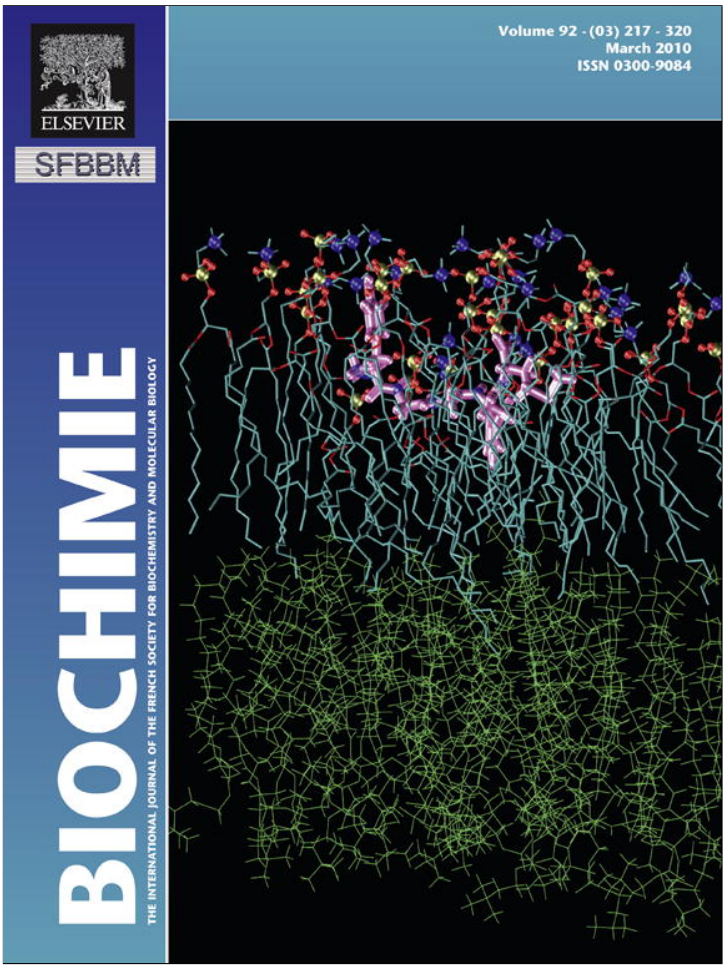

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Research paper

Isolation of an acidic phospholipase A_2 from the venom of the snake *Bothrops asper* of Costa Rica: Biochemical and toxicological characterization☆

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ABSTRACT

Phospholipases A_2 (PLA$_2$) are major components of snake venoms, exerting a variety of relevant toxic actions such as neurotoxicity and myotoxicity, among others. Since the majority of toxic PLA$_2$s are basic proteins, acidic isoforms and their possible roles in venoms are less understood. In this study, an acidic enzyme (BaspPLA$_2$-II) was isolated from the venom of *Bothrops asper* (Pacific region of Costa Rica) and characterized. BaspPLA$_2$-II is monomeric, with a mass of 14,212 ± 6 Da and a pI of 4.9. Its complete sequence of 124 amino acids was deduced through cDNA and protein sequencing, showing that it belongs to the Asp49 group of catalytically active enzymes. *In vivo* and *in vitro* assays demonstrated that BaspPLA$_2$-II, in contrast to the basic Asp49 counterparts present in the same venom, lacks myotoxic, cytotoxic, and anticoagulant activities. BaspPLA$_2$-II also differed from other acidic PLA$_2$s described in *Bothrops* spp. venoms, as it did not show hypotensive and anti-platelet aggregation activities. Furthermore, this enzyme was not lethal to mice at intravenous doses up to 100 μg (5.9 μg/g), indicating its lack of neurotoxic activity. The only toxic effect recorded *in vivo* was a moderate induction of local edema. Therefore, the toxicological characteristics of BaspPLA$_2$-II suggest that it does not play a key role in the pathophysiology of envenomings by *B. asper*, and that its purpose might be restricted to digestive functions. Immunochemical analyses using antibodies raised against BaspPLA$_2$-II revealed that acidic and basic PLA$_2$s form two different antigenic groups in *B. asper* venom.

1. Introduction

The snake *Bothrops asper* is responsible for most cases of envenomings in the Central American region [1,2]. Its venom contains proteins that belong to at least eight families: serine proteinases, disintegrins, metalloproteinases, L-amino acid oxidases, cysteine-rich secretory proteins, DC fragments, C-type lectin-like proteins, and phospholipases A_2 (PLA$_2$s) [3]. PLA$_2$s are ubiquitous enzymes that catalyze the hydrolysis of the C2 ester bond of 3-*sn*-phosphoglycerides, producing lysophospholipids and free fatty acids in a calcium-dependent reaction [4]. In snake venoms, PLA$_2$s have acquired during evolution the ability to exert different toxic activities *in vivo*, most notably neurotoxicity and myotoxicity [5–7]. The PLA$_2$ superfamily includes five types of enzymes (secreted PLA$_2$s, cytosolic PLA$_2$s, calcium-independent PLA$_2$s, lysosomal PLA$_2$s, and platelet-activating factor acetylhydrolases), classified within fifteen groups [8]. Snake venom PLA$_2$s are among the secreted PLA$_2$s, and those from *B. asper*, in similarity to PLA$_2$s of all viperids, belong to the subgroup IIA. Proteins of this subgroup can be further subdivided into two types: Asp49 PLA$_2$s, which are catalytically active, and PLA$_2$ homologues, which possess most commonly a Lys49 residue and do not have catalytic activity [9,10].

Both acidic and basic PLA$_2$s can be found in snake venoms, in variable proportions depending on the species. Nevertheless, the basic isoforms appear to have acquired the highest toxicity, especially in the case of neurotoxic and myotoxic enzymes [11,12]. To date, all acidic PLA$_2$s purified from viperid venoms present an Asp residue at position 49. These acidic isoforms usually have a higher catalytic activity than basic PLA$_2$s upon conventional substrates *in vitro* [11,13,14]. In spite of this, many acidic PLA$_2$s are not lethal or show a weak lethal potency in mice [15–17].

Toxic effects induced by acidic PLA$_2$s from *Bothrops* species were demonstrated in early studies by Nisenbom et al. [18], who isolated an enzyme from *Bothrops alternatus* causing severe tissue damage in the liver, kidneys, lungs and heart of mice. More recent studies have shown that acidic PLA$_2$s from *Bothrops* spp. venoms may express other toxic actions *in vivo*, such as myotoxicity and

☆ UniProt Knowledgebase accession number of BaspPLA$_2$-II is P86389.

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hypotensive activity, as well *in vitro*, such as neuromuscular blockade and inhibition of platelet aggregation [14,17,19–23]. In the case of *B. asper*, Ferlan and Gubenšek [24] purified an acidic enzyme from the venom of specimens from Costa Rica, PLA_2 I, which showed a lethal intravenous potency of 2 μg/g in mice. Alagón et al. [25] characterized three acidic isoforms from the venom of *B. asper* from Mexico, named PLA_2 1, PLA_2 2 and PLA_2 3. This multiplicity of acidic PLA_2 isoforms in the venoms of *B. asper* from the Pacific and Caribbean regions of Costa Rica has also been evidenced by isoelectrofocusing techniques [26], and more recently confirmed using a proteomic approach [3].

The potential toxic activities of acidic PLA_2s of *B. asper* venom have not yet been identified, and therefore their possible roles in the pathophysiology of envenoming are still unknown. In the present work, an acidic PLA_2 from *B. asper* venom (BaspPLA$_2$-II) was isolated and thoroughly characterized, to gain insights into its possible biological roles and relevance in the pathophysiology of envenomings by *B. asper*.

2. Materials and methods

2.1. Isolation of BaspPLA$_2$-II

Crude venom was obtained from more than twenty specimens of *B. asper* from the Pacific region of Costa Rica, kept at the serpentarium of Instituto Clodomiro Picado. The venom was pooled, centrifuged to remove debris, lyophilized, and stored at −20 °C. Batches of 500 mg of venom were dissolved in 6 ml of 0.1 M ammonium acetate buffer, pH 7.0, and applied to a CM-Sephadex C25 column (20 × 2 cm) equilibrated with the same buffer. Protein elution was monitored at 280 nm using an Econo-system chromatograph (Bio-Rad), at 0.4 ml/min. The unbound fraction was collected and applied to a DEAE-Sepharose column (23 × 3 cm), which was eluted at 0.5 ml/min with a linear gradient of ammonium acetate, from 0.1 to 1.0 M, at pH 7.0. Fractions were assayed for PLA_2 activity as described below, and freeze-dried. The fraction with highest activity was subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) on a semi-preparative C8 column (Vydac, 250 × 10 mm, 5 μm particle size), monitored at 280 nm on an Agilent 1100 chromatograph. The protein was dissolved in 1 ml of buffer A (0.1% trifluoroacetic acid [TFA], 5% acetonitrile, 95% water), injected, and eluted at 1 ml/min with a linear gradient from 0 to 70% buffer B (0.1% TFA, 95% acetonitrile, 5% water) in 55 min. The main peak was collected, dried by vacuum centrifugation, dissolved in 1 ml of 0.1 M ammonium acetate buffer, pH 5.0, and finally applied to a CM-Sephadex column (1 × 5 cm) equilibrated with the same buffer, to remove traces of a contaminant. The unbound fraction was collected, freeze-dried, and stored at −20 °C. Homogeneity of the final preparation was evaluated by sodium dodecylsulphate-polyacrylamide (15%) gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions, followed by Coomassie blue R-250 staining. In some experiments, two basic PLA_2s from *B. asper* venom were included for comparative purposes: myotoxin I is a catalytically active Asp49 enzyme [27], whereas myotoxin II is a catalytically inactive Lys49 homologue [28].

2.2. Isoelectric point and molecular mass determinations

Two-dimensional polyacrylamide gel electrophoresis of BaspPLA$_2$-II was performed on a Multiphor II (Amersham Bioscience) apparatus. For the first dimension, 5 μg of enzyme were loaded onto a 7 cm IPG Immobiline® Dry Strip of pH range 3–10, and focused at 200 V for 1 min, followed by 3500 V for 120 min. Second dimension was run on 12% SDS-PAGE and stained by Coomassie. The experimentally observed pI was compared with the theoretically predicted value based on the amino acid sequence, using the Compute pI/MW tool at the ExPASy Proteomics Server (www.expasy.ch/tools). The molecular mass of BaspPLA$_2$-II was determined by electrospray ionization (ESI-MS) on a QTrap 2000 instrument (Applied Biosystems).

2.3. Amino acid sequence

The N-terminal sequence of BaspPLA$_2$-II was obtained directly by automated Edman sequencing on a Procise Instruments Sequenator (Applied Biosystems). Then, protein fragments were generated with cyanogen bromide and separated by RP-HPLC using an Ettan LC system (Amersham) with a C18 column (250 × 4 mm, 5 μm particle size) eluted at a flow rate of 1 ml/min with a linear gradient of 0.1% TFA in water (buffer A) or in acetonitrile (buffer B): 5% B for 10 min, followed by 5–15% B over 20 min, 15–45% B for 120 min, and 45–70% B over 20 min. Detection of peptides was monitored at 215 nm, and the main fragments recovered were subjected to Edman sequencing. Additional internal peptides of BaspPLA$_2$-II were sequenced by tandem MS. Protein bands were excised from Coomassie-stained, reduced 15% gels (SDS-PAGE) and subjected to automated reduction with dithiothreitol, alkylation with iodoacetamide, and digestion with sequencing grade bovine pancreatic trypsin (Roche) using a Progest Digestion Station (Genomic Solutions), following manufacturer's instructions. A total of 0.65 μl of the tryptic peptide mixtures (total volume of 20 μl) was spotted onto a MALDI-TOF sample holder, mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% TFA, dried, and analyzed with a Voyager-DE Pro MALDI-TOF mass spectrometer (Applied Biosystems), operated in delayed extraction and reflector modes. For peptide sequencing, the protein digest mixture was loaded in a nanospray capillary column and subjected to ESI-MS analysis on a QTrap 2000 instrument equipped with a nanospray source (Protana). Doubly- or triply-charged ions of selected peptides from the MALDI-TOF mass fingerprint spectra were analyzed in Enhanced Resolution MS mode, and the monoisotopic ions were fragmented using the Enhanced Product Ion tool with Q0 trapping. Enhanced Resolution was performed at 250 amu/s across the entire mass range. Settings for MS/MS experiments were as follows: Q1, unit resolution; Q1-to-Q2 collision energy, 30–40 eV; Q3 entry barrier, 8 V; LIT (linear ion trap) Q3 fill time, 250 ms; and Q3 scan rate, 1000 amu/s. CID spectra were interpreted manually or using a licensed version of MASCOT (www.matrixscience.com) against a private database containing 927 viperid protein sequences deposited in the Swiss-Prot/TrEMBL database, plus the previously assigned peptide ion sequences from snake venomics projects carried out in the laboratory of J.J. Calvete. MS/MS mass tolerance was set to ± 0.6 Da. Carbamidomethylcysteine and oxidation of methionine were fixed and variable modifications, respectively.

2.4. cDNA cloning and nucleotide sequencing

The complete sequence of BaspPLA$_2$-II was deduced from the cloning and nucleotide sequencing of its cDNA. Total RNA was extracted from the venom glands of *B. asper* (Pacific Region) using specifications of the RNEasy Protect Mini kit (Qiagen). BaspPLA$_2$-II specific mRNA underwent reverse transcription to obtain cDNA with a FirstChoice® RLM-RACE Kit (Ambion) using a rapid amplification of 3′ cDNA ends polymerase chain reaction (3′ RACE-PCR). According to the kit specifications, the entire mRNA was first transformed into cDNA using an OligodT with the following adapter sequence: 5′-GCGAGCACAGAATTAATACGACTCACTATAGGT12VN-3′. From the cDNA obtained, BaspPLA$_2$-II sequence was amplified using

a gene specific primer for the enzyme. This primer was designed on the basis of the N-terminal sequence data. The primer 5′-TGGCAATTCGGGCAAATGATG-3′ corresponds to the N-terminal portion WQFGQMM of the protein. The 3′ RACE Outer Primer had the sequence 5′-GCGAGCACAGAATTAATACGACT-3′, and the 3′ RACE Inner Primer had the sequence 5′-CGCGGATCCGAATTAATACGA CTCACTATAGG-3′. The 3′ RACE-PCR was conducted using the M-MLV reverse transcriptase (Promega). The reaction occurred under the following conditions: 5 min at 65 °C and then 1 h at 42 °C. For the second part of the reaction a touchdown PCR was carried out from 60 to 50 °C. The touchdown 60/50 PCR protocol included an initial denaturation step at 95 °C for 10 min followed by 4 cycles of denaturation (30 s at 94 °C), annealing (30 s at 60 °C), and extension (30 s at 72 °C); 21 cycles starting with the above conditions and, in subsequent cycles, decreasing the annealing temperature by 0.5 °C (reaching 50 °C in cycle 21); 10 cycles of denaturation (30 s at 94 °C), annealing (30 s at 50 °C), and extension (30 s at 72 °C); and a final extension for 10 min at 72 °C. Products were identified on 2% agarose gel electrophoresis, searching for bands of approximately 0.4 kb. The cDNA obtained was treated with the ExoSAP-IT® kit (Affymetrix) for 15 min at 37 °C and 15 min at 80 °C to remove all contaminants. Then, dA tails were added to the cDNA for 30 min at 72 °C. Subsequently, the cDNA was cloned into the pGEM®-T vector (Promega) overnight at 4 °C. Once the cDNA was ligated to the vector, *Escherichia coli* strain DH5α (Novagen) were transformed by electroporation. A PCR was used to detect the presence of the vector with BaspPLA$_2$-II sequence in the colonies. Transformed bacteria were incubated overnight at 37 °C, and then the vector was extracted with the Wizard plus Minipreps DNA purification system (Promega) DNA extraction kit. Final nucleotide sequencing was performed with an Applied Biosystems model 377 instrument, using primers T7 and SP6.

2.5. Molecular modeling

Homology modeling using the Swiss-Model server (http://swissmodel.expasy.org) was utilized to predict the three-dimensional structure of BaspPLA$_2$-II using the acidic PLA$_2$ from *Bothrops jararacussu* (PDB code 1ZL7) as a template, which has a sequence identity of 81%, and has been crystallized and resolved at 1.6 Å [29]. Superposition of model and template structures, and r.m.s.d. calculations were performed with Swiss-PdbViewer [30] and DS ViewerPro (Accelrys).

2.6. Phospholipase A_2 activity

Enzymatic activity of BaspPLA$_2$-II was determined by the colorimetric method of de Araujo and Radvanyi [31], with phenol red as a pH indicator, upon micelles of 0.4% v/v Triton X-100 and 0.25% w/v *sn*-3-phosphatidylcholine as substrate. Twenty microliters of a solution of enzyme, containing 500, 250, 125, or 62.5 ng, in water, were added to 1 ml of substrate in a thermoregulated cuvette at 30 °C. After a stabilization period of 20 s, the decrease in absorbance at 558 nm was monitored continuously for 1 min. One unit of PLA$_2$ activity was defined as the change of 0.001 in absorbance per min. Results obtained with this method were additionally confirmed by means of the titrimetric assay of Dole [32] using egg yolk phospholipids, as described [33], and expressed as μEq/mg/min of enzyme activity.

2.7. Anticoagulant activity

Citrated (3.8% v/v) human plasma was obtained from the blood of healthy volunteers. Aliquots of 0.2 ml were dispensed into glass tubes and incubated in a water bath for 5 min at 37 °C. Then, 50 μl of a BaspPLA$_2$-II solution in phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2) containing 40 μg of enzyme were added, and further incubated for 10 min at 37 °C. Control tubes contained plasma incubated with PBS only. Finally, 50 μl of 0.25 M $CaCl_2$ was added to all tubes and the clotting time was determined, in duplicate assays.

2.8. Anti-platelet aggregating activity

Fresh platelet-rich human plasma was prepared by centrifugation of citrated blood from healthy volunteers, at 135 *g* for 15 min. Aliquots of 450 μl of this preparation were incubated with BaspPLA$_2$-II at final concentrations up to 20 μg/ml plasma, for 5 min at 37 °C. Then, platelet aggregation was initiated by adding 5 μl of 0.1 mM ADP and monitored through the increase in the light transmittance using a model 530-VS aggregometer (Chrono-Log Corporation). Platelet-poor plasma (450 μl), obtained after centrifugation at 1500 g for 15 min, was used as a blank. Platelet-rich plasma incubated with 50 μl of ADP alone served as a positive control for aggregation. Assays were performed in duplicate.

2.9. Cytotoxic activity

The cytotoxic activity of BaspPLA$_2$-II on C2C12 skeletal muscle cell cultures was determined as described [34]. Doses up to 40 μg of the enzyme were diluted in assay medium (Dulbecco's modified Eagle Medium supplemented with 1% fetal bovine serum) and added to cells growing in 96-well plates, in a volume of 100 μl/well. Control wells consisted of medium alone (0% toxicity), or 0.1% Triton X-100 in medium (100% toxicity). After 3 h at 37 °C, 40 μl of the supernatant were taken to determine the activity of lactic dehydrogenase released by damaged cells, using a kinetic assay (LDH-P Mono, Biocon Diagnostik). Assays were performed in duplicate.

2.10. Lethal activity

To evaluate the lethal activity of BaspPLA$_2$-II, four CD-1 mice (16–18 g body weight) received an intravenous injection of 100 μg of enzyme, dissolved in 100 μl of PBS. As a control, two mice were injected similarly with 100 μl of PBS alone. Animals were observed up to 24 h after injection to record deaths. All animal experiments were approved by the Institutional Committee for the Care and Use of Laboratory Animals of the University of Costa Rica (CICUA).

2.11. Myotoxic activity

A group of five mice (18–20 g) received an intramuscular injection of 50 μg of BaspPLA$_2$-II, dissolved in 50 μl of PBS, in their right gastrocnemius. A control group received an identical injection of PBS alone. After 3 h, a tail blood sample was collected into heparinized capillaries, centrifuged, and a plasma aliquot of 4 μl was utilized to determine the activity of creatine kinase (CK; E.C. 2.7.3.2) using a kinetic assay (CK-Nac, Biocon Diagnostik). Enzyme activity was expressed in U/L. Myotoxicity was also assessed by histological evaluation. Twenty-four hour after BaspPLA$_2$-II injection mice were sacrificed by inhalation of carbon dioxide, and samples of their right gastrocnemius were obtained, fixed in 3.7% formalin, and processed for hematoxylin–eosin staining of paraffin-embedded sections.

2.12. Histological evaluation of systemic toxicity

For histological assessment of the systemic toxicity of BaspPLA$_2$-II, two mice (16–18 g) received an intravenous injection of 100 μg of

the enzyme, dissolved in 100 μl of PBS. As a control, two mice were injected identically with PBS alone. Animals were euthanized by carbon dioxide inhalation 24 h after injection, and samples of liver, lungs, heart, and kidneys were obtained. Tissues were fixed and processed as described above.

2.13. Edema-forming activity

A group of four mice (18–20 g) received an injection of 10 μg of BaspPLA$_2$-II, dissolved in 50 μl of PBS, in the footpad. As a control, another group received an identical injection of PBS alone. Footpad thickness was measured with a low-pressure spring caliper (Oditest) before and at various intervals after injection (30, 60, 120, 180, 240, 300, and 360 min). Edema was expressed as the percentage increase in thickness relative to readings obtained before injection.

2.14. Hypotensive activity

A non-invasive blood pressure monitoring system (CODA®, Kent Scientific Corporation) was utilized to evaluate the hypotensive activity of BaspPLA$_2$-II in a group of five mice (18–20 g). Blood pressure was determined before, and at 5 and 30 min after the intravenous injection of 10 μg of BaspPLA$_2$-II, dissolved in 100 μl PBS. As a positive control, another group of mice received 4 μg of crude *B. asper* venom i.v., in 100 μl of PBS. A negative control group received an i.v. injection of 100 μl of PBS alone.

2.15. Preparation of rabbit antibodies against BaspPLA$_2$-II

Antibodies to BaspPLA$_2$-II were prepared by immunization of two rabbits with the purified enzyme, either intramuscularly or subcutaneously. An initial dose of 100 μg, emulsified in complete Freund's adjuvant, was followed by booster doses of 50 μg in incomplete adjuvant, at weeks 5 and 10. Rabbits were bled at week 12 and their sera were separated, aliquoted, and stored at −20 °C.

2.16. Immunochemical analyses

Rabbit antibodies raised against BaspPLA$_2$-II, together with previously obtained rabbit antibodies to *B. asper* myotoxin I [35], and the equine polyvalent (Crotalinae) antivenom produced at Instituto Clodomiro Picado [36] were utilized to analyze the immunochemical relationships between the acidic and basic PLA$_2$s of *B. asper*. Antibody characterization was performed by double immunodiffusion in gel, enzyme-immunoassay (EIA), and immunoblotting. Immunodiffusion was carried out in 1% agarose-PBS gels, loading 30 μl/well of undiluted sera, enzymes (0.2 mg/ml) or crude venom (2 mg/ml), and read after 24 h. For the EIA, 0.2 μg/well of enzymes (BaspPLA$_2$-II or myotoxin I) were adsorbed onto microplates as described [37]. After washing and blocking excess free sites with PBS containing 1% bovine serum albumin (BSA), varying dilutions of antisera were added to triplicate wells and incubated for 1 h. After five washings with FALC buffer (Tris 0.05 M, NaCl 0.15 M, $ZnCl_2$ 20 μM, $MgCl_2$ 1 mM, pH 7.4), bound antibodies were detected with either anti-horse IgG or anti-rabbit IgG–alkaline phosphatase conjugates (1:5000) and *p*-nitrophenylphosphate as substrate. Absorbances were recorded on a Multiskan RC microplate reader (Labsystems) at 405 nm. Normal sera of the corresponding animal species were utilized as negative controls. For immunoblotting, 30 μg of crude *B. asper* venom were separated by SDS-PAGE (15%) under reducing conditions, followed by electrotransfer to nitrocellulose in a Bio-Rad cell at 150 mA during 90 min. To assess transfer efficiency, membranes were previsualized by reversible Ponceau-S Red staining. Then, membranes were blocked in 1% BSA–PBS for 30 min, and incubated for 90 min with 1:1000 dilutions of antisera, or the corresponding normal sera for each species. After washing four times with PBS containing 0.1% BSA and 0.05% Tween-20, the membranes were incubated with the appropriate anti-IgG–alkaline phosphatase conjugates (1:2000) during 90 min. Membranes were finally washed four times, and color development was performed with the BCIP/NBT substrate (Chemicon).

2.17. Neutralization of BaspPLA$_2$-II enzymatic activity by rabbit and equine antibodies

BaspPLA$_2$-II was preincubated for 30 min at 37 °C with rabbit antiserum or equine antivenom, at ratios of 0.5, 1, 2, and 4 ml serum/mg enzyme. Then, aliquots containing 0.25 μg or 15 μg of enzyme were assayed for PLA$_2$ activity, as described above, using the colorimetric or the titrimetric assays, respectively. Controls included identical enzyme aliquots incubated with PBS alone, or with normal sera from the corresponding species. Assays were performed in duplicate.

2.18. Statistical analysis

Results are expressed as mean ± S.D. The significance of differences between the means of two experimental groups was analyzed by Student's *t*-test, where a p value <0.05 was considered significant.

3. Results

3.1. Isolation and biochemical properties of BaspPLA$_2$-II

To ensure the removal of basic PLA$_2$s and PLA$_2$ homologues of *B. asper* venom, the first chromatographic step was performed in CM-Sephadex at pH 7.0 (Fig. 1A), where such components were retained. The unbound material was subsequently resolved into several peaks by the DEAE-Sepharose step, where the highest PLA$_2$ activity eluted in fraction D1 (Fig. 1B). The subsequent RP-HPLC separation of this fraction (Fig. 1C) eliminated most contaminants, but traces of a procoagulant venom component still remained, only detectable by its clotting activity upon human plasma (data not shown). This minor contaminant, most likely a thrombin-like serine proteinase [38], was successfully removed from BaspPLA$_2$-II by a final fractionation step on CM-Sephadex at pH 5.0, where it was retained by the chromatographic support.

Electrophoretic analyses of BaspPLA$_2$-II by SDS-PAGE showed that this enzyme migrates as a monomer of approximately 15–16 kDa, both under reducing and non-reducing conditions (Fig. 1D), consistent with the molecular mass of 14,212 ± 6 Da determined by ESI-MS. Experimental assessment of the pI of this enzyme by 2D electrophoresis resulted in an estimated value of 4.9, close to the theoretical pI value of 5.05 predicted on the basis of its complete sequence.

The amino acid sequence of BaspPLA$_2$-II was obtained by a combination of Edman degradation, tandem mass spectrometry, and nucleotide sequencing of its cloned cDNA. It is composed of 124 amino acid residues, containing the conserved Asp49 of catalytically active enzymes (Fig. 2). The calculated isotope-averaged molecular mass for the amino acid sequence shown in Fig. 2 (14,179.97) is about 32 ± 6 Da lower than the experimentally determined mass, suggesting that the protein may contain modified residues. In line with this assumption, the C-terminal peptide was sequenced by MS/MS analysis of the doubly-charged peptidic ion at m/z 576.6 as NCQE(129)SEPC. The sequences of the b5 and y5 daughter ions were interpreted as NCQE(D-oMe) and (D-oMe)SEPC, respectively, indicating that Asp120 was o-methylated. The remaining 16 Da difference between experimental and calculated

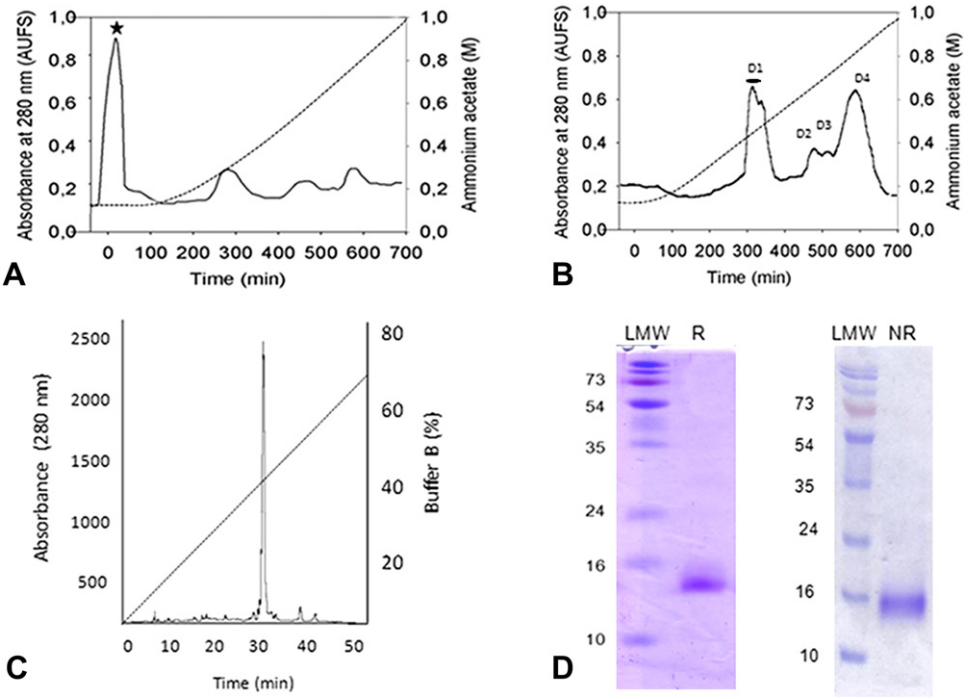

Fig. 1. Isolation of BaspPLA$_2$-II. (A) Fractionation of crude *B. asper* venom on CM-Sephadex at pH 7.0, eluted with ammonium acetate (0.1–1.0 M), as described in Materials and Methods. The unbound fraction (star) was subjected to separation on DEAE-Sepharose (B) using an identical gradient as in (A). Fraction D1 (thick horizontal line) was further purified by RP-HPLC on a semi-preparative C8 column (C), eluted with a 5–70% acetonitrile gradient over 55 min. (D) SDS-PAGE (15%) analysis of BaspPLA$_2$-II under reduced (R) and non-reduced (NR) conditions. LMW: low molecular weight markers, as indicated at the left, in kDa.

masses may correspond to oxidation of one of the 5 methionine residues of the protein.

The PLA$_2$ activity of BaspPLA$_2$-II was confirmed, as shown in Fig. 3. In comparison to myotoxin I, a basic Asp49 isoform from *B. asper* venom, this acidic enzyme was slightly more active in hydrolyzing phosphatidylcholine micelles.

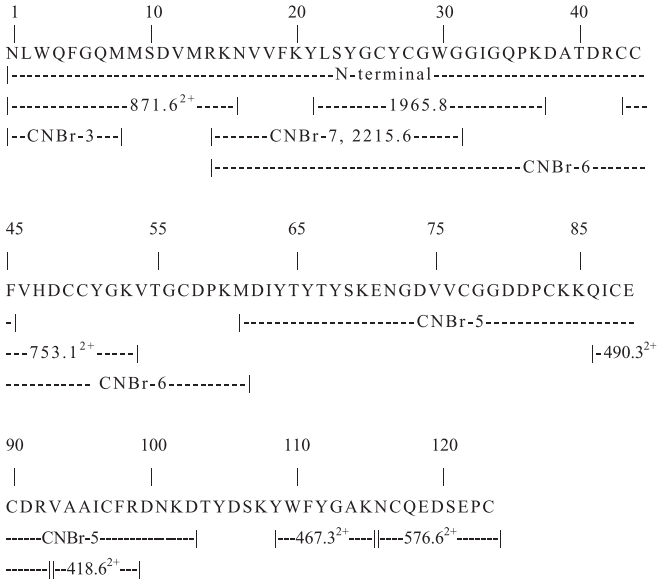

Fig. 2. Amino acid sequence of BaspPLA$_2$-II. The first 44 amino acid residues were determined by direct Edman degradation sequencing from the N-terminus. Overlapping peptides were generated by protein cleavage with CNBr. Other internal fragments, obtained after trypsin digestion, were sequenced *de novo* by ESI-MS/MS. Molecular mass values of the fragments are indicated.

Comparison of BaspPLA$_2$-II with similar proteins in the SwissProt database showed that its primary structure is closely related to several group IIA acidic PLA$_2$s of crotaline species, mostly to the enzymes isolated from *Bothrops jararaca, Bothrops insularis*, and *B. jararacussu* (Fig. 4). Multiple sequence alignment evidenced that only BaspPLA$_2$-II and the *B. jararaca* P81243 enzyme present 124 amino acids within this group of proteins, all others having 122, or 123 in the case of *Gloydius ussurensis* (Q7LZU4). This difference is caused by the insertion of two residues, Thr67 and Tyr68, in both BaspPLA$_2$-II and the *B. jararaca* PLA$_2$ (Fig. 4). A phylogenetic tree

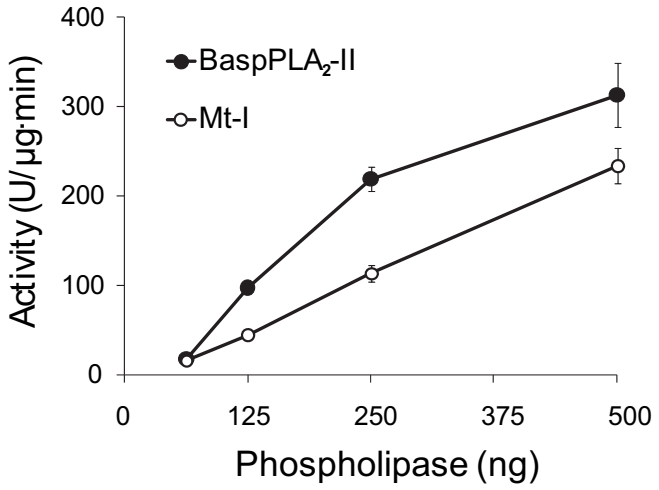

Fig. 3. Phospholipase A$_2$ activity of BaspPLA$_2$-II and myotoxin I (Mt-I) from *B. asper* venom upon phosphatidylcholine micelles, determined by the phenol red assay, as described in Materials and Methods. (●) BaspPLA$_2$-II; (○) myotoxin I. Each point represents mean ± SD of duplicates.

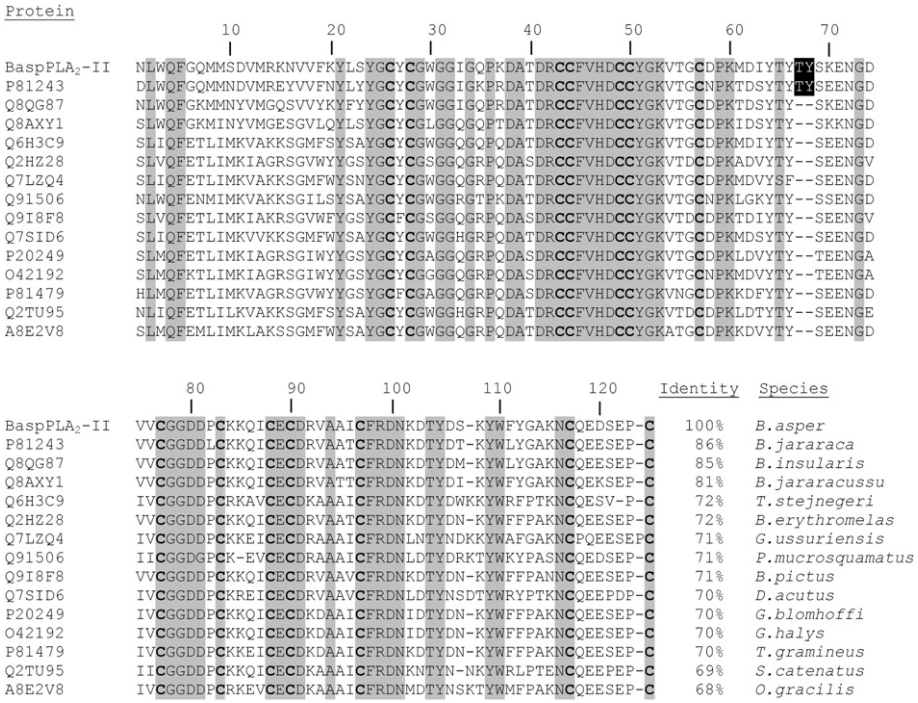

Fig. 4. Multiple sequence alignment of BaspPLA$_2$-II with related proteins in crotaline snake venoms. Protein codes correspond to the UniProtKB database at the ExPASy Proteomics Server. Alignments and percent identity calculations were performed with ClustalW [57]. Identical positions are shaded in gray, and cysteine residues are shown in boldface. A black background highlights the insertion of two amino acids at positions 69 and 70, only present in BaspPLA$_2$-II and the PLA$_2$ (P81243) of *B. jararaca*.

constructed with 15 acidic PLA$_2$s confirmed the close evolutionary relationship of BaspPLA$_2$-II with the enzymes of *B. jararaca*, *B. insularis*, and *B. jararacussu* from South America (Fig. 5), whereas the acidic PLA$_2$s from other South American *Bothrops*, such as *B. erythromelas* and *B. pictus*, were more distant from the clade of BaspPLA$_2$-II. On the other hand, the basic PLA$_2$ myotoxin I (P20474) from *B. asper* venom was markedly distant from BaspPLA$_2$-II in the cladogram, serving as an outgroup (Fig. 5), and confirming the divergent evolutionary pathways of acidic and basic PLA$_2$s even within the venom of a single viperid species.

A three-dimensional model of BaspPLA$_2$-II was built using as template the crystal structure of *B. jararacussu* acidic PLA$_2$. Both structures were superimposed, as shown in Fig. 6, resulting in average r.m.s.d. value for α-carbon backbones of 1.27 Å. The main structural deviation between the BaspPLA$_2$-II model and its template protein was predicted to occur immediately before the

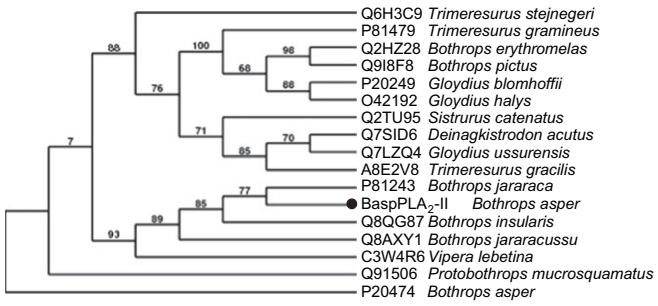

Fig. 5. Phylogenetic relationships of BaspPLA$_2$-II with other phospholipases A$_2$ from snake venoms. Protein codes are as described in Fig. 4. The cladogram was constructed using the maximum likelihood method implemented in the PhyML program at www.phylogeny.fr [58], and graphically represented with TreeDyn. Support values for branches are indicated as percentages. In addition to all proteins aligned in Fig. 4, a basic PLA$_2$ from *Bothrops asper* (P20474; myotoxin I), and an acidic PLA$_2$ from a non-crotaline viperid (*Vipera lebetina*; C3W4R6) were included as outgroups.

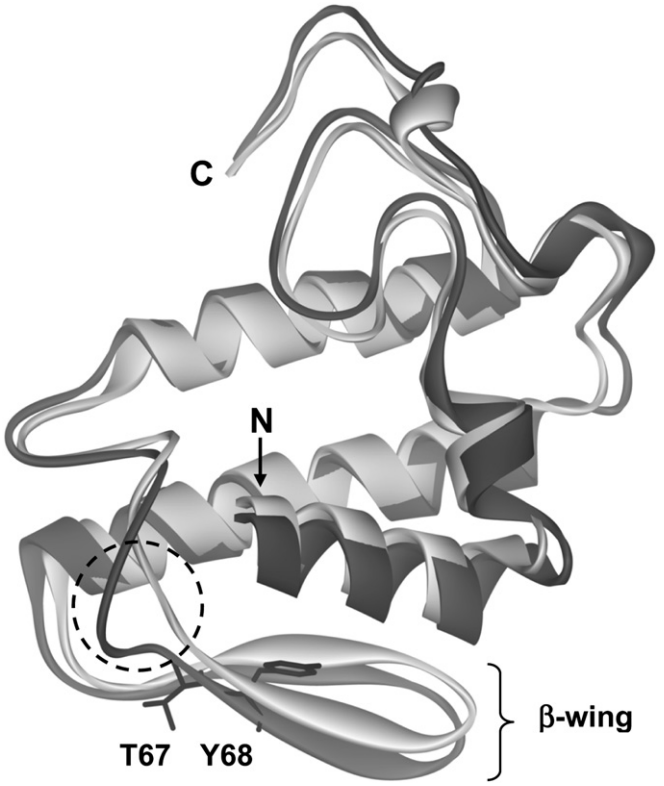

Fig. 6. Three-dimensional model of BaspPLA$_2$-II (dark gray), superimposed on the crystal structure of *B. jararacussu* acidic PLA$_2$ (PDB code 1ZL7; light gray), in ribbon representation. N- and C-termini are labeled. Amino acid side chains of T67 and Y68 of BaspPLA$_2$-II are shown in dark gray. The dashed circle highlights the large deviation in segment 62–65, immediately before the β-wing region, which bulges out in comparison to the structure of the template protein.

"β-wing" region, where residues 62–65 bulge out, probably due to the insertion of the additional Thr67 and Tyr68 (see alignments of Fig. 4) within the constraints of the relatively rigid scaffold of PLA_2s.

3.2. Biological activities of BaspPLA$_2$-II in vitro and in vivo

BaspPLA$_2$-II did not exert anticoagulant effect upon human plasma *in vitro*, up to a concentration of 100 μg/ml. The mean time for clot formation in plasma incubated with this enzyme was 230 ± 34 s, while plasma incubated with PBS clotted after 217 ± 21 s ($p > 0.05$). Under the same conditions, myotoxin I prolonged the clotting time of plasma to 2400 ± 50 s ($p < 0.05$). BaspPLA$_2$-II also lacked anti-aggregating activity for ADP-stimulated human platelets, up to a concentration of 10 μg/ml of enzyme. Similarly, this enzyme did not lyse skeletal muscle C2C12 myoblasts in culture, in contrast to the basic Lys49 myotoxin II used as a control (Fig. 7A). Exposure of these cells to BaspPLA$_2$-II, up to 40 μg/well (400 μg/ml) for 3 h, did not induce morphological alterations nor LDH release to the supernatants.

In vivo, the i.m. injection of BaspPLA$_2$-II (50 μg) did not increase plasma CK levels after 3 h (Fig. 7B), indicating its lack of myotoxic activity. This was also confirmed by histological evaluation of the injected gastrocnemius muscle, obtained after 24 h, which showed a normal tissue morphology (Fig. 7C and D). Similarly to observations made on skeletal muscle, the histological evaluation of other tissues, including liver, lungs, heart, and kidneys, after the i.v. injection of 100 μg of BaspPLA$_2$-II, indicated in all cases a normal morphology, similar to the corresponding tissues of control mice receiving a PBS injection (not shown). In addition, BaspPLA$_2$-II was not lethal to mice by the i.v. route, up to a dose of 100 μg (5.6–6.2 μg/g). No changes in the blood pressure of mice were recorded after the i.v. injection of this enzyme (10 μg), whereas injection of the crude venom (4 μg) under identical conditions caused a rapid and transient drop in this parameter (Fig. 8) One mouse was also injected with 25 μg of BaspPLA$_2$-II, and there were no changes in blood pressure (data not shown). The only toxic effect induced by BaspPLA$_2$-II was a transient induction of local edema in the mouse footpad assay (Fig. 9).

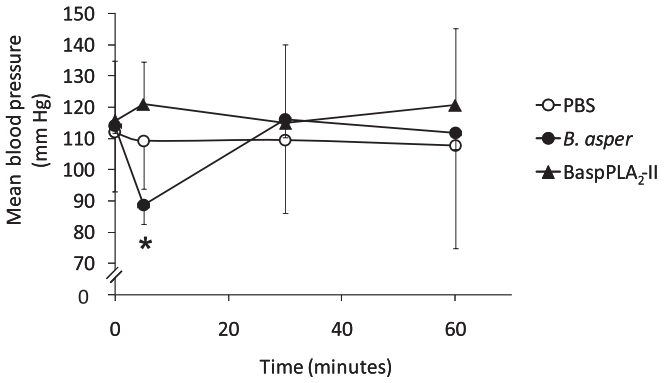

Fig. 8. Lack of hypotensive effect of BaspPLA$_2$-II. Mean blood pressure of mice was recorded before (0 min) and after (5 and 30 min) the i.v. injection of this enzyme (10 μg), crude *B. asper* venom (4 μg), or PBS alone, under identical conditions. Points represent mean ± SD of five animals per group. The asterisk indicates a statistically significant ($p < 0.05$) difference.

3.3. Immunochemical analyses of BaspPLA$_2$-II

Rabbits immunized with BaspPLA$_2$-II by i.m. or by s.c. routes, respectively, produced an antibody response to the enzyme, as shown by the ability of their sera to form a precipitin line against both the purified BaspPLA$_2$-II or crude *B. asper* venom by gel immunodiffusion (Fig. 10A). The sera of these two rabbits had similar titers by EIA (data not shown). These rabbit antibodies recognized a single band of 15–16 kDa in crude *B. asper* venom subjected to immunoblotting analysis (Fig. 10B), corresponding to the expected migration of the enzyme, and further supporting the homogeneity of the immunizing preparation as well as the monospecificity of the antiserum. By EIA, rabbit antibodies to BaspPLA$_2$-II recognized the homologous antigen, but not the basic PLA_2 myotoxin I, resulting in a signal close to that of non-immune sera (Fig. 10C). Reciprocally, rabbit antibodies to myotoxin I readily recognized this basic protein in the EIA, but did not cross-react with the acidic BaspPLA$_2$-II (Fig. 10C). On the other hand, the equine

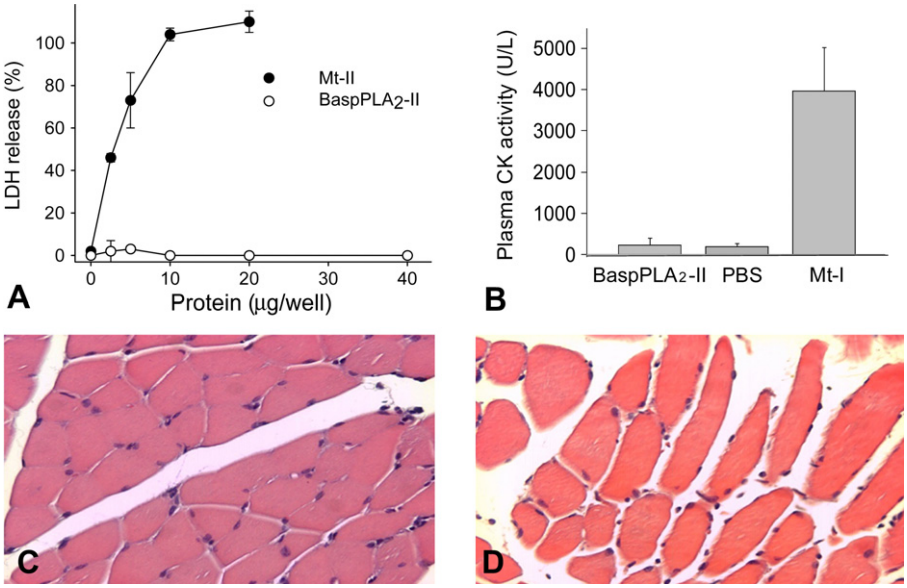

Fig. 7. Lack of muscle damaging activity of BaspPLA$_2$-II. (A) Cytotoxicity was evaluated upon cultured C2C12 skeletal muscle cells, exposed to BaspPLA$_2$-II or to *B. asper* myotoxin II as a control. Lactic dehydrogenase (LDH) release was determined after 3 h. Each point represents mean ± SD of duplicate assays. (B) Myotoxic activity was evaluated by determining plasma creatine kinase (CK) activity 3 h after i.m. injection of BaspPLA$_2$-II (50 μg/μl) or *B. asper* myotoxin I (50 μg/μl) or PBS (50 μl) as controls. (C) Histologic evaluation of hematoxylin–eosin stained sections of gastrocnemius muscle 24 h after the i.m. injection of BaspPLA$_2$-II (50 μg/50 μl) or (D) PBS (50 μl).

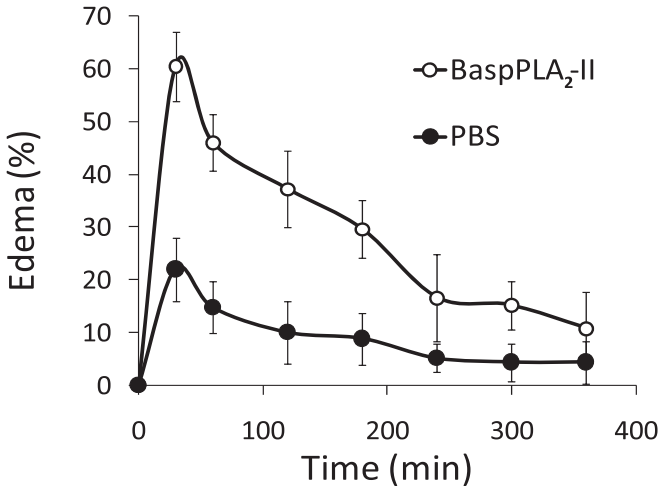

Fig. 9. Edema-forming activity of BaspPLA$_2$-II. Footpad thickness was determined before and after injection of the enzyme (10 μg dissolved in 50 μl PBS), and edema was expressed as the percentage increase relative to readings obtained before injection. (○) BaspPLA2-II; (●) PBS control. Each point represents mean ± SD of four animals.

polyvalent antivenom produced at Instituto Clodomiro Picado clearly recognized BaspPLA$_2$-II by EIA, resulting in a titration curve comparable to that corresponding to antibodies against myotoxin I (Fig. 11A). However, as shown in Fig. 11B and C, when the ability of equine and rabbit antibodies to neutralize the enzymatic activity of BaspPLA$_2$-II was tested in preincubation assays, neutralization was only partial in the case of the polyvalent antivenom, whereas inhibition by the rabbit serum was null, even at a very high serum/enzyme ratio (4 ml/mg).

4. Discussion

The first complete biochemical and toxicological characterization of an acidic PLA$_2$ from the venom of *B. asper*, here named BaspPLA$_2$-II, is reported. This enzyme is monomeric, with a pI of 4.9 and a molecular mass of 14,212 ± 6 Da. According to its structural characteristics, this protein corresponds to the fraction described as peak 12 in the venom proteome of *B. asper* (Pacific region of Costa Rica), which matches its molecular mass, N-terminal and internal peptide sequences, and pI on 2-D gel electrophoresis [3]. On this basis, and considering the quantitative data generated by the

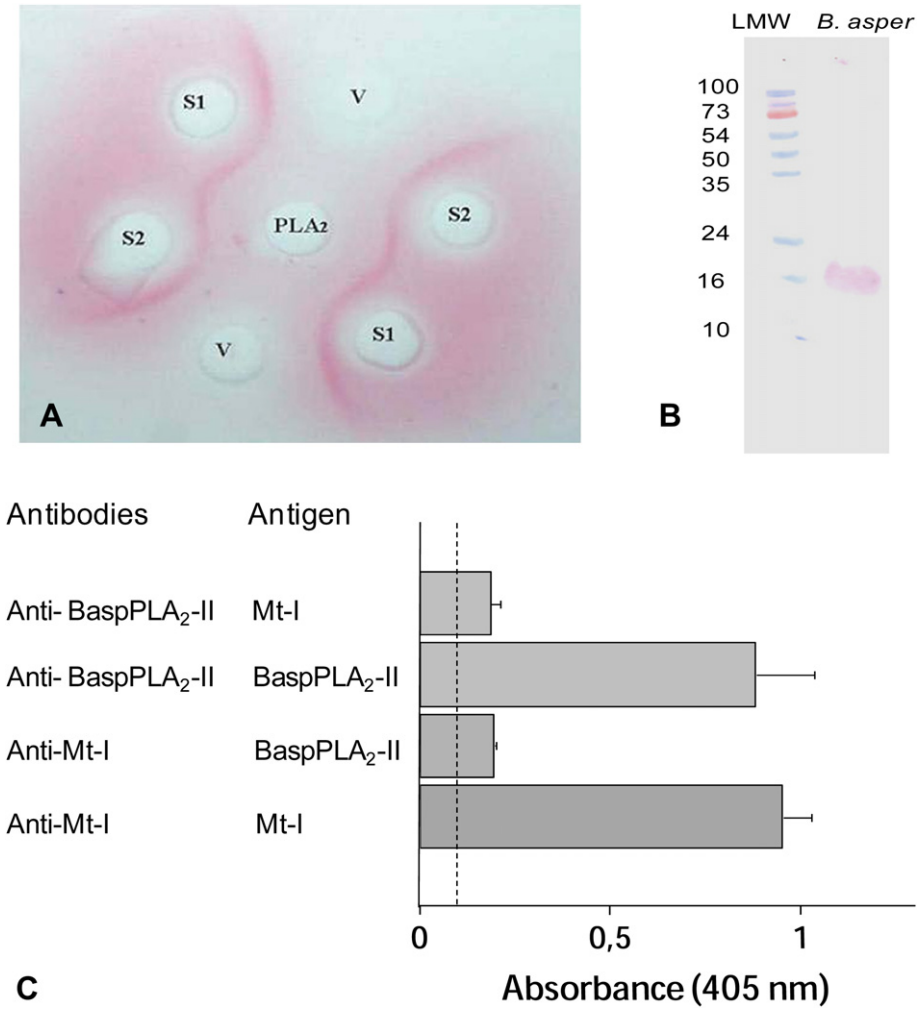

Fig. 10. (A) Gel immunodiffusion of rabbit antibodies to BaspPLA$_2$-II. Immune sera from two rabbits (S1, S2) precipitated BaspPLA$_2$-II, both in purified form (PLA$_2$) or in the crude *B. asper* venom (V), as identified by the fused precipitin lines. (B) Immunoblotting analysis of the rabbit serum to BaspPLA$_2$-II. Crude *B. asper* venom (30 μg, reduced) was subjected to SDS-PAGE (15%), transferred to nitrocellulose, and probed with the rabbit antibodies as described in Materials and Methods. (C) Antigenic comparison of BaspPLA$_2$-II and *B. asper* myotoxin I (Mt-I) by enzyme-immunoassay. Both antigens were adsorbed to microplates and probed with rabbit antibodies against BaspPLA$_2$-II or Mt-I, respectively. The dashed line represents the absorbance value of non-immune rabbit sera.

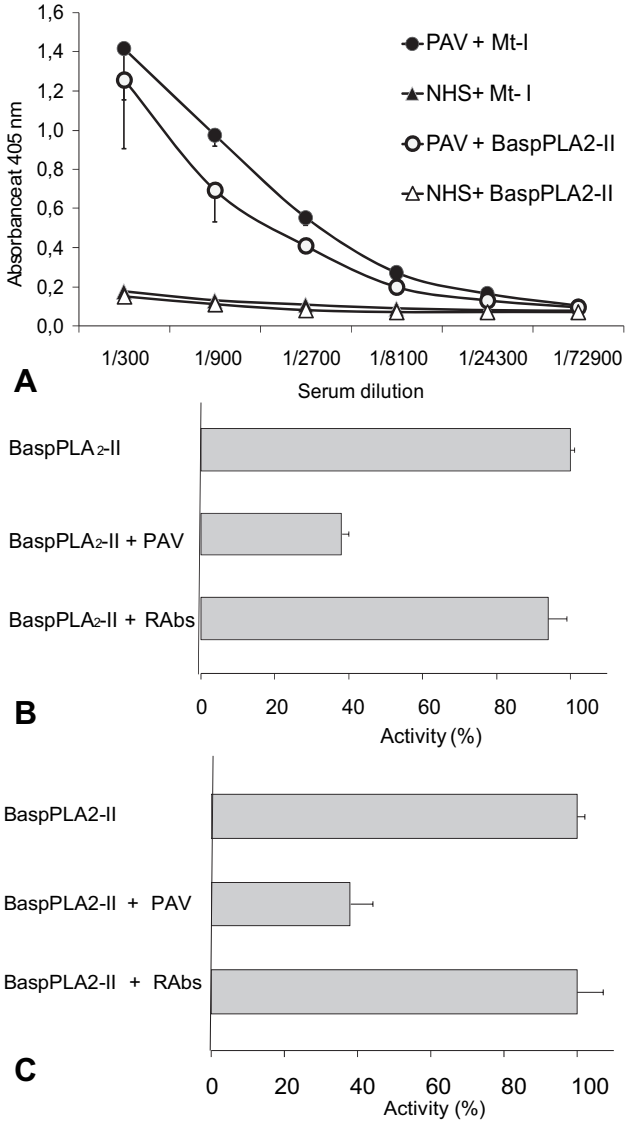

Fig. 11. Titration of antibodies to BaspPLA$_2$-II or to *B. asper* myotoxin I (Mt-I) in the equine polyvalent (Crotalinae) antivenom from Instituto Clodomiro Picado (PAV), determined by EIA (A). NHS: normal horse serum. Points represent mean ± SD of triplicate wells. Neutralization of PLA$_2$ activity of BaspPLA$_2$-II by polyvalent antivenom or rabbit immune serum (RAbs), determined by the phenol red (B) or titrimetric (C) assays. Bars represent mean ± SD of duplicates.

proteomic analysis of this venom, it is estimated that BaspPLA$_2$-II represents 6.3% of its proteins [3,39].

BaspPLA$_2$-II is composed of 124 amino acids, presenting Asp49 and the characteristic pattern of half-Cys residues of group IIA PLA$_2$s [8]. These findings place BaspPLA$_2$-II within the catalytically active enzymes, consistent with all acidic PLA$_2$s purified from viperid snake venoms, where Asp49 appears so far to be an absolutely conserved position. The phospholipolytic activity of BaspPLA$_2$-II was confirmed using phosphatidylcholine micelles as substrate, and shown to be slightly higher than the activity of myotoxin I, a basic PLA$_2$ of the same venom. In general, snake venom acidic PLA$_2$s tend to be more active in catalysis than basic isoforms, in spite of the stronger toxicity of the latter [11,13,14].

The primary structure of BaspPLA$_2$-II presents high identity values in comparison to other acidic enzymes within the genus *Bothrops*, particularly P81243 from *B. jararaca* [19], Q8QG87 from *B. insularis* [40], and Q8AXY1 from *B. jararacussu* [17]. Of these, only the PLA$_2$ from *B. jararaca* shares with BaspPLA$_2$-II the feature of having 124 amino acids (as opposed to the pattern of 122 residues of most of these enzymes), caused by two insertions at positions 67 and 68. Interestingly, the three proteins with highest similarity to BaspPLA$_2$-II express some toxic activities, such as inhibition of platelet aggregation (P81243 and Q8AXY1), myotoxicity (Q8QG87), and hypotensive effect (Q8AXY1), whereas the toxicological characterization of BaspPLA$_2$-II, here presented, evidenced none of these actions. Negative results were obtained for anticoagulant, anti-platelet aggregation, cytotoxic, myotoxic, hypotensive, and lethal effects of BaspPLA$_2$-II. The growing structural information on acidic PLA$_2$s that differ in their toxic activities, or even lack toxicity, may become of value to address the complex structure–function relationships that govern this highly diverse group of snake venom proteins. The elucidation of an increasing number of venom proteomes, or venomes, has revealed that PLA$_2$s constitute percentages as large as 30–60% in some species [39,41], strongly arguing for their relevance in such secretions. In the case of BaspPLA$_2$-II, with the exception of a transient, moderate edema-inducing effect, the observed lack of toxic activities implies that its contribution to the overall physiopathology of envenomings by *B. asper* is probably of marginal relevance. Rather, the present results suggest that this enzyme could play mainly a digestive function in this venom, by contributing to the hydrolysis of phospholipids of the prey, a hypothesis that would need to be addressed. Alternatively, this "non-toxic" enzyme could have yet unknown toxic actions upon the physiology of prey other than rodents. Since it is known that neonate and juvenile specimens of *B. asper* feed on ectothermic prey, i.e. frogs and lizards [42], it would be relevant to assess the toxic profile of BaspPLA$_2$-II in these prey.

The induction of edema by BaspPLA$_2$-II is consistent with reports of this activity being expressed by a number of acidic PLA$_2$s from snake venoms [14,16,17,20–22,40,43,44]. Mechanisms that underlie this effect have been attributed to phospholipid hydrolysis, resulting in the release of precursors of eicosanoids and platelet-activating factor, or to the degranulation of mast cells, with subsequent release of vasoactive amines [14,45,46].

BaspPLA$_2$-II was devoid of myotoxic activity *in vivo*, as determined by the lack of plasma CK increase and by histological observation. This result was in agreement with the absence of cytolytic action upon C2C12 cells, known to represent a good correlate for myotoxicity in the case of group IIA PLA$_2$s [34]. These findings are consistent with observations in most of the acidic PLA$_2$s purified from snake venoms, which generally lack myotoxicity. However, some recently isolated acidic PLA$_2$s display myotoxicity *in vivo* [14,20,21,40,47]. Although it is clear that the catalytic activity of PLA$_2$s is not a sufficient requirement to generate myotoxicity *per se*, the structural determinants of such differences in myotoxicity among acidic enzymes are unknown, and their identification poses a challenging question.

The fact that BaspPLA$_2$-II was not lethal up to a dose of 5.9 μg/g, and the lack of systemic toxicity to major organs, is also in line with literature reports for several acidic PLA$_2$s which display a very low or no lethal activity. It also indicates that BaspPLA$_2$-II does not correspond to the acidic PLA$_2$ isolated from *B. asper* venom by Ferlan and Gubenšek [24], which had a lethal intravenous activity of 2 μg/g. A comparison of partial amino acid sequences between BaspPLA$_2$-II and another acidic PLA$_2$ isolated from the venom of *B. asper* from Panamá (to be named BaspPLA$_2$-I, personal communication of A.M. Soares, University of São Paulo, Brasil) revealed several structural differences. Therefore, the different acidic PLA$_2$ isoforms that are present in *B. asper* venom may vary in the expression of toxic effects.

BaspPLA$_2$-II did not present anticoagulant activity, an effect which has been reported for a number of acidic PLA$_2$s from viperid

snake venoms [20,22]. Moreover, anti-platelet aggregating activity and hypotensive effect were also absent in BaspPLA$_2$-II, again at variance with other acidic PLA$_2$s [14,17,19–23,48–50]. It will be important to determine in future studies if the hypotensive effect of whole *B. asper* venom is induced by other acidic PLA$_2$ isoforms or by proteins/peptides from other families. Interestingly, the proteomic analyses of *B. asper* venoms from both versants of Costa Rica (Caribbean and Pacific) indicate the absence of bradykinin-potentiating peptides [3], known as important mediators of hypotension in other *Bothrops* species [51].

Immunochemical analyses with antibodies raised against BaspPLA$_2$-II revealed that this acidic enzyme differs antigenically from the basic PLA$_2$s in *B. asper* venom. This conclusion was also supported by the reciprocal analyses using antibodies to myotoxin I, which did not recognize BaspPLA$_2$-II. The antigenic divergence between snake venom PLA$_2$s from elapids and viperids has been demonstrated in previous studies [52,53]. However, the present results offer a first evidence of antigenic divergence among acidic and basic PLA$_2$s within viperids, in this case, within the venom of a single species. Thus, despite a sequence identity of 52% between *B. asper* myotoxin I and BaspPLA$_2$-II, antibodies against each of the two were not cross-reactive.

Interestingly, despite the clear presence of antibodies to BaspPLA$_2$-II in both its homologous rabbit serum and in the equine polyvalent antivenom, their ability to neutralize its catalytic activity was poor. This suggests the possibility that BaspPLA$_2$-II may induce antibodies towards immunodominant non-neutralizing epitopes, in sharp contrast with the case of basic PLA$_2$s of this venom, which are well neutralized by antibodies from various sources [35,54–56]. Nevertheless, the lack of major toxic activities of BaspPLA$_2$-II, as shown in the present study, predicts that its poor neutralization by antivenoms should not be of concern from a therapeutic point of view.

In conclusion, the newly characterized protein BaspPLA$_2$-II is a monomeric, acidic PLA$_2$ from *B. asper* (Pacific versant of Costa Rica) venom which, due to its lack of major toxic actions reported for this type of proteins, probably has a marginal role in envenomings by this snake species. This enzyme might play a digestive function. The elucidation of its complete primary structure, here reported, will be of value in future comparative studies attempting to identify the molecular determinants of toxic activities by some acidic PLA$_2$s in crotaline venoms.

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