid sequences.

Number	Sequenced Fragment	Monoisotopic		Accuracy (ppm)
		Measured	Theoretical	
1	DLMQFETLIMK	1367.6829	1367.6802	1.97
2	DLMQFETLIMKIAK	1679.9190	1679.9287	−5.77
3	QWYSAYGCYCGWGGQGKPQDATDR	2810.1670	2810.1674	−0.14
4	CCFVHDCCYGK	1504.5356	1504.5417	−4.05
5	RCCFVHD	992.4041	992.4028	1.31
6	CCFVHDCCYGKVTGCDPK	2261.9085	2261.9050	1.55
7	VTGCDPK	775.3654	775.3607	6.06
8	TYTYSVE	861.3844	861.3829	1.74
9	IVCGGNPCKKE	1375.6256	1375.6297	−2.98
10	LDTYTYSVENGDIVCGGNDPCK	2476.0685	2476.0593	3.72
11	EICECDR	980.3892	980.3887	0.51
12	AAAICFR	807.4161	807.4191	−3.72
13	EICECDRAAAICFR	1769.7647	1769.7800	−8.65
14	AAAICFRDNK	1164.5910	1164.5956	−3.95
15	DNKVTYDNK	1095.5396	1095.5452	−5.11
16	VTYDNKYWR	1243.6186	1243.6229	−3.46
17	FPPQNCKEESEPC	1620.6548	1620.6518	1.85

The enzymatic activity of AplTx-I was assayed using the chromogenic substrate, 4-nitro-3-(octanoyloxy) benzoic acid (Fig. 5). The effect of the substrate on the AplTx-I is shown in Fig. 5A. Under the conditions used in this experiment, AplTx-I showed a Michaelian behavior. The K*m* and Vmax kinetic parameters of the purified enzyme were calculated from a reciprocal double graph, being 1.7488 mM and 14.43 nmol/min, respectively (Fig. 5B). Maximum enzyme activity was obtained at 37 °C (Fig. 5C) and pH 8.0 (Fig. 5D). Additionally, AplTx-I was strictly dependent on the Ca^{2+} cation for its enzymatic activity. In contrast to the divalent Ca^{2+}cation, none of the other positive ions evaluated (Cd^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}) demonstrated the ability to activate the purified acidic PLA_2. These clearly indicate that the substitution of Ca^{2+} by other positive divalent ions significantly decreases the PLA_2 activity of AplTx-I. Curiously, when Ca^{2+} was added in a kinetic assay with Mg^{2+} and Mn^{2+}, AplTx-I presented significant PLA_2 activity; however, a considerable inhibition of catalytic activity was observed for Cd^{2+} and Zn^{2+}, even in the presence of 10 mM Ca^{2+} (Fig. 5E).

3.3. Pharmacological characterization of AplTx-I

In order to identify the probable functional roles of the

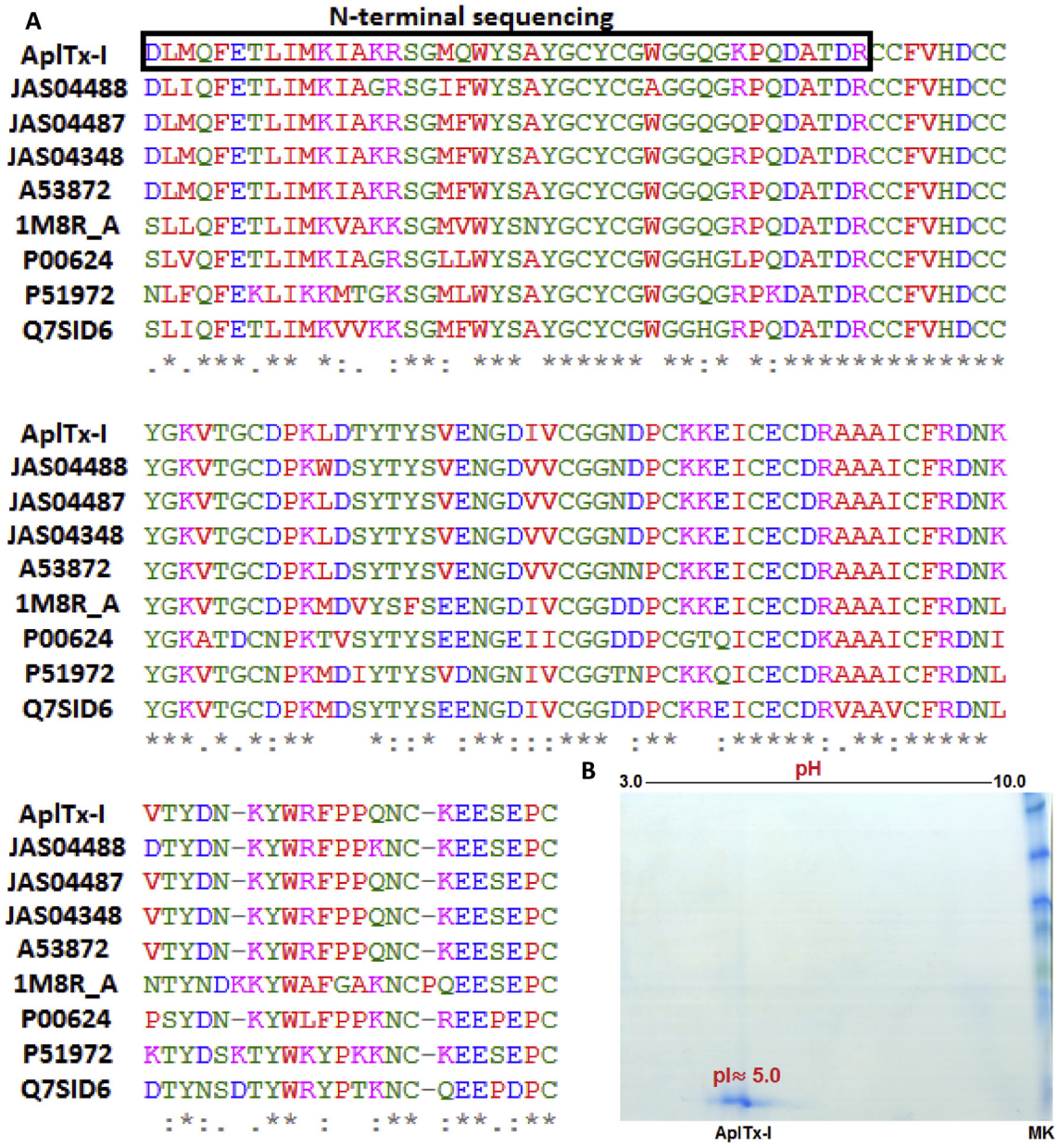

Fig. 3. Comparison alignment of the amino acid sequence of AplTx-I with similar Asp49 PLA_2s from snake venoms and determination of experimental pI. (A) Asterisks represent identical amino acids. Two dots demonstrate conservation between groups with highly similar properties, while dots correspond to conservation between groups with weakly similar properties. Protein codes in the BLAST database are shown on the left of each primary structure. The theoretical pI shown in the legend was calculated using the Compute pI/Mw bioinformatic tool using the amino acid sequence of each protein reported in the multiple alignment. AplTx-I: acidic PLA_2 from *Agkistrodon piscivorus leucostoma*, pI: 5.12; JAS04488: acidic PLA_2 from *Agkistrodon piscivorus*, pI: 5.12 (Rokyta et al., 2015); JAS04487: acidic PLA_2 from *Agkistrodon piscivorus*, pI: 4.93 (Rokyta et al., 2015); A53872: acidic PLA_2 from *Agkistrodon piscivorus piscivorus*, pI: 5.38 (Welches et al., 1993); JAS04348: acidic PLA_2 from *Agkistrodon contortrix contortrix*, pI: 4.95 (Rokyta et al., 2015); 1M8R: acidic PLA_2 from *Agkistrodon halys pallas*, pI: 4.82 (Zhao et al., 1998); Q7SID6.2: acidic PLA_2 from *Deinagkistrodon acutus*, pI: 4.68 (Wang et al., 1996b); P51972: basic PLA_2 from *Agkistrodon piscivorus piscivorus*, pI: 8.62 (Welches et al., 1993); P00624: acidic PLA_2 from *Crotalus atrox*, pI: 4.64 (Brunie et al., 1985). (B) 2D SDS-PAGE showing the isolated Asp49 PLA_2 with a pI of 5.0.

abundant acidic PLA_2 in the venom of *A. p. leucostoma*, *in vivo*, *ex vivo* and *in vitro* assays of the pharmacological activities of AplTx-I were carried out to investigate its systemic and local myotoxicity, edema, effects on plasma coagulation, antibacterial potential and neurotoxicity activity. *In vivo*, intramuscular and intravenous injections of AplTx-I did not induce a significant increase in plasma creatine kinase (CK) concentrations, indicating the very low and no ability of the purified acidic PLA_2 to induce muscular cell damage in sites both near (Fig. 6A) or distant (Fig. 6B) from the toxin inoculation site, respectively. When the local myotoxic activity of AplTx-I was compared with the myotoxicity of a basic Aps49 PLA_2 provided by Almeida et al. (2016b), the myotoxic activity of AplTx-I was found not to be significant.

The subplantar injection of AplTx-I also presented an low-level effect on local edema in the mice footpad assay (Fig. 7). Its highest value of edema-forming activity (44%) was induced by a concentration of 20 μg of AplTx-I, 30 min after toxin inoculation; similarly to inflammatory activity promoted by 5 μg ColTx-I, used as positive control in this assay.

A qualitative test was carried out to acquire information about

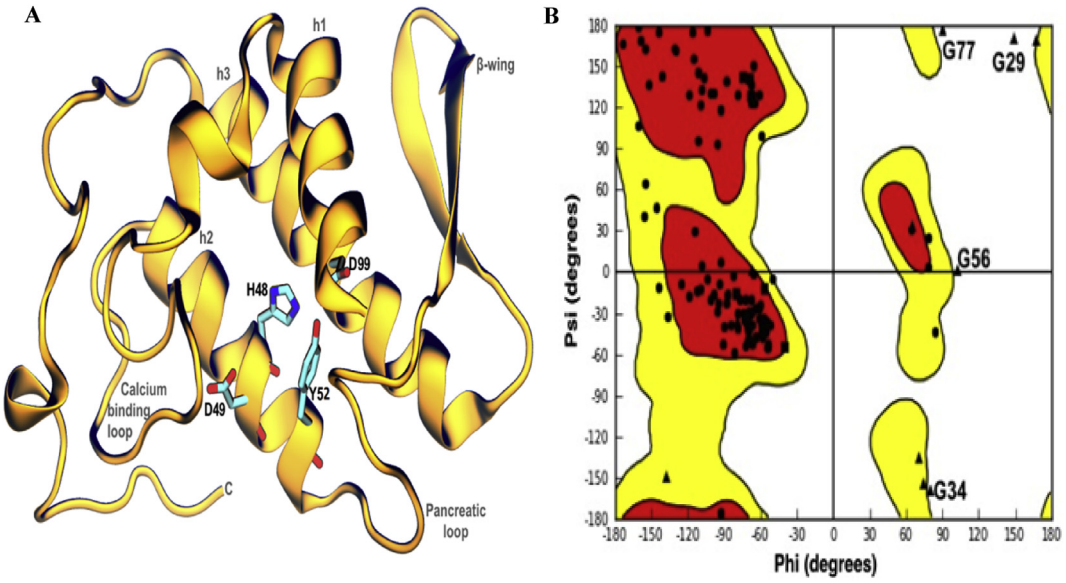

Fig. 4. Three-dimensional scaffold of the AplTx-I, determined by homology modeling. The crystal structure of *Agkistrodon halys pallas* acidic PLA_2 (PDB access: 1M8R) was employed as a protein template. **(A)** AplTx-I demonstrated a similar molecular architecture to that of other Asp 49 PLA_2s. The conserved regions and the amino acids important for enzymatic activity are highlighted. **(B)** Ramachandran plot: Most amino acid residues in the AplTx-I structure presented a favorable stereochemistry.

the ability of isolated acidic PLA_2 on plasma coagulation. The data obtained showed that AplTx-I does not show coagulant activity. The clotting time was 15.09 ± 1.26 s for the positive control group (thrombin), whereas the negative control group (saline) demonstrated no coagulation up to 10 min. In the presence of acidic toxin, no clotting was observed up to 10 min, similarly to the negative control. AplTx-I also did not cause anticoagulant effect upon rat plasma *in vitro*, up to a concentration of 160 μg/mL. The mean clotting time in rat plasma incubated with purified toxin is similar to mean clotting time of control group as determined by *in vitro* assay (data not shown).

The evaluation of the potential of AplTx-I to inhibit the growth of Gram-negative and Gram-positive bacteria, assayed by the agar diffusion method, demonstrated that the purified acidic PLA_2 was unable to mediate an antibacterial effect against the bacteria tested (*Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* 31NM, *E. coli* ATCC 25922, *Staphyloccocus aureus* BEC9393 and *Staphyloccocus aureus* Rib1), even at 3 mg/mL.

The ability of AplTx-I to mediate neurotoxicity activity was performed using two different animal models; chick *biventer cervicis* (BC) nerve-muscle preparations and mouse phrenic nerve-diaphragm (PND) preparations. In relation to mouse phrenic nerve-diaphragm preparations (PND), AplTx-I (at all concentrations tested) did not induce significant blockade of contractile responses, presenting similar effects to those of the preparations incubated with the negative control (Tyrode solution) during the 120 min of incubation (Fig. 8A; shows result of the highest concentration tested). The myographic register (Fig. 8B) confirms the lack of neurotoxic activity in PND preparations with a stimulation of 1 g.

Otherwise, the acidic toxin, AplTx-I, induced irreversible time- and concentration-dependent neuromuscular blockade in indirectly *biventer cervicis* (BC) preparations, with complete blockade of twitch responses occurring after approximately 70 min, at the highest concentration of toxin tested (30 μg/mL) (Fig. 9A). The neuromuscular blockade triggered by different amounts of AplTx-I under indirect stimulation at 37 °C can also be visualized in representative recordings showed in Fig. 9B. The neurotoxic findings evidenced that the neuromuscular blockade in BC preparations obtained with purified toxin was not accompanied by significant effect in muscle contractures to exogenous acetylcholine (ACh), to carbachol (CCh) and or to potassium (KCl) (Fig. 9C).

The mean time required for AplTx-I to induce 50% and 90% neuromuscular blockade in chick *biventer cervicis* preparations was approximately 31 and 42 min, respectively (Fig. 10A), at the highest concentration tested (30 μg/mL). This neuromuscular blockade in BC preparations was not reversed after washing the muscle preparations (Fig. 10B). There was a slight decrease in contracture responses in muscle preparations that received low concentrations of the purified toxin (0.1; 0.3; 1 and 3 μg/mL).

4. Discussion

Snake venoms are recognized in biomedicine and biotechnology as relevant and rich sources of new molecular tools and drugs, particularly owing to the ability of venom compounds to affect physiological of the victim, sometimes leading to death (Da Silva et al., 2011; Almeida et al., 2016c). Among the most toxic components of these intriguing secretions are the PLA_2s toxins, which often present a remarkable diversity of pharmacological effects in spite of their uniform and conserved molecular architectures (Gutierrez and Lomonte, 2013; Jimenez-Charris et al., 2016). When compared with each other, snake venoms exhibit variable proportions of PLA_2s, and a single snake species can produce distinct combinations of the PLA_2 variants, such as acidic and basic isoforms (Lomonte et al., 2014).

In most reports in the literature, acidic PLA_2s present a higher ability to hydrolyze synthetic chromogenic substrates than basic isoforms. However, basic PLA_2s are more toxic and mediate a number of biological activities that are often not significantly induced by the acidic PLA_2s with higher enzymatic activity (Fernandez et al., 2010; Vargas et al., 2012). The functional role of acidic isoforms in pathophysiology of snakebites is not completely elucidated. Therefore, the structure-activity and enzymatic-pharmacology relationship of PLA_2s have been extensively studied, but still present challenging questions, thus highlighting the importance of understanding the structural and pharmacological proprieties of new PLA_2s, particularly the less-described acid

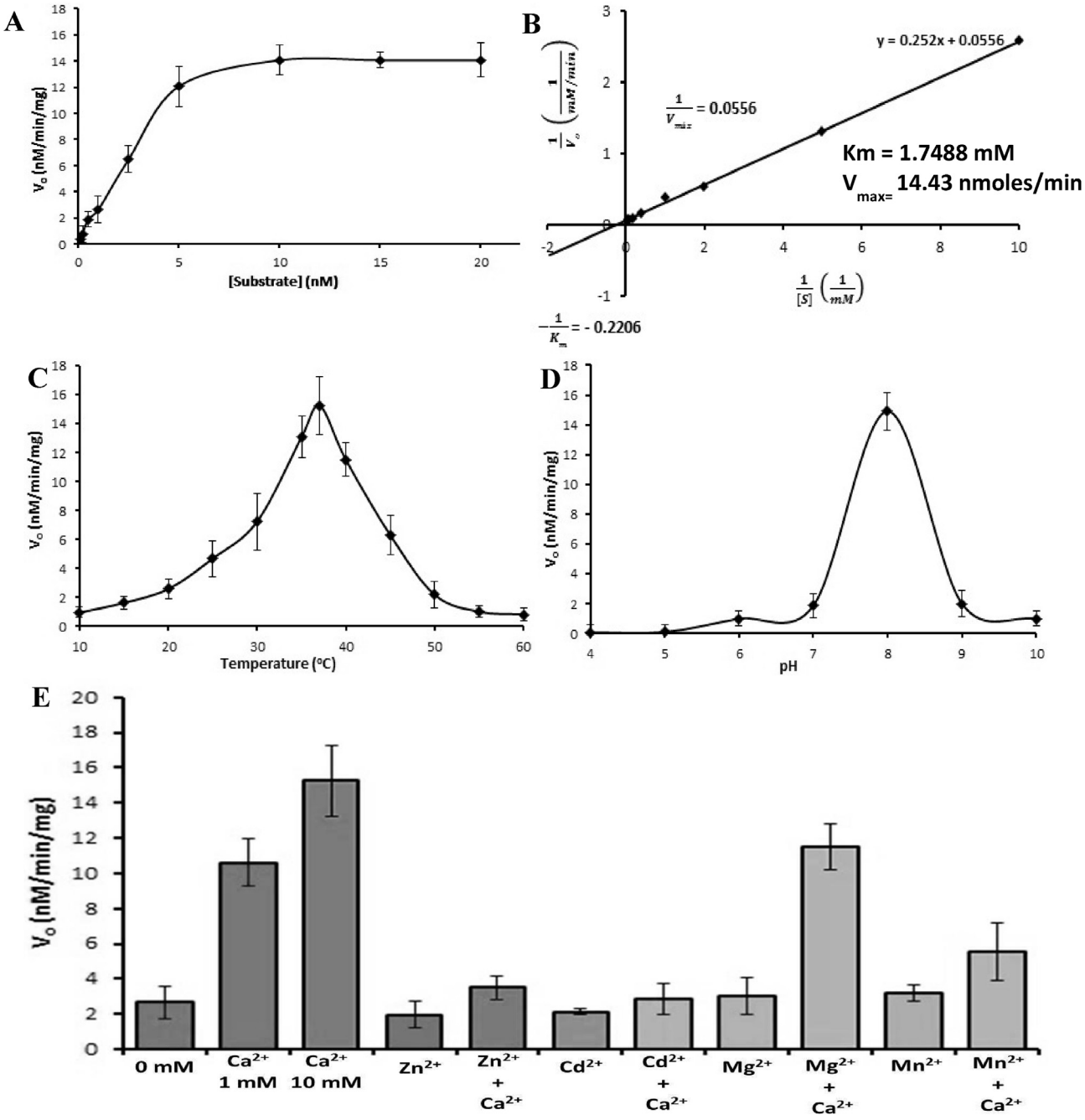

Fig. 5. Kinetic assays for AplTx-I. (A) Effect of substrate concentration on the enzymatic activity. AplTx-I obeys Michaelis-Menten kinetics, where the rate of catalysis increases with the increasing substrate concentration, until all the enzyme molecules are saturated with substrate. **(B)** Linewaever-Burk plot (reciprocal double). From the reciprocal of V_o values and [S] the kinetic K_m and V_{max} parameters can be determined more precisely. **(C)** Effect of temperature on the enzyme's activity. AplTx-I is active when incubated at 37 °C, but its activity is reduced as the temperature changes from this value. **(D)** AplTx-I has an optimal pH for enzymatic activity of 8.0 **(E)** Effects of different divalent ions on phospholipase activity. AplTx-I presented a strong dependence on calcium for its catalytic activity. The same does not occur in the presence of other divalent cations.

isoforms. Such data could shed light on several aspects of the roles of these proteins, such as their molecular determinants of toxicity, biological roles and evolutionary emergence (Fernandez et al., 2010; Jimenez-Charris et al., 2016).

Previous molecular and venom studies have confirmed the high amount of PLA_2 toxins in the venom of *A. p. leucostoma*, a snake subspecies that has not been fully described clinically in the scientific literature and with very limited information about its purified components, toxicity, pharmacologic and potential therapeutic (Jia et al., 2008; Lomonte et al., 2014). Considering the metabolic cost to protein expression and the large percentage of PLA_2s (~35%) in this snake venom, there is a strong evidence for a clinical relevance of these abundant proteins in this secretion. As such, we isolated and biochemically, structurally and pharmacologically characterized an acidic PLA_2 from *A. p. leucostoma* snake venom to gain insights into its general features and properties.

A combination of two chromatographic steps, size exclusion and RP-HPLC, allowed the effective and high-yield isolation of this abundant acidic PLA_2. From 800 mg snake venom, 15.8 mg of pure AplTx-I was obtained. The isolation procedure was followed by an

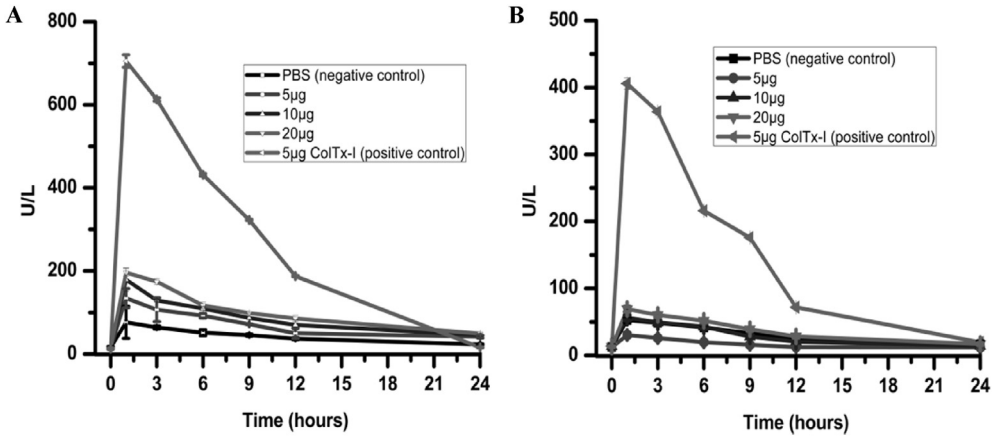

Fig. 6. Evaluation of the ability of AplTx-I to induce local and systemic myotoxic activity. The local **(A)** and systemic **(B)** myotoxicity were determined by quantification of plasma creatine kinase levels at different times after intramuscular or intravenous injection of different doses of toxin, respectively. Negative control animals received PBS buffer alone. The basic Asp49 PLA_2, ColTx-I, provided by Almeida et al. (2016a) was used as positive control. Myotoxic activity was expressed in U/L, where 1 U is defined as the amount of toxin that produces 1 µmol of NADH/min at 37 °C.

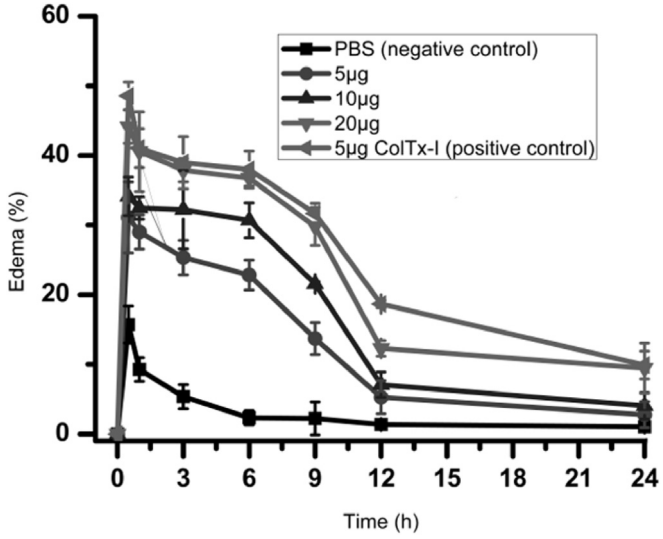

Fig. 7. Time-course of paw edema induced by AplTx-I in 18–20 g male Swiss mice. Edema was determined as the percentage increase in volume of the right footpad relative to the reading obtained in the left footpad (control) at each time interval and before the injection. The edema-inducing percentage is expressed as the mean ± SD of three independent experiments performed in triplicate (n = 5). The basic Asp49 PLA_2, ColTx-I, provided by Almeida et al. (2016a) was used as control.

increase in PLA_2 activity in each chromatography step, as revealed by kinetic assays. The protein's low tendency to form aggregates, as well as homogeneity of the purified protein, was demonstrated by its electrophoretic mobility in SDS-PAGE, indicating its monomeric nature and relative molecular mass of 14 kDa, which was confirmed by mass spectrometry (13,885.7930 Da), in accordance with data for other acidic PLA_2s isolated and characterized from viperid venoms (which are also known to be composed of a single polypeptide chain of approximately 14 kDa) (Fernandez et al., 2010; Vargas et al., 2012).

AplTx-I is an acidic enzyme composed of 122 amino acids residues and a theoretical pI of 5.12 (experimental pI of 5.0). All acidic snake venom PLA_2s previously characterized in the literature have demonstrated a pI varying from 4.0 to 5.5 and none of these identified isoforms has a pI value of greater than 5.5 (Menaldo et al., 2015). The determination of the amino acid sequence of AplTx-I identified a conserved aspartic acid amino acid residue at position 49 and the characteristic pattern of a half-Cys group IIA PLA_2 (7 disulfide bridges), which is relevant for protein stability (Gutierrez and Lomonte, 2013). The active site of the PLA_2s is constituted of the amino acid residues, His48, Asp49, Tyr52 and Asp99, which exhibit a catalytically essential water molecule that is attached to the proteins' side-chains by hydrogen bonds (Breithaupt, 1976; Kang et al., 2011). The presence of the Asp49 amino acid in the primary structure of AplTx-I (an essential amino acid residue of the catalytic site) corroborates its ability to catalyze the hydrolysis of chromogenic substrates in colorimetric enzymatic assays. Similarly to the catalytic site, the calcium-binding site formed by X27CGXGG32, is also a characteristic molecular region in the primary structure of all Group II Asp49 PLA_2s (Scott et al., 1990) and was identified in the amino acid sequence of AplTx-I.

The comparison of the primary structure of AplTx-I with other related proteins demonstrated its high identity with acidic Asp49 PLA_2s, particularly proteins from viperid venoms. AplTx-I exhibits well-conserved functional motifs, such as the calcium-binding site, catalytic site and N-terminal region (Schaloske and Dennis, 2006), and consequently has a conserved structural scaffold that is similar to other crystal structures of acidic PLA_2s (Wang et al., 1996a; Xu et al., 2003), as demonstrated by its three-dimensional structure, determined by homology modeling, confirming its good structural and energy quality.

Breithaupt (1976) studied the enzymatic characteristics of snake venom Asp49 PLA_2s and demonstrated that these biomolecules show classic Michaeles-Menten behavior. AplTx-I exhibited similar enzymatic behavior, with a Vmax of 14.43 nmol/min and Km value of 1.7488 mM, in agreement with other PLA_2s that have already been purified and enzymatically characterized (Almeida et al., 2016b). Maximum PLA_2 activity of AplTx-I occurs at pH 8 and 37 °C. The Asp49 PLA_2s PhTX-III from *Porthidium hyoprora* (Marques et al., 2015), CoaTx-I from *Crotalus oreganus lutosus* (Almeida et al., 2016b) display optimal pHs that are similar to that of AplTx-I.

The kinetic assays performed with divalent cations confirmed a strong dependence of AplTx-I enzymatic activity on the Ca^{2+} positive ion. Several studies have demonstrated that Ca^{2+} plays important roles in both enzymatic activity and PLA_2 binding to substrate (Scott et al., 1990; Yu et al., 1998). Its replacement by other ions of the same charge, such as Cd^{2+}, Zn^{2+}, Mg^{2+} and Mn^{2+}, abolished PLA_2 activity, demonstrating insufficient activity, or an inability to bind to the enzyme or to competitively inhibit Ca^{2+}

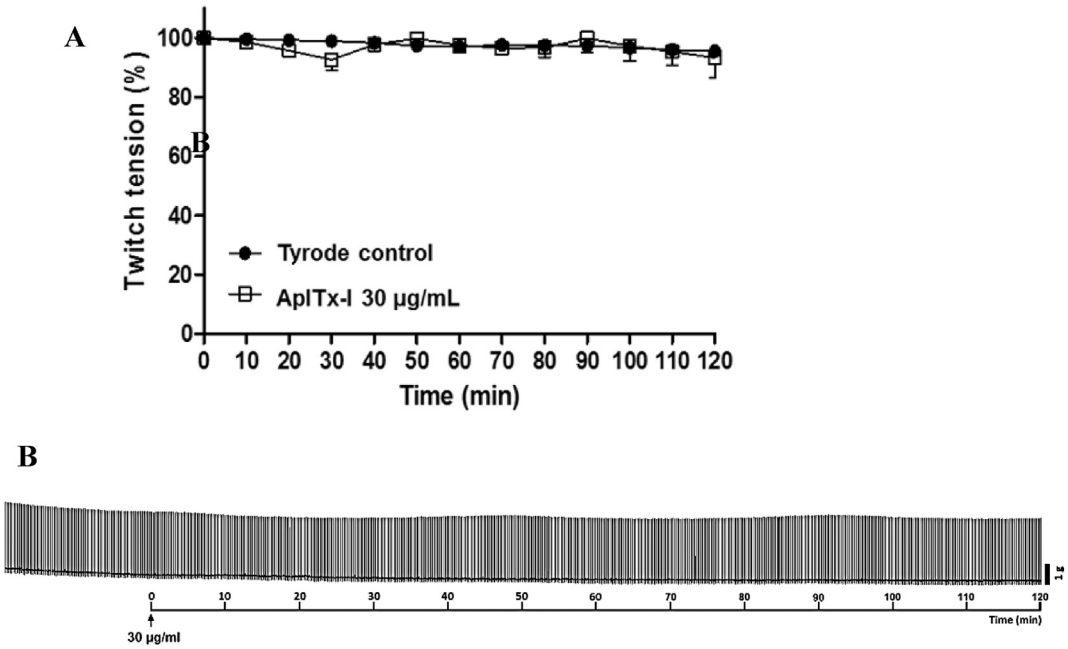

Fig. 8. AplTx-I does not cause neuromuscular blockade in mouse phrenic-nerve diaphragm (PND) preparations. **(A)** Twitch tensions (%) of the PND preparations under indirect stimuli during a 120-min incubation with Tyrode control (n = 6) or the toxin (30 µg/mL; n = 3), at 37 °C. Each point represents the mean ± SEM. **(B)** Myographic register of PND preparations under indirect stimuli, incubated with 30 µg/mL for 120 min, at 37 °C.

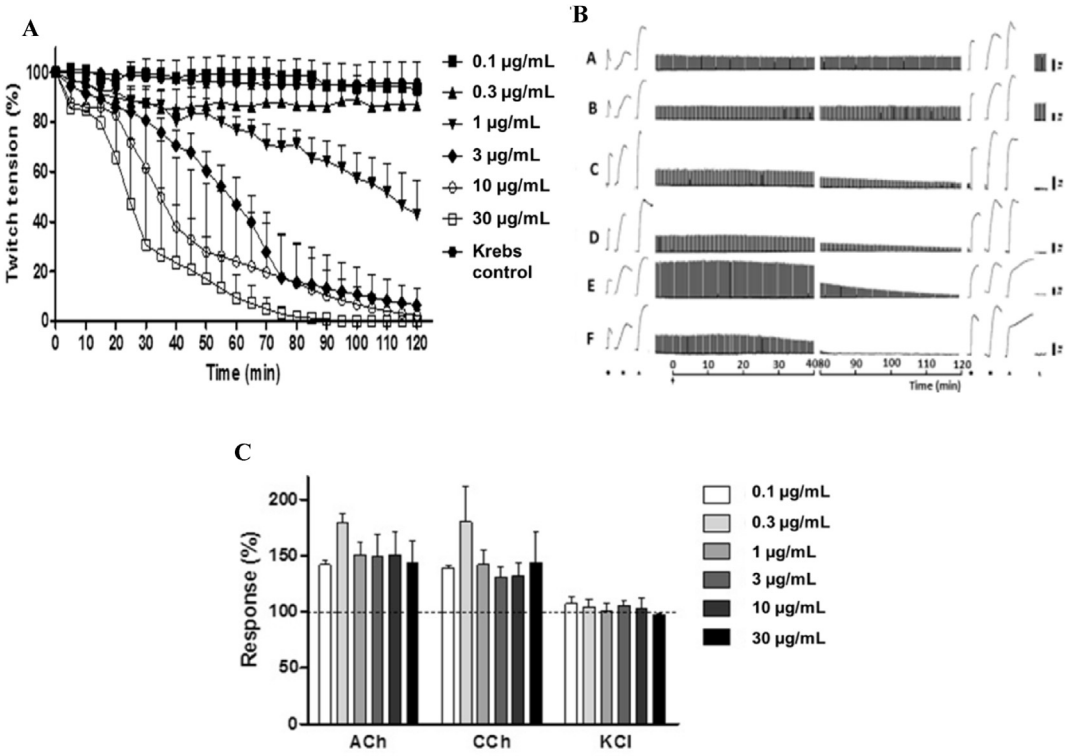

Fig. 9. Neurotoxic activity of AplTx-I in chick *biventer cervicis* (BC) preparations. (A) Neuromuscular blockade promoted by different concentrations of AplTx-I (0.1–30 µg/mL), in BC preparations at 37 °C. **(B)** Representative recordings of BC after incubation with different concentrations of AplTx-I under field stimulation for 120 min at 37 °C. A: 0.1 µg/mL; B: 0.3 µg/mL; C: 1 µg/mL; D: 3 µg/mL; E: 10 µg/mL; F: 30 µg/mL. The responses to 55 µM ACh (●), 8 µM CCh (■) 40 mM KCl (▲) were obtained. **(C)** Muscle contractures to exogenous: 1 mM acetylcholine (Ach), 20 µM carbachol (CCh) and 40 mM KCl after 120 min of incubation with AplTx-I (0.1–30 µg/mL). Each column represents mean ± SEM of the contraction after incubation with isolated toxin, compared to the contracture before incubation (dashed line at 100%).

mediated enzymatic activity, probably due to the size and number of electron shells on these ions (Mezna et al., 1994; Almeida et al., 2016b).

When compared with basic PLA_2s identified in the literature,

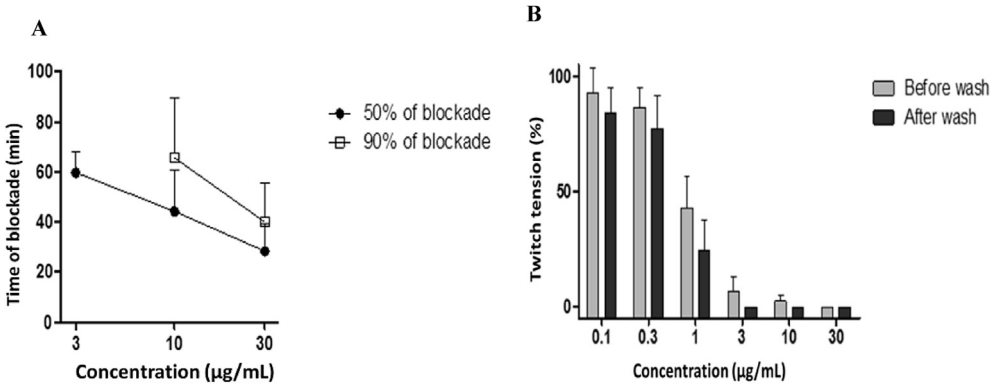

Fig. 10. Neuromuscular blockade of the *biventer cervicis* preparations after incubation with AplTx-I. (A) Time required for different concentrations of AplTx-I I (3–30 µg/mL) to induce 50% and 90% neuromuscular blockade. Each point represents the mean ± SEM. **(B)** Reversal of neuromuscular blockade produced by acidic PLA_2 (0.1–30 µg/mL) by washing of the muscle preparations. The BC preparations was washed after 120 min of incubation (0.1–3 µg/mL) or after total blockade (10 and 30 µg/ml). Each column represents the mean ± SEM.

AplTx-I shows a higher enzymatic activity, especially when compared to enzymes characterized in our laboratory (Huancahuire-Vega et al., 2011; Almeida et al., 2016b). However, this acidic PLA_2 present in *A. p. leucostoma* snake venom and characterized herein did not exert high or significant local or systemic myotoxicity activity, induction of edema, effect on blood coagulation or antibacterial activity, similarly to the pharmacological properties of other acidic PLA_2s (Fernandez et al., 2010; Marques et al., 2015). These enzymatic and pharmacological findings of AplTx-I are in line with data in the literature suggesting that the catalytic or PLA_2 activity *per se* generally is not enough to trigger or induce pharmacological effects (Montecucco et al., 2008; Jimenez-Charris et al., 2016). This hypothesis can be exemplified by Lys49 PLA_2s, which are catalytically inactive or unable to hydrolyze membrane phospholipids, but mediate a diversity of biological effects (Angulo et al., 2005; Lomonte and Rangel, 2012). Another example demonstrating that the pharmacological effects caused by PLA_2s do not always result from enzymatic activity or the action of its reaction products, are studies with basic Asp49 PLA_2 that have shown biological activity even when treated with specific inhibitors (Bazaa et al., 2010; Huancahuire-Vega et al., 2013). The features of these proteins indicate that additional steps (not only enzymatic activity), such as the recognition and interfacial interaction of PLA_2s with cell receptors are required for the stimulation of its pharmacological activities (Jimenez-Charris et al., 2016). Therefore, these findings indicate an essential role for other molecular regions of toxin primary structures (different of catalytic site) in the pharmacological effects (Lomonte et al., 2010).

Many scientific works have highlighted the relevance of C-terminal region of PLA_2s for the antibacterial and myotoxic activity (Paramo et al., 1998; Costa et al., 2008). The ability of this molecular region (C-terminal) of PLA_2s to mediate these pharmacological effects has been proposed to be related to its high density of cationic amino acids surrounded by hydrophobic and aromatic residues (Lomonte et al., 2010; Lomonte and Gutierrez, 2011). The primary structure analysis of AplTx-I (C-terminal) evidenced the presence of negatively charged residues, which are probably responsible for the very low or lack of myotoxic and antibacterial activity of purified toxin.

The *Agkistrodon piscivorus leucostoma* snake feeds on fish, mammals, birds and small reptiles (Rose et al., 2010). Most PLA_2s that present neurotoxic activity act in both animal models; birds and mammals, including the PLA_2s isolated from *Laticauda colubrina* (Rowan et al., 1989), *Lachesis muta muta* (Damico et al., 2005), *Bothriopsis bilineata smargadina* (Floriano et al., 2013) and *Daboia russelli* (Silva et al., 2017). On the other hand, some venoms may exhibit different effects for birds and mammals, presenting greater or less intensity or even the presence of neurotoxicity in one and absence in another (Carreiro da Costa et al., 2008; Torres-Bonilla et al., 2016). Among the neuromuscular preparations used for the characterization of neurotoxic activity, the mouse phrenic-diaphragm and the chick *biventer cervicis* (used in our study) are the most suitable, valuable tools and are frequently used as they allow comparisons to be made between functional studies (Gallacci and Cavalcante, 2010). Interestingly, in the case of AplTx-I, there was no toxicity in PND preparations from mice, in contrast to observations for chick BC preparations. This indicates a probable selectivity of this PLA_2 in birds, without neurotoxicity in mammals. The *ex vivo* neurotoxicity of this protein should also be compared to those of basic PLA_2s to understand the determinants and diversification of acidic and basic isoforms. This toxin was able to induce strong and irreversible neuromuscular blockade (*biventer cervicis* muscles), which decreased the contractile response after application of the toxin. These neurotoxic results showed that the inhibition obtained with 30 µg/mL of toxin was not accompanied by inhibition of contraction responses to acetylcholine (ACh), to carbachol (CCh) and or to potassium (KCl). These data indicate that, in the chick *biventer cervicis* preparation, the AplTx-I toxin exhibits *ex vivo* neurotoxic effects that are mainly presynaptic. Presynaptically-active neurotoxins are able to abolish the contractile response without affecting the response to cholinergic agonists (Lewis and Gutmann, 2004).

A probable mechanism for the presynaptic neurotoxic action of the catalytically active PLA_2 was proposed by Montecucco and Rossetto (2000), reviewed some years ago by Pungercar and Krizaj (2007), and recently discussed and updated by Sribar et al. (2014). According to these review articles, the PLA_2 molecules penetrate the lumen of synaptic vesicles of nerve terminals at the neuromuscular junction, where exocytosis of acetylcholine into the synaptic cleft occurs. After the release of the neurotransmitter, the vesicle is internalized by endocytosis in the nerve terminal; PLA_2 may hydrolyze phospholipids in the inner leaflet of the membrane vesicle, generating an excess of fatty bioactive lipids, which promotes the exocytosis of synaptic vesicles and conductance of Ca^{2+} channels. There are many possible internalization pathways, one of which is the recycling of synaptic vesicles. At the molecular level, studies have demonstrated an interaction of presynaptic PLA_2s with calmodulin and other proteins in the cytosol. Mitochondria

uncoupling and degeneration affecting the production of ATP has also been shown. The high concentration of bioactive lipids influences Ca^{2+} homeostasis, the cell membrane permeability and, consequently, intracellular phospholipase and proteinase activities. The integration of these several molecular signals (messenger molecules), prevents the closure of the lumen of the vesicles, leading to blockage of neurotransmission (Montecucco and Rossetto, 2000; Pungercar and Krizaj, 2007; Sribar et al., 2014).

Despite the high abundance of acidic PLA_2s in snake venoms, their functional role and relevance for envenoming and the survival of snakes, as well as evolutionary emergence has not been completely characterized (Vargas et al., 2012; Marques et al., 2015). Considering the abundance of AplTx-I and the metabolic cost to express a protein, it seems intriguing that there is no significant participation of this acidic protein in the toxic effects of venom (considering mammals). However, other acidic Asp49 PLA_2s purified, such as BaspPLA$_2$-II from *Bothrops asper* and PhTX-III from *Porthidium hyoprora* (Fernandez et al., 2010; Marques et al., 2015) have demonstrated similar toxicological profiles, presenting little or no toxicity when tested in isolation and alone. Other toxicological effects not explored in this study, such as renal damage, hemolytic and hypotensive activity should be evaluated in future to understand the relevance of this abundant enzyme in this venom. Some studies have suggested an important digestive role for the acidic PLA_2s from snake venom, contributing to the hydrolysis of phospholipids of prey (Fernandez et al., 2010). Thus, the abundant AplTx-I, despite demonstrating a lack of major toxic activity, could play a digestive function in *A. p. leucostoma* snake venom.

One interesting hypothesis that should be considered when analyzing the lack of toxicity of AplTx-I for mammals is the synergistic action between venom proteins; however, this was not tested in this work. Some studies have demonstrated that the Asp49 PLA_2s can act synergistically with other Aps49 or Lys49 PLA_2s proteins (Mora-Obando et al., 2014; Jimenez-Charris et al., 2016) or also with other types of venom toxins such as metalloproteinases (Bustillo et al., 2015) and cytotoxins (Laustsen, 2016). Jimenez-Charris et al. (2016), in a recent study about the divergent functional profiles of acidic and basic PLA_2s, showed a synergetic action between the two types of PLA_2s isoforms (acidic and basic). The ability of a basic PLA_2 isoform to trigger muscle damage was enhanced two-fold in the presence of non-toxic acidic PLA_2. Thus, future assays should evaluate the toxicological effects of AplTx-I together with other proteins of *A. p. leucostoma* snake venom (i.e. its synergic effects).

In conclusion, the first abundant acidic Asp49 PLA_2 from *A. p. leucostoma* snake venom, AplTx-I, was isolated by two chromatographic steps. AplTx-I, despite its very high enzymatic activity, presents very low or insignificant myotoxicity, neurotoxicity for mammals, edema-inducing and antimicrobial activities, and has no effect on blood clotting. The only notable effect triggered by this acidic toxin was a selective presynaptic *ex vivo* irreversible neuromuscular activity, with significant inhibition of the contraction of *biventer cervicis* preparations. These data, together with the elucidation of the complete primary structure of AplTx-I will be of value for future comparative studies attempting to understand the function of acidic PLA_2s in snake venoms, their potential biomedical applications and to identify the molecular determinants of certain biological activities. New variant isoforms of this snake venom (*A. p. leucostoma*) should be isolated, studied in isolation and in assays of synergetic actions with AplTx-I. Such data may enable the elucidation of the relevance and functional role of this abundant acid protein (in relation to mammals, since the functional role in chicken has been described herein), as well as those of other previously identified acidic PLA_2s.

Conflict of interest

The authors declare no relevant conflicts of interest.

Acknowledgments

The authors wish to express their gratitude to the Coordination for the Improvement of Higher Education Personnel (CAPES), the National Counsel of Technological and Scientific Development (CNPq) and Fundação Rondônia de Amparo ao Desenvolvimento das Ações Científicas e Tecnológicas for financial support. The authors thank the Program for Technological Development in Tools for Health-PDTIS-FIOCRUZ for the use of its facilities. The authors thank Mr. Paulo A. Baldasso for his helpful technical assistance. This work forms part of the PhD thesis of Letícia Monteiro de Resende.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2017.01.002.

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