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Anti-platelet aggregation activity of two novel acidic Asp49-phospholipases A_2 from *Bothrops brazili* snake venom

Juliana C. Sobrinho[a], Anderson M. Kayano[a], Rodrigo Simões-Silva[a], Jorge J. Alfonso[a,b], Ana F. Gomez[a,b], Maria C. Vega Gomez[b], Fernando B. Zanchi[a], Laura A. Moura[c], Vivian R. Souza[c], André L. Fuly[c], Eliandre de Oliveira[d], Saulo L. da Silva[e], José R. Almeida[e], Juliana P. Zuliani[a], Andreimar M. Soares[a,f,*]

[a] Centro de Estudos de Biomoléculas Aplicadas à Saúde, CEBio, Fundação Oswaldo Cruz, FIOCRUZ, unidade Fiocruz Rondônia e Departamento de Medicina, Núcleo de Saúde, Universidade Federal de Rondônia, UNIR, Porto Velho, RO, Brazil
[b] Centro para el Desarrollo de la Investigación Científica, CEDIC, Asunción, Paraguay
[c] Departamento de Biologia Celular e Molecular (GCM), Instituto de Biologia, Universidade Federal Fluminense, Niterói, RJ, Brazil
[d] Proteomics Platform, Barcelona Science Park, Baldiri Reixac 10-12, 08028 Barcelona, Spain
[e] Universidade Regional Amazónica, IKIAM, Via Muyuna Km 7, Tena, Napo, Ecuador
[f] Centro Universitário São Lucas, UNISL, Porto Velho, RO, Brazil

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ABSTRACT

Phospholipases A_2 (PLA_2s) are important enzymes present in snake venoms and are related to a wide spectrum of pharmacological effects, however the toxic potential and therapeutic effects of acidic isoforms have not been fully explored and understood. Due to this, the present study describes the isolation and biochemical characterization of two new acidic Asp49-PLA_2s from *Bothrops brazili* snake venom, named Braziliase-I and Braziliase-II. The venom was fractionated in three chromatographic steps: ion exchange, hydrophobic interaction and reversed phase. The isoelectric point (pI) of the isolated PLA_2s was determined by two-dimensional electrophoresis, and 5.2 and 5.3 pIs for Braziliase-I and II were observed, respectively. The molecular mass was determined with values of 13,894 and 13,869 Da for Braziliase-I and II, respectively. Amino acid sequence by Edman degradation and mass spectrometry completed 87% and 74% of the sequences, respectively for Braziliase-I and II. Molecular modeling of isolated PLA_2s using acid PLA_2BthA-I-PLA_2 from *B. jararacussu* template showed high quality. Both acidic PLA_2s showed no significant myotoxic activity, however they induced significant oedematogenic activity. Braziliase-I and II (100 μg/mL) showed 31.5% and 33.2% of cytotoxicity on *Trypanosoma cruzi* and 26.2% and 19.2% on *Leishmania infantum*, respectively. Braziliase-I and II (10 μg) inhibited 96.98% and 87.98% of platelet aggregation induced by ADP and 66.94% and 49% induced by collagen, respectively. The acidic PLA_2s biochemical and structural characterization can lead to a better understanding of its pharmacological effects and functional roles in snakebites pathophysiology, as well as its possible biotechnological applications as research probes and drug leads.

1. Introduction

The venomous snake *Bothrops brazili* [1] known as "Jergón íShushupe" is distributed throughout South America and is found mainly in Brazil (the Amazon region), Colombia, Ecuador, Guyana, Peru, Suriname and French Guiana [2]. It has an average size of approximately 1.2 m in length and is found mainly in the primary forest [3–5].

One of the main local effects is myonecrosis, which is mainly caused by phospholipases A_2 (PLA_2) present in the venom [6–8]. Other actions triggered by venom PLA_2s are oedema, neurotoxicity, cardiotoxicity and hemostatic disorders [9–11]. Snake venom PLA_2s are secreted enzymes belonging to groups IA (Elapidae) and IIA (Viperidae). PLA_2s from Viperidae venoms (including the genus *Bothrops*) have been divided into two main subgroups: the enzyme Asp49 (D49), catalytically active and the Lys49 (K49) PLA_2-homologue, catalytically inactive [12,13]. Most PLA_2 described

* Corresponding author at: Centro de Estudos de Biomoléculas Aplicadas à Saúde – CEBio, Fiocruz Rondônia, Rua da Beira, 7176, Bairro Lagoa, 76812-245 Porto Velho, Rondônia, Brazil.
E-mail address: andreimar.soares@fiocruz.br (A.M. Soares).

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from the genus *Bothrops* are basic proteins with isoelectric points (pI) between 7 and 10, presenting or not catalytic activity and various pharmacological effects such: as anticoagulant action and myotoxic and oedema activity [8,14].

On the other hand, acidic PLA_2s present in the venom of *Bothrops* snakes have not been well studied and little is known about the physiopathological and therapeutic effects triggered by them. Generally, these acidic isoforms are not myotoxic and its functional roles in snakebite envenomings have not been determined. In the venom, the proportion of acidic and basic PLA_2s is variable for each species; however, in bothropic snakes, the content of acidic PLA_2s is markedly lower than that of the basic ones. This is one of the reasons for the smaller number of studies performed with these acidic toxins. Some examples of acidic PLA_2s described are BthA-I-PLA_2 from *B. jararacussu* [15], BaspPLA$_2$-II from *B. asper* [16], Bmoo-I-PLA_2 from *B. moojeni* [17] and BaPLA$_2$ from *B.atrox* [18], which do not share a pharmacological and toxicological uniformity, and highlight the need of isolate and characterize new acidic PLA_2s from snake venoms. Recently, Resende et al. [19] purified and identified an acidic PLA_2 from *Agkistrodon piscivorus leucostoma* snake venom, which is not toxic for mammals, but induced a selective neuromuscular toxicity in chick *Biventer cervicis* that plays a significant role for the snake feeding. This finding emphasizes the functional diversification and relevance of these acidic isoforms for snake evolution and ecology, and advances in toxinology research.

From *B. brazili* venom four basic PLA_2s have already been isolated, two Lys49-PLA_2 homologues and two Asp49-PLA_2s [19,20], but the acidic isoforms had not been isolated and characterized until now. Thus, owing to the lack of information's, in this study we have isolated and characterized the first two acidic PLA_2s, named Braziliase-I and Braziliase-II, from *B. brazili* snake venom with potential anti-platelet aggregation activity and cytotoxicity on *L. infantum* and *T. cruzi*.

2. Material and methods

2.1. Snake venom and animals

The venom of the specie *B. brazili* was acquired from Serpentário Proteínas Bioativas Ltda, Batatais-SP and kept refrigerated (8 °C) in the Bank of Amazon Venoms at the Center of Biomolecular Studies Applied to Health, CEBIO-UNIR-FIOCRUZ-RO (authorization: CGEN/CNPq 010627/2011-1 and IBAMA 27131-1). The use of mice (18–22 g) for the toxicological assays was performed according to the Ethical Principles of Animal Experimentation (CONCEA) and was approved by the Ethics Commission on the use of animals FIOCRUZ/RO (CEUA/RO) under the number 2013/07. Experiments using human blood (obtained from healthy volunteer donors) were approved by the Federal Fluminense University Committee for Ethical in Experimentation – CEP-UFF, CAAE: 28941314.0.0000.5243.

2.2. Biochemical and structural characterization

2.2.1. Isolation of the PLA_2s

Braziliases-I and II were purified by three chromatographic steps: cation exchange, hydrophobic interaction and reversed phase. Dried *B. brazili* venom (50 mg) was diluted in 1 mL of ammonium bicarbonate buffer (Ambic) 0.05 M, pH 7.8 and centrifuged at 480 *xg* for 10 min and applied to a CM-Sepharose GE® column (1 × 40 cm) preequilibrated with Ambic. Elution of the samples was performed with a continuous gradient up to a concentration of 0.5 M of Ambic at a flow rate of 1 mL/minute. Fractions 2 and 3 were pooled and lyophilized, and subjected to hydrophobic interaction chromatography on a Butyl-Sepharose GE® column (1 × 15 cm). Fractions were diluted in 1 mL of 4 M NaCl and 20 mM Ambic pH 7.8 and then applied to the column equilibrated with the same buffer. The elution took place under a decreasing gradient (100, 75, 50, 25 and 0%, respectively) of NaCl in a buffered environment (4 M NaCl and 20 mM Ambic) ending the process in an electrolyte-free environment (deionized water), at flow rate of 1 mL/minute. The fraction that presented phospholipase activity (data not shown) was subjected to reverse phase chromatography on a C18 column (Discovery – Sigma-Aldrich®) using 0.1% trifluoracetic acid (TFA) as solution A and 0.1% trifluoracetic acid (TFA) and 99.9% acetonitrile as solution B in a 0–70% gradient with a flow of 1 mL/minute. In all chromatographies, the absorbance was monitored at a wavelength of 280 nm and graphics were registered in UNICORN Control Chromatography System 5.0 software.

2.2.2. Determination of the isoelectric point

The polyacrylamide gel (PAGE) was carried out in the presence of sodium dodecyl sulfate (SDS-PAGE) and isoelectric focusing with pH range used was 3.2–10.3, as previously described [21].

2.2.3. Molecular mass determination

Mass spectrometry was performed in MALDI equipment (matrix-assisted laser desorption ionization), with two TOF analyzers (AXIMA TOF2 Shimadzu Biotech) operating in linear mode using a saturated solution of sinapinic acid as the ionization matrix and 1 μg of protein (3:1 ratio, respectively).

2.2.4. Amino acid sequencing

For amino acid sequencing Edman's chemical degradation technique (PPSQ-33A Shimadzu Corp., Kyoto, Japan) and the "*de novo*" sequencing technique were used. The spectrometric analysis was performed in a NanoAcquity (Waters) HPLC coupled to an OrbitrapVelos spectrometer (Thermo Scientific). An aliquot of the triptych digest was injected and separated in a C18 column (75 μm Oi, 10 cm, nano Acquity, 1.7 μm BEH column, Waters). The peptides were eluted using the following gradient: 1–40% B in 20 min, followed by a gradient of 40% to 60% B in 5 min under a flow of 250 nL/minute. Solution A contained 0.1% formic acid (v/v) in water and solution B contained 0.1% formic acid (v/v) in acetonitrile). The mass of the peptides (*m*/*z*: 300–1700) were measured in the Orbitrap with a resolution of 60,000 at *m*/*z* 400. The most abundant peptides were selected and fragmented in an HCD collision cell with 40% normalized collision energy (NCE) with nitrogen used as the collision gas. The fragments were detected in the Orbitrap with a resolution of 7500FWHM at 400 *m*/*z*. Data were collected with a Thermo Xcalibur (v.2.1.0.1140). After obtaining the partial sequences of the acidic PLA_2s, a similarity search was performed using the BLAST algorithm and multiple alignment was performed using the Clustal W program available at http://www.ebi.ac.uk/Tools/msa/clustalw2/

2.2.5. Enzymatic activity

Phospholipase A_2 activity was performed in an M4 Spectra Max (Molecular Devices®) using a 96-well plate and 5 μM of the fluorescent phospholipids from Avanti Polar Lipids®: Phosphatidic Acid (NBD-PA), NBD-Phosphatidilcoline (NBD-PC) and Phosphatidil Glycerol (NBD-PG). The phospholipids were mixed with a solution containing 8 mM $CaCl_2$, 20 mM Tris-HCl pH 7.5 and the samples (0.5 μg). The influence of pH was performed using such buffer solutions: 0.1 M acetate pH 5.5 and 1 M Tris pH 6.8, 7.5, 8.5, 11 and 12. Excitation and emission wavelengths were 460 and 534 nm, respectively. The kinetic assays were performed for 5 min and RFU (Relative Fluorescence Units) were measured. Results were represented as mean ± standard deviation.

2.2.6. Molecular modeling

The homology modeling of isolated PLA_2s was conducted using the crystal structure of acidic PLA_2 BthA-I-PLA_2 from *B. jararacussu* as a template (PDB code: 1UMV) with resolution of 1.79 Å. After multiple alignment using Protein Blast (http://blast.ncbi.nlm.nih.gov) [22] the residues to complete the primary sequence of isolated PLA_2s were predicted. The residues considered conserved were inserted to obtain a theoretical complete sequence: NLWQFEMLIMKIALTSGFMFYSSYGCYCGWGGHGR PKDASDRCCFVHDCCYGKVTTCNPKFDVYTYSEENGVVVCGGDDPCK KQICECDRVAATCFRDNKDTYDNKYWFYGAKNCQEESDPC (Braziliase-I) and NLWQFEMLIMKIAKTSGFMFYSSYGCYCG-WGGH GRPQDASDRCCFVHDCCYGKVTXXNPKXDXYTYSEENGVVVC GGDDPCKKQICECDRVAATCFRDNKDTYDNKYWFFPAKNCQEESEPC (Braziliase-II). The aligned residues are equivalent to obtained sequence. Protein Blast and Protein Data Bank (PDB) (http://www.pdb.org) to search and retrieval the template structure was used. The sequence alignments were carried out in MODELLER v9.16 [23] and ClustalW [24] softwares. The model building was carried out in MODELLER v9.16. A total of 1000 models were generated and the final model was selected based on the lowest DOPE scores calculated by MODELLER software. The overall stereochemical quality of the final model for PLA_2s were assessed by the program PROCHECK [25]. Interactive visualization and comparative analysis of molecular structures were carried out in UCSF Chimera [26].

2.3. Characterization of biological activity

2.3.1. Oedematogenic activity

Male Swiss mice de 18–22 g (n = 4) were inoculated in the subplantar right paws with 50 μg of isolated PLA_2s or *B. brazili* venom, solubilized in 50 μL of 0.15 M phosphate buffered saline (PBS). As a control, PBS was used in the left paws. The oedematogenic activity was determined by measuring the animal paws, using a thickness gauge (Mitutoyo 7301) at 0, 30, 60 and 120 min. Results were represented in percentage related to negative control.

2.3.2. Myotoxic activity

Groups of four male Swiss mice (18–22 g) were injected in the gastrocnemius muscle with 50 μg/50 μL of *B. brazili* venom, Braziliase-I, Braziliase-II or positive control (MTX-II), dissolved in 0.15 M PBS. For the negative control, animals received an injection of 0.15 M PBS. After 3 h, blood was collected from plexus orbital puncture in heparinized tubes and centrifuged at 3000 *xg* for 5 min at room temperature to separate the plasma. CK Total Kit (CK- NAC kit Life Biotechnology) was used to analyze the myotoxic activity. Absorbances were read in a spectrophotometer (Biotek). The activity was expressed as U/L, where one unit corresponds to the phosphorylation of 1 nMol of creatine/min at 25 °C. For statistical analysis, the Tukey's multiple comparisons test was carried out using the GraphPad Prism (v.6.01) program.

2.3.3. Evaluation of cytotoxicity on epimastigote forms of T. cruzi

For the evaluation of trypanocidal activity, *T. cruzi* epimastigotes of the strain CL clone B5 were cultured at a concentration of 2.5×10^5 parasites/mL in 96 well plates using Liver Infusion Tryptose culture medium (LIT) supplemented with 10% Fetal Bovine Serum (FBS). The plate was incubated at 26 °C for 72 h with different concentrations of *B. brazili* venom and the purified PLA_2s (6.25–100 μg/mL). Each concentration was tested in triplicate. Thereafter a 200 μM Chlorophenol red β-D-*galactopyranoside* (CPRG) solution was added. The plate was incubated at 37 °C for 4 h and the absorbance was monitored at 575 nm using a multimodal Synergy H1 spectrophotometer (Biotek). The efficacy of each test compound was estimated by calculating the percentage of anti-epimastigote activity. A positive control, the reference drug Benznidazole, at a concentration of 100 μg/mL, was used.

2.3.4. Evaluation of cytotoxicity on promastigote forms of L. infantum

For this test, promastigotes of *L. infantum* (MCAN/ES/92/BNC 83) were cultured at a concentration of 1.5×10^6 parasites/mL in 96 well plates using Schneider culture medium supplemented with 10% FBS. The plate was incubated at 26 °C for 48 h with different concentrations of *B. brazili* venom and the purified PLA_2s (6.25–100 μg/mL). Each concentration was tested in triplicate. Subsequently, a 3 mM Resazurin solution was added. The plate was subjected to incubation at 26 °C for 4 h and finally the absorbance was monitored at 570 and 600 nm using a multimodal Synergy H1 spectrophotometer (Biotek). The efficacy of each compound was estimated by calculating the percentage of anti-promastigote activity. The drug Pentamidine, at a concentration of 100 μg/mL was used as a positive control.

2.3.5. Platelet aggregation assay

Platelet aggregation assay was carried out according to methodology reported in previous studies [27,28], with modifications, using human platelet-rich plasma (PRP). PRP was obtained after citrated (0.31%, v/v) whole blood centrifugation at 340 *xg* for 12 min at 25 °C. Platelet aggregation was measured turbidimetrically using a Whole Blood Aggregometer (Model 490 2D – Chrono-Log Corporation, Pennsylvania, USA). Assays were performed at 37 °C in siliconized glass cuvettes using 300 μL of PRP under stirring, and aggregation was triggered by the addition of adenosine diphosphate (ADP) or collagen (Chrono Log Corp). One hundred percent (100%) of platelet aggregation was determined as the full platelet response obtained 6 min after the addition of a supramaximal concentration of the agonists (concentration that gives 70–80% of aggregation), and the 0% (base line) of platelet aggregation was the light transmittance recorded in the presence of PRP alone. Different concentrations of isolated PLA_2s (2,5; 5 and 10 μg) were incubated with PRP for 2 min at 37 °C, and then, platelet aggregation was triggered by adding ADP (10 μM) or collagen (16 μg/mL). Inhibitory effect on platelet aggregation was expressed as the difference in the maximal responses of platelets in the presence or absence of PLA_2s, after challenge with agonists. Control experiments were performed in the presence of saline, instead of PLA_2s.

3. Results and discussion

The *B. brazili* venom fractionation by cation exchange chromatography resulted in 11 main fractions (Fig. 1A). The fractions identified as 2 and 3 presented catalytic activity (data not shown) and were subjected to the second purification step using hydrophobic interaction chromatography (Fig. 1B), which resulted in six major fractions. The sixth fraction showed enzymatic activity (data not shown) and then was subjected to reversed phase chromatography (Fig. 1C). The fractions with PLA_2 activity were eluted at 42% and 43% of solution B, named Braziliase-I and II, respectively. PLA_2s purity was analyzed by SDS-PAGE (insert Fig. 1C) and showed the homogeneity of the purified toxins and their monomeric nature. The molecular mass for Braziliase-I was 13,894 Da and for Braziliase-II was 13,869 Da (Fig. 1D). The isoelectric point (pI) of the isolated PLA_2s was determined by two-dimensional electrophoresis, and 5.2 and 5.3 pIs for Braziliase-I and II were observed, respectively (Fig. 1E). As expected, the pIs and the molecular weights are similar to published data for other species of the genus *Bothrops* [16,17,29]. So far, four basic PLA_2s were characterized from the venom of *B. brazili*: MTX-I (Asp49-PLA_2), MTX-II (Lys49-PLA_2-homologue), BbTX-II (Lys49-PLA_2-homologue) and BbTX-III (Asp49-PLA_2) [19,20].

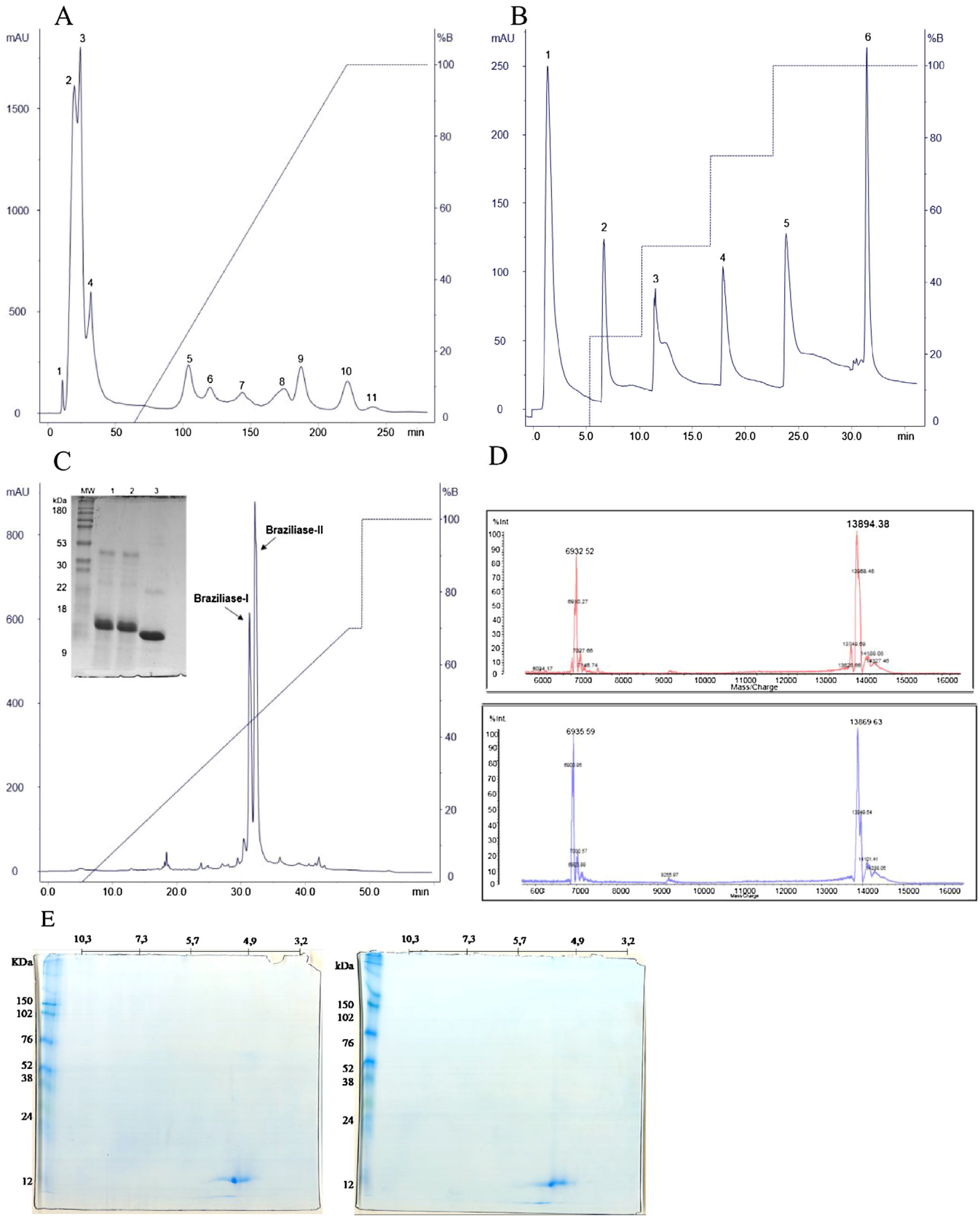

Fig. 1. Isolation and mass determination of PLA_2s.
B. brazili venom was subjected to cation exchange chromatography (**A**) and fractions 2 and 3 were selected and subjected to hydrophobic interaction chromatography (**B**). Fraction 6 was selected, and then subjected to reverse phase resulting in two fractions (1 and 2) which are displayed in SDS-PAGE in columns 1 and 2, in column 3 (MTX-II), a PLA_2 used as a molecular weight standard, MW corresponds to Weight Marker (**C**). Mass spectrum of Braziliase-I (superior) and II (inferior), the spectrum represents the average of the laser pulses (**D**). 2D electrophoresis of Braziliase-I (left), showing a pI of 5.2 and Braziliase-II (right), showing a pI of 5.3 **(E)**.

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Braziliase-I              NLWQFEMLIMKIALTSGFMFYSSYGCYCGWGGHGRPKDASDRCCFVHDCCYGKVTTCNPKF 61
Braziliase-II             NLWQFEMLIMKIAKTSGFMFYSSYGCYCGWGGHGRPQDASDRCCFVHDCCYGKVT------
G3DT18.1 B. moojeni       NLWQFEMLIMKIAKTSGFLFYSSYGCYCGWGGHGRPQDATDRCCFVHDCCYGKVTGCNPKT
AFJ79208.1 B. diporus     NLVQFETLIMKIAGRSGVWYYGSYGCYCGSGGQGRPQDASDRCCFVHDCCYGKVTGCNPKA
AGG39584.1 B. neuwiedi    SLVQFDTLIMKIAGRSGIIFYSSYGCYCGLGGQGRPQDASDRCCFVHDCCYGKVTGCNPKA
C9DPL5.1 B. pirajai       NLWQFGKLIMKIAGESGVFKYLSYGCYCGLGGQGQPTDATDRCCFVHDCCYGKVTGCDPKI
Q2HZ28.1 B. erythromelas  SLVQFETLIMKIAGRSGVWYYGSYGCYCGSGGQGRPQDASDRCCFVHDCCYGKVTDCDPKA
AFJ79207.1 B. diporus     SLVQFETLIMKIAGRSGVWYYGSYGCYCGSGGQGRPQDASDRCCFVHDCCYGKVTDCDPKA
Q7ZTA8.1 C. v. viridis    NLVQFELLIMKVAKRSGLLSYSAYGCYCGWGGHGRPQDATDRCCFVHDCCYGKVTDCNPKT
Q9I8F8.1 B. pictus        SLVQFETLIMKIAKRSGVWFYGSYGCFCGSGGQGRPQDASDRCCFVHDCCYGKVTDCDPKT
P86907.1 B. ammodytoides  HLMQFETLIKKIAGRSGVWFYGFYGCYCGSGGRGKPKDATDRCCFVHDCCYGKVTGCDPKM
P20249.1 G. blomhoffii    SLMQFETLIMKIAGRSGIWYYGSYGCYCGAGGQGRPQDASDRCCFVHDCCYGKVTGCDPKL
1UMV B. jararacussu      SLWQFGKMINYVMGESGVLQYLSYGCYCGLGGQGQPTDATDRCCFVHDCCYGKVTGCNPKI
                           * **  :*  :   **.  *  ***:** **:*:* **:***************

Braziliase-I              ---------GVVVCGGDDPCKKQICECDRVAATCFRDN------KYWFYGAK-CQEESDPC 122
Braziliase-II             ---------GVVVCGGDDPCK-QICECDRVAATCFRDN------KYWFFPAK---------
G3DT18.1 B. moojeni       DSYTYSEENGDVVCGGDDPCKKQICECDRVAATCFRDNKDTYDNKYWFYPAKNCQEESEPC
AFJ79208.1 B. diporus     DTYTYSEENGVVVCGGDDPCKKQICECDRVAATCFRDNKDTYDNKYWFFPAKNCQEESEPC
AGG39584.1 B. neuwiedi    DVYTYSEDNGDIVCGGDDPCKKQICECDRVAATCFRDNKDTYDNKYWLFPAKNCQEESEPC
C9DPL5.1 B. pirajai       DSYTYSKENGDVVCGGDDPCKKQICECDRVAATCFRDNKDTYDIKYWFYGAKNCQEESEPC
Q2HZ28.1 B. erythromelas  DVYTYSEENGVVVCGGDDPCKKQICECDRVAATCFRDNKDTYDNKYWFFPAKNCQEESEPC
AFJ79207.1 B. diporus     DVYTYSEENGVVVCGGDDPCKKQICECDRVAATCFRDNKDTYDNKYWFFPAKNCQEESEPC
Q7ZTA8.1 C. v. viridis    ASYTYSEENGEIVCGGDDPCKKQVCECDRVAAICFRDNIPSYDNKYIQFPAKNCQEKPEPC
Q9I8F8.1 B. pictus        DIYTYSEENGVVVCGGDDPCKKQICECDRVAAVCFRDNKDTYDNKYWFFPANNCQEESEPC
P86907.1 B. ammodytoides  DFYTYSEENGVVVCGGDDPCKKQICECDRVAATCFRDNKTYDNNKYWFYPAKNCQEESEPC
P20249.1 G. blomhoffii    DVYTYTEENGAIVCGGDDPCKKQICECDKDAAICFRDNIDTYDNKYWFFPAKNCQEESEPC
1UMV B. jararacussu      DSYTYSKKNGDVVCGGDNPCKKQICECDRVATTCFRDNKDTYDIKYWFYGAKNCQEKSEPC
                                  * :*****:*** *:****: *: *****      **  : *:
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Fig. 2. Alignment of Braziliase-I and Braziliase-II sequences with PLA_2s of snake venoms.
The amino acid residues highlighted in light gray (His-48, Asp-99 and Tyr-52) form the active site of the enzyme. The residues highlighted in dark gray (Asp-49, Tyr-28, Gly-30 and Gly-32) are those that bind to a calcium ion. The residues highlighted in bold font are cysteines , responsible for the formation of disulfide bridges.

This is the first publication describing the structural and pharmacological characterization of acidic isoforms of PLA_2s from *B. brazili* venom. The amino acid sequence obtained showed 106 amino acids corresponding to 87% for Braziliase-I's and 91 amino acids corresponding to 74% of Braziliase-II's primary sequences (Fig. 2). The sequence alignment analysis confirmed that both proteins are Asp49-PLA_2s. Multiple alignment showed high similarity with others acidic PLA_2s such as BmooPLA$_2$ (*B. moojeni*) and PLA$_2$ (*B. diporus*) (Fig. 2).

From theoretical complete sequence, the Braziliase-I and Braziliase-II PLA_2s alignment showed 76% and 74% of identity with BthA-I-PLA_2, respectively. The residues percentage lying in the favored regions of a Ramachandran plot [30] is one of the best guides to check stereochemical quality of a protein model based on the assumption that an adequate model should have more than 90% of the residues in the allowed regions [25]. Ramachandran plot analysis for both PLA_2s structure showed that more than 91.4% of the amino acids are in favorable regions. The quality of the model was also assessed by comparing the predicted structure with the template structure via superimposition and atoms RMS deviation (RMSD) assessment. The RMSD of Cα trace between Braziliase-I and Braziliase-II PLA_2s and template is 0.13 Å and 0.23 Å respectively. Thus, the models are reasonably adequate and quite similar to template (Fig. 3). Both models presented a typical structure of group II PLA$_2$ enzymes with: a N-terminal α-helix (residues Leu2 to Ala13), a "short" α-helix (residues Thr15 to Ser22), a Ca^{2+}-binding loop (Ser23 to Asp38 and Asp49), an α-helix2 (residues Ala39 to lys53), a short two-stranded antiparallel β-sheet known as β-wing (residues Thr65 to Glu69 and Val72 to Gly76 of Braziliase-I; Thr65 to Glu68 and Val73 to Gly76 of Braziliase-II), an α-helix3 (residues Pro80 to Asp98) and a C-terminal loop region (residues Cys115 to Cys122 for Braziliase-I; Gln116 to Cys122 for Braziliase-II). It is important to note that the α-helix 2 and 3 presented in an anti-parallel form. In addition, seven disulfide bonds are formed (Cys26–Cys115, Cys28–Cys44, Cys43–Cys95, Cys49–Cys122, Cys50–Cys88, Cys57–Cys81 and Cys75–Cys86), a common characteristic structure for this group of PLA_2s.

The proportion of acidic and basic PLA_2s is variable for each species; however, in most bothropic snakes, the content of acidic PLA_2s is smaller than the basic ones, which is one of the factors for a few number of published studies. These multiple isoforms were generated by gene duplication and the accelerated evolution of exons resulting in changes mainly in the surface of the amino acids of these molecules and enabling the development of new functions [9]. To date, all acidic PLA_2s purified from viperid snake venoms present an Asp residue in position 49, but not all Asp49-PLA_2s are acidic. These acidic isoforms are always catalytically active and their activity is generally more intense than that of basic PLA_2s [31]. Corroborating this information, both PLA_2s Braziliases-I and II presented elevated phospholipase activity on the fluorescent phospholipid NBD-PC. However, the activity was less intense on NBD-PG and absent on NBD-PA (Fig. 4A). The preference of venoms PLA_2s by different phospholipid substrates is determined by the surface topology specific to the region of the protein that interacts with the membrane surface. In PLA_2s from Groups I and II (viperid), this region consists of a hydrophobic surface slit (highly conserved) which binds the acyl ester chain of the phospholipid substrate with a ring around varying polar and charged residues, thus forming the interfacial recognition site, also called the "i" face [32]. Variations in the topology of the "i" face determine the specificity for one phospholipid or another [33,34].

The substrate NBD-PC was used to evaluate the influence of pH and calcium ion on the enzymatic activity (Fig. 4B e C). Both isolated PLA_2s, hydrolyzed the NBD-PC substrate over a wide pH range (4.5–11) with the highest enzymatic activity in the pH range of 5.5–8.5 (Fig. 4B). However, at pH values below 5.5 or above pH 8.5,

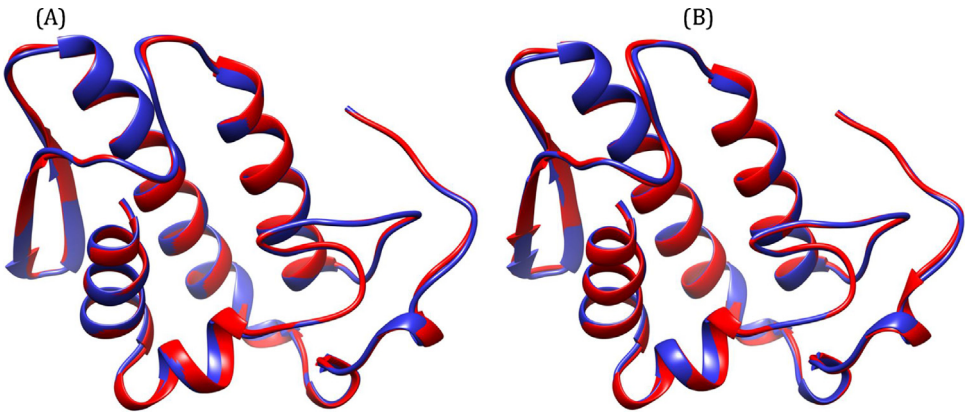

Fig. 3. Superimposition of template (1M8R in blue) between (**A**) Braziliase-I and (**B**) Braziliase-II in red. Representation in ribbon performed at the UCSF Chimera. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

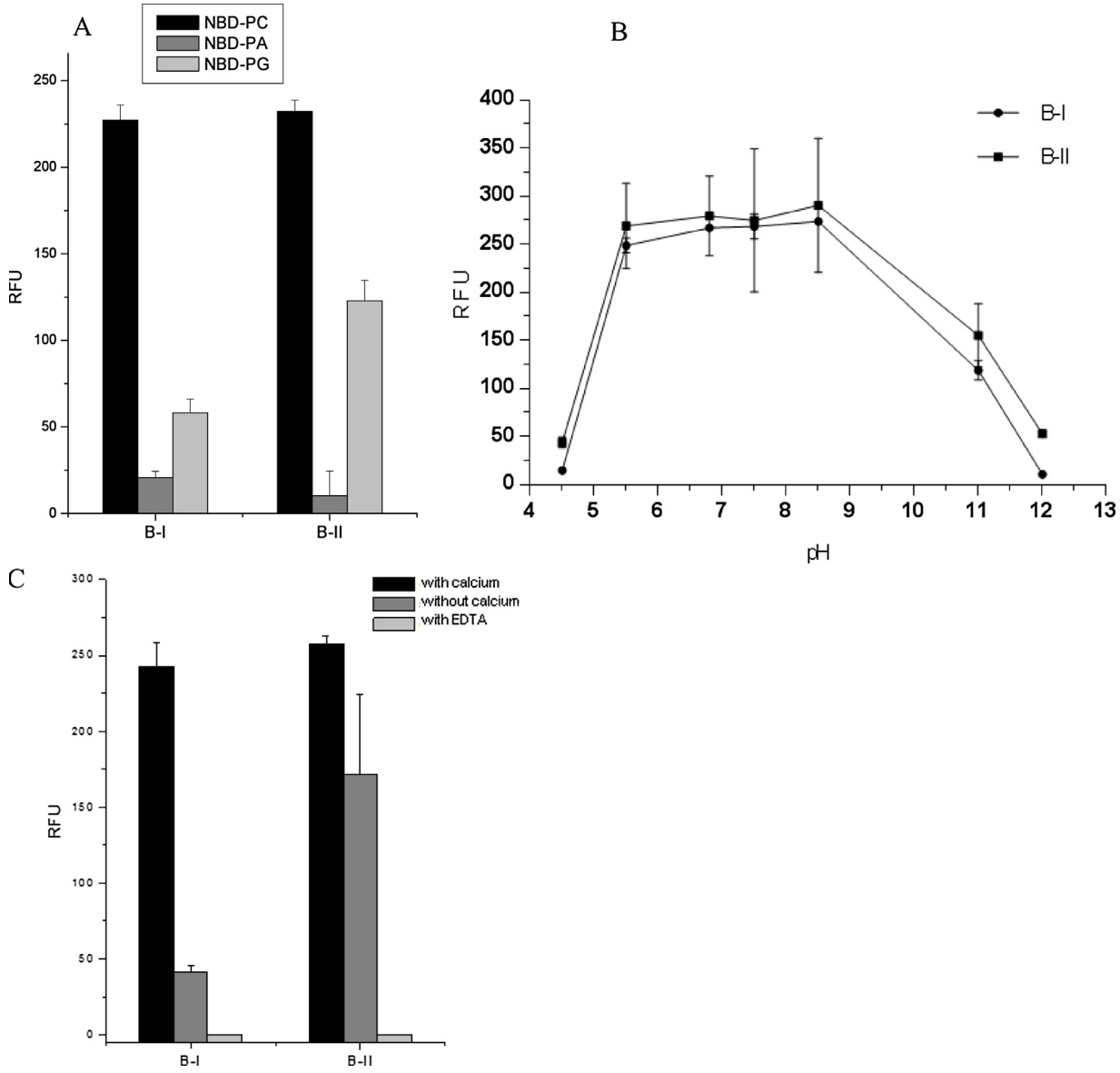

Fig. 4. Phospholipase activity using the isolated PLA_2s Braziliase-I (B-I) and Braziliase-II (B-II).
(A): Using the fluorescent substrates NBD-PC, NBD-PA and NBD-PG, and 0.5 μg of PLA_2s. NBD-PC was the chosen substrate for the other trials. (B): Influence of the pH range from 4.5 to 12 on enzymatic activity. (C): influence of the cofactor Ca^{2+} and EDTA on enzymatic activity.

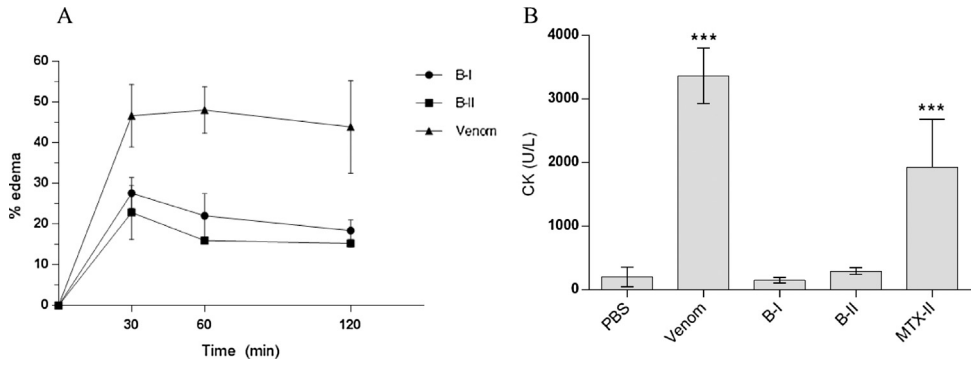

Fig. 5. Biological assays in mice injected with isolated PLA_2s and *Bothrops brazili* venom.
(**A**): Oedema activity using 30 μg of Braziliases I (B-I), II (B-II) and the *B. brazili* venom. Mice received an intraplantar injection in right paw and oedema formation was measured at 30, 60 and 120 min after the administration. PBS was injected in left paw as a control. (**B**): Evaluation of the myotoxic activity of 50 μg of Braziliase-I and II. Myotoxicity was evaluated by creatine kinase (CK) levels in mice plasma serum after 3 h of proteins injection. MTX-II and *B. brazili* venom were used as positive controls for myotoxic activity and PBS for negative control. Results are represented as mean ± standard deviation ($p < 0.05$).

their enzymatic activity dropped significantly. A similar result is described for the acidic PLA_2 BmooTX-I from *B. moojeni* [35] that presented elevated enzymatic activity at pHs 7–11 and for Bp-PLA_2 from *B. pauloensis* [36]. The activity of both enzymes on NBD-PC was dependent on calcium ions. A reduction of their activity was observed without adding calcium and a total loss when added to medium reaction the metal chelator, EDTA (Fig. 4C). Calcium ion is essential for enzymatically active PLA_2s, since it is required to stabilize the conformation of the catalytic calcium binding site which is formed by the amino acid residues Tyr28, Gly30, Gly32 and Asp49. Two water molecules structurally complement the calcium coordination sphere, thus forming a bi-pentagonal pyramid. The Asp residue in position 49 is essential for calcium binding to the protein [37].

In a murine model of oedematogenic activity (Fig. 5A), Braziliase-I and II induced an increase of 27.6% and 22.8% of paw volume, respectively, at 30 min of their injection. After this period, the paw volume returned to basal levels. *B. brazili* venom reached maximum oedema formation after 60 min of its injection with 48% of oedema formation and after 120 min maintained at high levels. Thus, results showed that both PLA_2s, Braziliases-I and II, may contribute or act synergistically to trigger the inflammatory process contributing to the oedema activity observed. Literature, also showed that other acidic bothropic PLA_2s induced oedema formation using the same experimental model such as BthA-I-PLA_2 [15], BpirPLA$_2$-I [29] and BmooPLA$_2$ [17]. Local oedema induced by bothropic venom can be mediated by inflammatory mediators such as vasoactive substances like histamine and serotonin [38]. There is not in the literature studies about the mediators involved in oedema induced by acidic bothropic PLA_2s. Moreover, several *in vitro* studies describe basic snake venom PLA_2s capable to induce mast cells degranulation, to induce microvascular leakage and inflammatory cell accumulation at the inflammatory sites [39–41].

The myotoxic activity assay (Fig. 5B) demonstrated that the purified and characterized Braziliases in our work are not myotoxic. Acidic bothropic snake PLA_2s, usually, do not cause myotoxicity when tested in murine models. The same result was found in literature [15–17]. On the other hand, the basic isoforms seem to have acquired the highest toxicity, especially in the case of neurotoxic and myotoxic enzymes [9]. Fernández et al. [16] suggested a secondary role for acidic PLA_2s with moderate or low toxicity (as seems to be the case of Braziliases), which should be related and restricted to digestive functions. Currently, Resende et al. [19], reported an acidic PLA_2 from *Agkistrodon piscivorus leucostoma* that despite do not induce toxic effects in mammals, plays a significant role for the toxicity in chick, unraveling new functions for these acidic isoforms.

Other works have considered the synergetic action between snake venom toxins. Jimenez-Charris et al. [44], for example, have demonstrated the myotoxic effect triggered by a basic PLA_2 is significantly enhanced by adding acidic PLA_2. Thus, the acidic isoforms isolated from *B. brazili* snake venom in our work may contribute to toxicity for other animals, such birds, or act synergistically with other toxins for mammalian toxicity. However, future studies should be conducted to characterize the pharmacological effects of the acidic PLA_2s together with basic isoforms.

Snake venom, along with other animal venoms, is a source of natural biological compounds that should be evaluated for their potential therapeutic use [45]. Given the advancement in resistance of various parasites to the drugs administered, investigation into alternative therapies is proposed, mainly for the treatment of neglected diseases [42]. *B. brazili* venom presented high cytotoxicity against *T. cruzi* epimastigotes and *L. infantum* promastigotes presenting up to 91.2% and 75.6% (100 μg/mL), respectively (Fig. 6A e B). In the assay with the isolated PLA_2s, Braziliase-I and II induced trypanocidal activity of 31.5% and 33.2% (100 μg/mL) and leishmanicidal activity of 26.2% and 19.2% (100 μg/mL), respectively. In *B. brazili* venom literature documented that there are molecules with anti-leishmania activity. This was observed using the basic PLA_2s from *B. brazili* MTX-I and II, as well as synthetic peptides derived from their primary structure, and both showed activity against *L. amazonensis* and *L. braziliensis* [19]. The leishmanicidal activity reported by Costa et al. [20] work, were related to the C-terminal region composed by hydrophobic and cationic amino acids, from which the peptides were derived.

The mechanism of action, along with other biological activities, is still not well explained. However, Nunez et al. [43] demonstrated that the Lys49-PLA_2-homologue BnSP-7 from *B. pauloensis* causes changes in *L. braziliensis* promastigotes shape, mitochondrial swelling, nuclear alteration, vacuolization, acidocalcisomes, multiflagellar aspects and blebbing effect in the plasma membrane. BmatTX-I (Lys49-PLA_2-homologue) and BmatTX-III (Asp49-PLA_2) isolated from *B. mattogrossensis* showed cytotoxicity against *L. amazonensis* with values very similar to those found in this study (100 μg – 25% and 30%) [44], which could indicate that the mechanism by which the PLA_2s exert cytotoxicity on parasites is not dependent on the enzymatic activity, and thus, it is possible that Lys49-PLA_2-homologues and Asp49-PLA_2s may differ in their mechanism of action on the parasites to promote cytotoxicity. On the other hand, possibly this is the first study reporting trypanocidal activity of an isolated snake venom acidic PLA_2 against *T. cruzi*. The ability of several snake species venoms to cause different effects on intracellular and ultrastructural levels in *T. cruzi*, causing

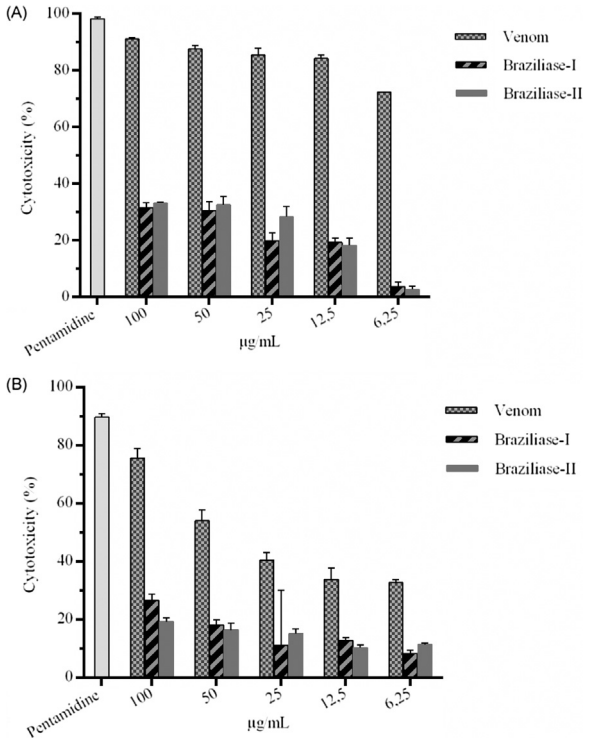

Fig. 6. Evaluation of the cytotoxic activity against *L. infantum* and *T. cruzi* of the isolated PLA_2s and *B. brazili*.venom.
(**A**): Activity against *L. infantum* promastigotes. Pentamidine was used as a positive control. (**B**): Activity against *T. cruzi* epimastigotes. Pentamidine was used as a positive control. The results are represented as mean ± standard deviation ($p < 00.05$).

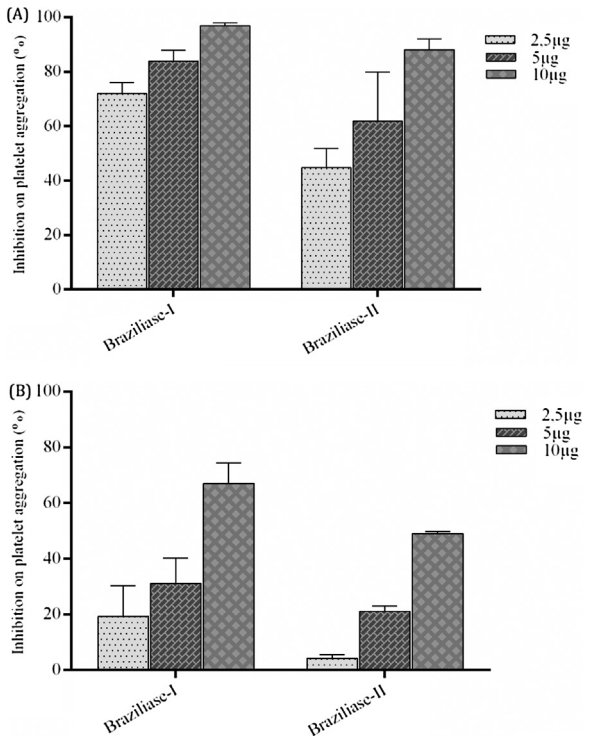

Fig. 7. Evaluation of the ability of acidic PLA_2s from B. *brazili* to inhibit the platelet aggregation.
The platelet aggregation was induced by ADP (A) or collagen (B). The results are represented as mean ± standard deviation ($p < 0.05$).

alterations in infectivity, apoptosis and growth inhibition has been documented [45–48].

Both acidic PLA_2s inhibited platelet aggregation induced by ADP and collagen in a concentration dependent manner (Fig. 7A and B). However, it is important to mention that the ADP agonist was more efficient than collagen in the inhibitory effect. The platelet aggregation inhibitory effect is frequently mentioned in studies with acidic bothropic PLA_2s such as BmooTX-I isolated from *B. moojeni* [17], BpirPLA$_2$-I isolated from *B. pirajai* [29], BE-I-PLA$_2$ isolated from *B. erythromelas* [49] and BthA-I-PLA$_2$ isolated from *B. jararacussu*, the protein used for molecular modeling template [15]. However, the mechanism of this action is not fully elucidated. Some works, such as the study performed by Teixeira et al. [31] have demonstrated that this pharmacological effect induced by toxin can be reproduced by small synthetic peptides that mimicking the C-terminal region of the primary structure of PLA_2s. These researchers reported that the synthetic peptide (^{105}IKYWFYGAKNCQEK^{117}) triggers inhibitory effect similar to the PLA$_2$ used as structural template [29]. The C-terminal region of the Braziliase-I (^{105}-KYWFYGAK-CQEE^{117}), shares high identity with the peptide cited above, suggesting that this region could be responsible for the effect observed in our study.

A very small number of acidic PLA_2s from viperid snake venoms have been characterized. In *B. brazili* venom case there are not published studies characterizing such proteins. The isolation, biochemical and structural characterization of acidic PLA_2s from *B. brazili* venom provide important information to a better understanding of the toxicological effects caused by these proteins, as well as, its potential biotechnological and therapeutic applications.

4. Conclusion

This study was carried out to characterize the structure and biochemistry of two acidic snake venom PLA$_2$ isolated from *B. brazili* venom. The method of purification was effective and resulted in two purified acidic PLA_2s called Braziliase-I and –II. Both Braziliases have molecular weight of approximately 14 kDa. The obtained complete primary sequences of both isolated proteins confirmed that they are Asp49-PLA_2s, catalytically active. The preferred substrate, of the three analyzed, was the phosphatidylcholine. The molecular modeling using BthA-I-PLA$_2$ as a template, showed efficient and the isolated PLA$_2$ were capable to acquire a typical conformation of PLA$_2$ from *Bothrops* venoms. Both Braziliases induced oedema formation, but it was a moderate effect. However, they are not myotoxic PLA_2s and they have low action on analyzed parasites. Nevertheless, both isolated acidic PLA$_2$ showed strong inhibitory effect on platelet aggregation induced by ADP and collagen. These insights are relevant for the elucidation of the functional roles of acidic PLA_2s in envenomings, as well as for identification of its biomedical and biotechnological values, which are essential elements for design and development of new drug leads.

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