Isolation of an acidic phospholipase A2 from the venom of the snake Bothrops asper of Costa Rica: Biochemical and toxicological characterization

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A B S T R A C T

Phospholipases A2 (PLA2) 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 PLA2s are basic proteins, acidic isoforms and their possible roles in venoms are less understood. In this study, an acidic enzyme (BaspPLA2-II) was isolated from the venom of Bothrops asper (Pacific region of Costa Rica) and characterized. BaspPLA2-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 BaspPLA2-II, in contrast to the basic Asp49 counterparts present in the same venom, lacks myotoxic, cytotoxic, and anticoagulant activities. BaspPLA2-II also differed from other acidic PLA2s 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 BaspPLA2-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 BaspPLA2-II revealed that acidic and basic PLA2s 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, metallocproteinases, s-αmino acid oxidases, cysteine-rich secretory proteins, DC fragments, C-type lectin-like proteins, and phospholipases A2 (PLA2) [3]. PLA2s 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, PLA2s have acquired during evolution the ability to exert different toxic activities in vivo, most notably neurotoxicity and myotoxicity [5–7]. The PLA2 superfamily includes five types of enzymes (secreted PLA2s, cystolic PLA2s, calcium-dependent PLA2s, lysosomal PLA2s, and platelet-activating factor acetylhydrolases), classified within fifteen groups [8]. Snake venom PLA2s are among the secreted PLA2s, and those from B. asper, in similarity to PLA2s of all vipers, belong to the subgroup IIA. Proteins of this subgroup can be further subdivided into two types: Asp49 PLA2s, which are catalytically active, and PLA2 homologues, which possess most commonly a Lys49 residue and do not have catalytic activity [9,10]. Both acidic and basic PLA2s 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 PLA2s purified from viperid venoms present an Asp residue at position 49. These acidic isoforms usually have a higher catalytic activity than basic PLA2s upon conventional substrates in vitro [11,13,14]. In spite of this, many acidic PLA2s are not lethal or show a weak lethal potency in mice [15–17].

Toxic effects induced by acidic PLA2s 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 PLA2s from Bothrops spp. venoms may express other toxic actions in vivo, such as myotoxicity and...
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 Gubensek [24] purified an acidic enzyme from the venom of specimens from Costa Rica, PLÁ2 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 PLÁ2 1, PLÁ2 2 and PLÁ2 3. This multiplicity of acidic PLÁ2 isoforms in the venoms of B. asper from the Pacific and Caribbean regions of Costa Rica has also been evidenced by iso-electrofocusing techniques [26], and more recently confirmed using a proteomic approach [3].

The potential toxic activities of acidic PLÁ2s 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 enzyme [27], whereas myotoxin II is a catalytically inactive Lys49 homologue [28].

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2. Materials and methods

2.1. Isolation of BaspPLÁ2-II

Crude venom was obtained from more than twenty specimens of B. asper from the Pacific region of Costa Rica, kept at the serpentinum 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 Ernoo-system chromatography (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 PLÁ2 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 (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 BaspPLÁ2-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 Z-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 (Pro-tana). 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 Q1 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.

2.2. Isoelectric point and molecular mass determinations

Two-dimensional polyacrylamide gel electrophoresis of BaspPLÁ2-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 pl was compared with the theoretically predicted value based on the amino acid sequence, using the Compute pl/mw tool at the ExPaSy Proteomics Server (www.expasy.org/tools). The molecular mass of BaspPLÁ2-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 BaspPLÁ2-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 BaspPLÁ2-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 Z-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 (Pro-tana). 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 Q1 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 BaspPLÁ2-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 RNAEasy Protect Mini kit (Qiagen). BaspPLÁ2-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′-CGGACGAGAGGATATAGAGTACGCTCCTAGTGGT25-3′. From the cDNA obtained, BaspPLÁ2-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’-GCCAGCAGAATTAATCAGCT-3’, and the 3’ RACE Inner Primer had the sequence 5’-CCCCGGATCCGATTATACTGGA 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 BaspPLA2-II sequence in the colonies. Transformed bacteria were incubated overnight at 37 °C, and then the vector was extracted with the Wizard plus Miniprep 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-dimen- sional structure of BaspPLA2-II using the acidic PLA2 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 A2 activity

Enzymatic activity of BaspPLA2-II was determined by the colorimetric method of de Araujo and Radvanji [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 PLA2 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 BaspPLA2-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 CaCl2 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 centrifuga- tion of citrated blood from healthy volunteers, at 135 g for 15 min. Aliquots of 450 μl of this preparation were incubated with Basp- PLA2-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 centri- fugation 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 BaspPLA2-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 lactate 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 BaspPLA2-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 BaspPLA2-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 BaspPLA2-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 BaspPLA2-II, two mice (16–18 g) received an intravenous injection of 100 μg of...
the enzyme, dissolved in 100 \mu{l} 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 \mu{g} of BaspPLA2-II, dissolved in 50 \mu{l} 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 BaspPLA2-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 \mu{g} of BaspPLA2-II, dissolved in 100 \mu{l} PBS. As a positive control, another group of mice received 4 \mu{g} of crude B. asper venom i.v., in 100 \mu{l} of PBS. A negative control group received an i.v. injection of 100 \mu{l} of PBS alone.

2.15. Preparation of rabbit antibodies against BaspPLA2-II

Antibodies to BaspPLA2-II were prepared by immunization of two rabbits with the purified enzyme, either intramuscularly or subcutaneously. An initial dose of 100 \mu{g}, emulsified in complete Freund’s adjuvant, was followed by booster doses of 50 \mu{g} 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 BaspPLA2-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 PLA2 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 \mu{l} of undiluted sera, enzymes (0.2 mg/ml) or crude venom (2 mg/ml), and read after 24 h. For the EIA, 0.2 \mu{l} of enzymes (BaspPLA2-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 antiserum were added to triplicate wells and incubated for 1 h. After five washings with FALC buffer (Tris 0.05 M, NaCl 0.15 M, ZnCl2 20 \mu{M}, MgCl2 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 \mu{g} 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 antiserum, 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 BaspPLA2-II enzymatic activity by rabbit and equine antibodies

BaspPLA2-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 \mu{l} or 15 \mu{l} of enzyme were assayed for PLA2 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 BaspPLA2-II

To ensure the removal of basic PLA2s and PLA2 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 PLA2 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 BaspPLA2-II by a final fractionation step on CM-Sephadex at pH 5.0, where it was retained by the chromatographic support.

Electrophoretic analyses of BaspPLA2-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 pl of this enzyme by 2D electrophoresis resulted in an estimated value of 4.9, close to the theoretical pl value of 5.05 predicted on the basis of its complete sequence.

The amino acid sequence of BaspPLA2-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 tryptic peptide ion at m/z 576.6 as NQCE[129]SEPC. The sequences of the b5 and y5 daughter ions were interpreted as NQCE[D-oMe] and (D-oMe)SEPC, respectively, indicating that Asp120 was o-methylated. The remaining 16 Da difference between experimental and calculated
masses may correspond to oxidation of one of the 5 methionine residues of the protein.

The PLA2 activity of BaspPLA2-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.

Comparison of BaspPLA2-II with similar proteins in the Swiss-Prot database showed that its primary structure is closely related to several group IIA acidic PLA2s of crotaline species, mostly to the enzymes isolated from Bothrops jararaca, Bothrops insularis, and B. jararacussu (Fig. 4). Multiple sequence alignment evidenced that only BaspPLA2-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 BaspPLA2-II and the B. jararaca PLA2 (Fig. 4). A phylogenetic tree

Fig. 1. Isolation of BaspPLA2-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 BaspPLA2-II under reduced (R) and non-reduced (NR) conditions. LMW: low molecular weight markers, as indicated at the left, in kDa.

Fig. 2. Amino acid sequence of BaspPLA2-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.

Fig. 3. Phospholipase A2 activity of BaspPLA2-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. (●) BaspPLA2-II; (○) myotoxin I. Each point represents mean ± SD of duplicates.
constructed with 15 acidic PLA2s confirmed the close evolutionary relationship of BaspPLA2-II with the enzymes of B. jararaca, B. insularis, and B. jararacussu from South America (Fig. 5), whereas the acidic PLA2s from other South American Bothrops, such as B. erythromelas and B. pictus, were more distant from the clade of BaspPLA2-II. On the other hand, the basic PLA2 myotoxin I (P20474) from B. asper venom was markedly distant from BaspPLA2-II in the cladogram, serving as an outgroup (Fig. 5), and confirming the divergent evolutionary pathways of acidic and basic PLA2s even within the venom of a single viperid species.

A three-dimensional model of BaspPLA2-II was built using as template the crystal structure of B. jararacussu acidic PLA2. 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 BaspPLA2-II model and its template protein was predicted to occur immediately before the C-terminal residues.
“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 PLA₂s.

3.2. Biological activities of BaspPLA₂-II in vitro and in vivo

BaspPLA₂-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). BaspPLA₂-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 BaspPLA₂-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 BaspPLA₂-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 BaspPLA₂-II, indicated in all cases a normal morphology, similar to the corresponding tissues of control mice receiving a PBS injection (not shown). In addition, BaspPLA₂-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 blood pressure (data not shown). The only toxic effect induced by BaspPLA₂-II was a transient induction of local edema in the mouse footpad assay (Fig. 9).

3.3. Immunological analyses of BaspPLA₂-II

Rabbits immunized with BaspPLA₂-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 BaspPLA₂-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 BaspPLA₂-II recognized the homologous antigen, but not the basic PLA₂ 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 BaspPLA₂-II (Fig. 10D). On the other hand, the equine

![Fig. 7](image-url)  
Fig. 7. Lack of muscle damaging activity of BaspPLA₂-II. (A) Cytotoxicity was evaluated upon cultured C₂C₁₂ skeletal muscle cells, exposed to BaspPLA₂-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 BaspPLA₂-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 BaspPLA₂-II (50 µg/50 µl) or (D) PBS (50 µl).
polyvalent antivenom produced at Instituto Clodomiro Picado clearly recognized BaspPLA₂-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 BaspPLA₂-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 PLA₂ from the venom of B. asper, here named BaspPLA₂-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
proteomic analysis of this venom, it is estimated that BaspPLA2-II represents 6.3% of its proteins [3,39].

BaspPLA2-II is composed of 124 amino acids, presenting Asp49 and the characteristic pattern of half-Cys residues of group IIA PLA2s [8]. These findings place BaspPLA2-II within the catalytically active enzymes, consistent with all acidic PLA2s purified from viperid snake venoms, where Asp49 appears so far to be an absolutely conserved position. The phospholipolytic activity of BaspPLA2-II was confirmed using phosphatidylcholine micelles as substrate, and shown to be slightly higher than the activity of myotoxin I, a basic PLA2 of the same venom. In general, snake venom acidic PLA2s 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 BaspPLA2-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 PLA2 from B. jararaca shares with BaspPLA2-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 BaspPLA2-II express some toxic activities, such as inhibition of platelet aggregation (P81243 and Q8AXY1), myotoxicity (Q8QG87), and hypotensive effect (Q8AXY1), whereas the toxicological character-ization of BaspPLA2-II, here presented, evidenced none of these activities. Negative results were obtained for anticoagulant, anti-platelet aggregation, cytotoxic, myotoxic, hypotensive, and lethal effects of BaspPLA2-II. The growing structural information on acidic PLA2s 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 PLA2s constitute percentages as large as 30–60% in some species [39,41], strongly arguing for their relevance in such secretions. In the case of BaspPLA2-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 BaspPLA2-II in these prey.

The induction of edema by BaspPLA2-II is consistent with reports of this activity being expressed by a number of acidic PLA2s 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 degradation of mast cells, with subsequent release of vasoactive amines [14,45,46].

BaspPLA2-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 PLA2s [34]. These findings are consistent with observations in most of the acidic PLA2s purified from snake venoms, which generally lack myotoxicity. However, some recently isolated acidic PLA2s display myotoxicity in vivo [14,20,21,40,47]. Although it is clear that the catalytic activity of PLA2s 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 BaspPLA2-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 PLA2s which display a very low or no lethal activity. It also indicates that BaspPLA2-II does not correspond to the acidic PLA2 isolated from B. asper venom by Ferlan and Guben [24], which had a lethal intravenous activity of 2 μg/g. A comparison of partial amino acid sequences between BaspPLA2-II and another acidic PLA2 isolated from the venom of B. asper from Panamá (to be named BaspPLA2-1, personal communication of A.M. Soares, University of São Paulo, Brasil) revealed several structural differences. Therefore, the different acidic PLA2 isoforms that are present in B. asper venom may vary in the expression of toxic effects.

BaspPLA2-II did not present anticoagulant activity, an effect which has been reported for a number of acidic PLA2s from viperid
snake venoms [20,22]. Moreover, anti-platelet aggregating activity and hypotensive effect were also absent in BaspPLA2-II, again at variance with other acidic PL A2s [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 PL A2s 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].

Immunoochemical analyses with antibodies raised against BaspPLA2-II revealed that this acidic enzyme differs antigenically from the basic PL A2s within viperids, in this case, within the venom of Bothrops asper. Bothrops species [51]. Bothrops species [51].

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References


