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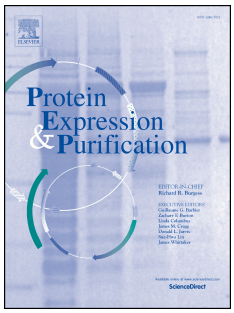

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cDNA cloning, heterologous expression, protein folding and immunogenic properties of a phospholipase A_2 from *Bothrops ammodytoides* venom

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Abstract

A mRNA transcript that codes for a phospholipase (PLA_2) was isolated from a single venom gland of the *Bothrops ammodytoides* viper. The PLA_2 transcript was cloned onto a pCR®2.1-TOPO vector and subsequently expressed heterologously in the *E. coli* strain M15, using the pQE30 vector. The recombinant phospholipase was named rBamPLA2_1, and is composed of an N-terminal fusion protein of 16 residues, along with 122 residues from the mature protein that includes 14 cysteines that form 7 disulfide bonds.

Following bacterial expression, rBamPLA2_1 was obtained from inclusion bodies and extracted using a chaotropic agent. rBamPLA2_1 had an experimental molecular mass of 15,692.5 Da that concurred with its theoretical molecular mass. rBamPLA2_1 was refolded in *in vitro* conditions and after refolding, three main protein fractions with similar molecular masses, were identified. Although, the three fractions were considered to represent different oxidized cystine isoforms, their secondary structures were comparable. All three recombinant isoforms were active on egg-yolk phospholipid and recognized similar cell membrane phospholipids to be native PLA_2s, isolated from *B. ammodytoides* venom. A mixture of the three rBamPLA2_1 cystine isoforms was used to immunize a horse in order to produce serum antibodies (anti-rBamPLA2_1), which partially inhibited the indirect hemolytic activity of *B. ammodytoides* venom. Although, anti-rBamPLA2_1 antibodies were not able to recognize crotoxin, a PLA_2 from the venom of a related but different viper genus, *Crotalus durissus terrificus*, they recognized PLA_2s in other venoms from regional species of *Bothrops*.

Keywords: antibodies; *Bothrops ammodytoides*; phospholipase; protein expression; snake; venom; viper.

Introduction

Bothrops ammodytoides is the world's most southerly situated viper. This snake inhabits a geographical region that stretches from the warm desert regions of "El Gran Chaco" and the dry "Pampeana" region, as far as the cold Patagonia region of Argentina. It is a small viper, averaging 70 cm in length, able to inflict the typical complications resulting from *Bothrops* envenomations [*1*]. *B. ammodytoides* venom provokes hemorrhagic, dermonecrotic, and inflammatory-edematogenic effects in mice, potentially causing death [*1*]. Likewise it is myotoxic, showing procoagulant activity on human plasma, but manifests low or absent thrombin type activity over bovine fibrinogen [*1*]. The venom also manifests phospholipase and indirect hemolytic activity [*1*, *2*]. Previously, the isolation and identification of an aspartic-49 (D49) phospholipase A_2, (PLA_2) was described as having low toxicity for mice (LD_{50}=117 μg/mouse of 20 g) [*2*] but to be similar to other PLA_2s such as myotoxin-I from *Bothrops moojeni* (LD_{50}=170 μg/mouse of

20 g) [3]. Although viper venoms represent complex mixtures of toxic proteins, including phospholipases, serine-proteases and metalloproteinases, the recombinant expression of these toxic enzymes may help to produce neutralizing antibodies for studying the toxic mechanisms of venom and anti-venom neutralization, related to these enzymes and eventually to improve the production of viper anti-venom. In this work, we describe the cDNA cloning and heterologous expression of a phospholipase A_2 from *B. ammodytoides*. Furthermore, the recombinant PLA_2, named rBamPLA2_1, was used as immunogen to produce anti-rBamPLA2_1 antibody (anti-rBamPLA2_1), which reduces the indirect hemolytic activity of *B. ammodytoides* venom and recognizes venom PLA_2s from other species of *Bothrops*. For the first time in this work, we describe a method for generating antibodies against PLA_2s from pit viper venoms, using recombinant PLA_2s as antigens; these are useful for anti-venom study.

Materials and Methods

Venom and venom gland

Adult *B. ammodytoides* specimens were kept in optimal health conditions and in plastic cages, at a constant temperature of 27º C. Animals were fed with a mouse every fortnight and tap water was provided *ad libitum*. Light-dark cycles consisted of 12 h. Venom was extracted manually and immediately vacuum dried and stored at -20º C until use. To obtain one of the two venom glands, a healthy specimen was selected and after being anesthetized, a venomous gland was removed by chirurgical extraction with ketamine-xylazine. Immediately after being extracted, the venom gland was treated with RNAlater® (Thermofisher, Asheville, NC, USA) and stored at -20º C until use. After, surgical intervention, the specimen recovered itself, and remained healthy.

Bacterial strains, enzymes and plasmids

We used XL1-Blue *Escherichia coli* strain for DNA cloning and plasmid propagation. The M15 *E. coli* strain was employed for the expression of recombinant PLA_2. Plasmids pCR®2.1-TOPO® (Invitrogen, CA, USA), and pQE30 (Qiagen, CA, USA) were used for cloning the PLA_2 gene, and for production of the 6His-tagged recombinant

rBamPLA2_1, respectively. Restriction enzymes, *Taq* polymerase, Factor Xa protease (FXa) and T4 DNA ligase were purchased from New England Biolabs (New England Biolabs, MA, USA).

RNA extraction and gene assembly

As mentioned, total RNA was extracted from a single venom gland of *B. ammodytoides*, using the "Total RNA Isolation System" (Quiagen, CA, USA). Based on the N-terminal sequence of the previously reported PLA_2, specific oligonucleotides were designed to amplify the corresponding transcript (Clement *et al*., 2012). The oligonucleotides were named Oligo1 Fw PLA2 (CAC CTG ATG CAA TTT GAG, Tm 52 ºC) and Oligo2 Rv PLA2 (GCA TGG CTC TGA CTC CTC, Tm 58 ºC).

Positive clones were selected, based on the blue/white selection scheme, and the integrity of gene construction was verified by DNA sequencing of the plasmidic minipreps, obtained using the High Pure Plasmid Isolation Kit (Roche, Basel, Switzerland).

Plasmid construction for expression

Gene construction made to express the rBamPLA2_1, included recognition sequences for restriction enzymes used for cloning (*Bam*HI and *Pst*I) and a sequence encoding for FXa cleavage site. The designed transcript was subcloned into the pQE30 expression vector, through the *Bam*HI and *Pst*I sites. The pQE30 vector introduces a polyhistidine-tag (6His) to facilitate purification of the product by affinity chromatography. The FXa cleavage sequence was conveniently placed between the 6His and the mature toxin to allow the cleavage of the full recombinant toxin, should this be necessary. The new pQE30-derived constructs were verified by sequencing from both sides. Competent *E. coli* M15 cells were transformed with the corresponding plasmids by incubation for 30 min on ice, heat-shocked for 1 min at 42º C, followed by 5 min in ice, recovered for 30 min at 37º C in SOC medium, and plated in LB, containing 100 µg/mL of ampicillin, and 30 µg/mL of kanamycin. The constructions were named pQE30rBamPLA2_1 and their expression product was abbreviated here to rBamPLA2_1.

Expression and purification of rBamPLA2_1

E. coli strain M15 expressing the plasmid pQE30rBamPLA2_1 was grown in Luria

Broth (LB) medium. Once absorbance at 600 nm had reached 0.8 of absorption units, cultures were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 24 h at 16º C. Cells were harvested by centrifugation (8,000 rpm for 20 min in JA-14 rotor), using a Beckman centrifuge model J2-21, recovered in washing buffer (0.05 M Tris-HCl, pH 8.0), and lysed with a BugBuster® protein extraction reagent (Novagen, Germany). This material was centrifuged again (12,000 rpm for 20 min) and the supernatant was discarded.

The insoluble fraction was rinsed twice with washing buffer and centrifuged again at 12,000 rpm for 20 min. The insoluble fraction having the inclusion bodies were treated with the chaotropic agent guanidinium chloride (GndHCl) at 6M in a Tris-base 0.05 M buffer (pH 8.0) to extract the recombinant PLA_2. It was then centrifuged for 20 min, using a Beckman centrifuge model J2-21 (12,000 rpm for 20 min in a JA-20 rotor) to remove any insoluble material. The supernatant that contains the recombinant protein was purified by Ni-NTA (Ni-nitrilotriacetic acid) affinity column chromatography, which was performed according to manufacturer's instructions (Qiagen, CA, USA), using denaturing conditions with buffer A (6M GndHCl in a 0.05M Tris-base buffer, pH 8.0) and buffer B (6M GndHCl in 0.05M Tris-base buffer, containing 400 mM imidazole, pH 8.0). Buffer B was eliminated by applying a second purification step under reverse-phase HPLC (RP-HPLC). An analytical C_4 reversed-phase column (Vydac 214 TP 4.6x250mm, USA) was run from solvent A (0.1% trifluoroacetic acid, TFA, in water) to solvent B (0.1% TFA in acetonitrile). The previously described HPLC system was used for this separation, and the gradient was run from 10 to 60% solvent B, for a 50 min period and after 1 mL/min, proteins were detected at 230 nm. The rBamPLA2_1 product was vacuum dried. The recombinant product was allowed to fold under controlled conditions using 2M GndHCl in 0.05M Tris-base buffer, pH 8.0, containing 1 mM reduced glutathione (GSH)/0.1 mM oxidized glutathione (GSSG).

Molecular mass determination

The molecular mass identities of the recombinant proteins and the enzymatically digested peptides were confirmed by mass spectrometry analysis. The protein fractions were reconstituted to a final concentration of 500 pmol/5 μL of 50% acetonitrile with 1% acetic acid and directly applied into a Thermo Scientific LCQ Fleet ion trap mass

spectrometer (San Jose, CA) with a Surveyor MS syringe pump delivery system. The eluate at 10 μL/min was split out in order to introduce only 5% of the sample into the nanospray source (0.5 μL/min). The spray voltage was set from 1.5 kV and the capillary temperature was set at 150 °C. The fragmentation source was operated at 25–35 V of collision energy, 35–45% (arbitrary units) of normalized collision energy and the scan with wide band was activated. All spectra were obtained in the positive-ion mode. The data acquisition and the deconvolution of data were performed on Xcalibur Windows NT PC data system

Phospholipase and hemolytic activity

The phospholipase activity of the purified enzyme was determined by the hydrolysis of egg-yolk phospholipids in agarose plates, as described by Bernheimer et al. [*4*]. The hemolytic activity of native phospholipases in presence of bivalent viper snake serum, and anti-rBamPLA2_1 antibodies were assayed, as described by Lanari *et al.* (2014) [*5*].

Protein-lipid interactions

Dot blots were carried out using P-6001 PIP Strips (2x6 cm nitrocellulose membranes, Echelon Biosciences, Salt Lake City, UT, USA), spotted with 100 pmol of phosphoinositides and other biologically important lipids. Interaction of rBamPLA2_1 with lipids was tested as follows; the membrane was blocked with 5 mL of blocking buffer, TBS-T (0.01M Tris-HCl+ 0.15M NaCl + 0.05% of Tween-20, pH 7.5) plus 5% fat free milk and gently agitated for 1h at room temperature (rt). The blocking buffer was discarded and final protein concentration of 5 μg/mL of rBamPLA2_1 was added to 5 mL of TBS-T buffer for 1h at rt, with gentle agitation. After contact, the protein solution was discarded and the membrane washed three times with 5 mL TBS-T, with gentle agitation. Then, 5 mL of TBS-T containing horse anti-rBamPLA2_1 (0.1 mg) was added, and gently agitated for 1h at rt. After washed again three times with 5 mL TBS-T with gentle agitation, 5 mL of TBST-T containing anti-horse conjugated with alkaline phosphatase was included, and the phospholipid membrane was incubated again for 1h at rt. Following these washing stages, membrane was developed using the BCIP/NTB substrate kit (Invitrogen Cat. 00-2209), according to manufacturer's instructions.

Biological activity

The protocol used for assaying the activity of rBamPLA2_1 *in vivo*, using the mice model, was followed according to the guidelines of our Institute Committee of Animal Welfare, maintaining the number of animals at a necessary minimum, in order to validate experiments. Male mice (CD-1, 20 g body weight) were tested by intravenous injection.

Enzyme-Linked ImmunoSorbent Assay (ELISA)

Solid-phase-adsorbed rBamPLA2_1, native PLA_2 and *Bothrops* venoms were prepared by treating wells of MaxiSorp plates (NUNC™, thermo scientific) with 100 µL solution of 5 µg/mL of recombinant protein in 100 mM sodium carbonate buffer (pH 9.6). Following overnight incubation at 4° C, wells were aspirated and washed three times with 200 µL of washing buffer (50 mM Tris-HCl, pH 8, containing 0.5 mg/mL of Tween 20 and 150 mM NaCl). Subsequently, wells were filled with 200 µL of blocking buffer (5 mg/mL gelatin and 2 mg/mL between 20 and 50 mM Tris-HCl buffer, at pH 8). After 2 h of incubation at 37° C, wells were washed as described above and filled with 100 µL aliquots, with serially diluted horse IgGs anti- rBamPLA2_1 in incubation buffer (50 mM Tris-HCl buffer at pH 8, containing 1 mg/mL gelatin, 0.5 mg/mL between 20 and 0.5 M NaCl). Dilution initiated at 1:30 and incubation time was 1 h at 37° C. After washing, the bound horse IgGs were allowed to react with 100 µL per well of 0.1 mg/mL of anti-horse IgGs, labeled with horseradish peroxidase (Roche), in incubation buffer. After another hour at 37° C, wells were washed and filled with 100 µL of ABTS solution (Roche), as the substrate for peroxidase. The color development reaction was arrested by the addition of 25 µL of 20% SDS, and the plate was read at 405 nm in a Microplate Reader (Tecan Sunrise IVD version). Data were analyzed by nonlinear regression, using the sigmoidal dose-response equation from the Prism program (Graph Pad Prism v. 6.0c). Conventional titers were calculated from the midpoint of the curve and correspond to the IgGs dilution for half of maximal binding, which was considered as the half maximal effective concentration (EC_{50}).

Circular dichroism

Secondary structure content of native and recombinant PLA_2s was calculated by applying circular dichroism spectroscopy (CD). Spectra of the native PLA_2 and the recombinant isoforms were recorded at room temperature in quartz cells (1 mm-path), manifesting a wavelength ranging from 190 to 260 nm, detected using a spectropolarimeter Jasco J-710 (Jasco, Japan). Data were collected every 1 nm at a speed of 20 nm/min. Each protein was dissolved in 60% trifluoroethanol, up to a concentration of 0.6 mg/mL. Trifluoroethanol improves secondary structure. The CD values correspond to the mean of three CD recordings. Finally, percentages of secondary structure were analyzed, using the algorithms hosted on line at K2D3 web server (http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d3/).

Animal immunizations

A horse was hyperimmunized subcutaneously with 7 mg of a mixture of the three rBamPLA2_1 cystine isoforms for producing serum antibodies (anti-rBamPLA2_1). We initiated immunization protocol by administering a dose of 0.1 mg total protein in Complete Freud´s Adjuvant (CFA), subsequently increasing doses up to 0.5 mg, and alternating with incomplete Freud´s (IFA) and aluminum hydroxide (AH), over a 1 month period. Immunizations were continued using 0.5 up to 1 mg weakly, for 3 months. The horse serum antibodies were purified from plasma by acid precipitation, using 5% caprylic acid. New Zealand rabbits were also immunized for three months, at intervals of fifteen days, administering increasing doses of the basic subunit of crotoxin (CB) (10-300 µg/rabbit). Primarily, immunization started with CFA, subsequently alternating this with incomplete Freund's adjuvant and AH. Serum from three rabbits was pooled and antibodies were purified by affinity chromatography in a Sepharose 4B resin, activated with cyanogen bromide, coupled to CB.

Statistics

Results were expressed as mean and standard deviation, or as mean with 95% confidence intervals. For all statistical methods, we used the software Prism 4.0 (Graph Pad Inc., San Diego, CA).

Results and Discussion

Isolation and sequence determination of rBamPLA2_1

Oligonucleotides were designed, based on the N-terminal sequence of the previously isolated PLA_2 from *B. ammodytoides* (see Materials and Methods). The cDNA coding region for rBamPLA2_1 with a predicted size of 122 bp was enlarged by PCR, cloned into plasmid pCR®2.1-TOPO® and then into the bacterial vector pQE30 (Fig. 1). The amino acid sequence of the cloned rBamPLA2_1 differs from the one previously isolated, in 6 out of 122 amino acids; that is, rBamPLA2_1 is 95% identical to that isolated from the venom (**Table 1**). rBamPLA2_1 retains the four highly conserved residues His48, Asp49, Tyr52 and Asp99, pertaining to the common PLA_2 active site (here His47, Asp48, Tyr51 and Asp98); apparently a characteristic of type II phospholipases [*6*]. The designation of type I and type II, old world snakes (cobras and mambas) and new world snakes (rattlesnakes and pit vipers), respectively, refers to the six disulfide bonds they have in common, but an extra one (see underlined C in table 1) is located in distinctly different locations for Type II phospholipases [*7, 8*].

Expression, purification and protein folding of rBamPLA2_1

The gene encoding rBamPLA2_1 was assembled and cloned into the expression vector pQE30. This vector is used to produce N-terminal 6His-tagged proteins, which facilitate the rapid purification of the recombinant products by IMAC. We added a cleavage site for FXa between the 6His-tag and the mature sequence of the **BamPLA2_1,** in anticipation of a possible deleterious effect of the 6His-tag on the biological activity of **BamPLA2_1**, which could if necessary, then be untagged from the mature protein. Heterologous expression of the rBamPLA2_1 peptide was achieved using the *E. coli* M15 strain (**Figure 2**). **rBamPLA2_1** was predominantly found in inclusion bodies (**Figure 2A**, **lane 3**), and was recovered by using agarose nickel affinity (**Figure 2A**, **lanes 8-14**). The heterologous expression of rBamPLA2_1 in inclusion bodies, as well as its purification from agarose nickel columns was confirmed by western-blot assays, using an anti-6His-tag antibody, coupled to alkaline phosphatase. rBamPLA2_1 was folded *in vitro* and purified by RP-HPLC. Three protein fractions with retention times ranging from 34 to 40 min

(linear gradient, 0-60% of B in 60 min) were collected (**Figure 3**). An analysis by 15% SDS-PAGE showed that all three fractions had similar apparent molecular mass, and also they were equally active on egg-yolk phospholipids (**Figure 3, inset**). Furthermore, the three fractions showed an experimental molecular mass of 15,692.5 Da in its reduced form, which was obtained by mass spectrometry and corresponds to the expected molecular mass for the 6His-tagged **rBamPLA2_1**; this data may indicate that the three main components obtained from RP-HPLC, correspond to oxidized cystine isoforms of **rBamPLA2_1**. There are reports of multiple cysteine-rich protein isoforms being generated during heterologous expression [*9-12*]. **rBamPLA2_1** contains 14 cysteines and could theoretically form up to 135,135 isoforms, considering scrambling disulfide pairing possibilities. Clearly, the molecular machinery in the snake venom gland, in charge of PLA2s expression, directs the formation of correct folding. The protein yield of the three **rBamPLA2_1** fractions together was 0.5 mg/L.

Secondary structure of rBamPLA2_1 isoforms

Native and recombinant phospholipases were analysed by CD, in order to compare the secondary structures of the three oxidized cystine isoforms of rBamPLA2_1. They all showed high absorption for α-helix secondary structure, and relatively low content in terms of β-strands (**Figure 4**). According to a CD deconvolution program, the secondary structure content was 90.2, 87.8, 90.1, 90.5 % respectively and 0.5, 0.4, 0.5, 0.5 %, of α-helix and β-strands, for the native PLA2 and the three rBamPLA2_1 isoform, respectively. So far, all pit viper venom PLA_2s contain a greater proportion of α-helix, which is characterized as having a positive band at 198 nm and negative ellipticities at 208-222 nm [*13*]. Here, the rBamPLA2_1 isoform 1 showed the lowest percentage of α-helix, and based on its CD spectrum, its secondary structure could be slightly different to the native PLA_2. Overall, the three rBamPLA2_1 isoforms resemble the canonical secondary structures of viper venom PLA_2s [*13*].

Evidence of different disulfide pairings in rBamPLA2_1 isoforms

According to descriptions of the three-dimensional structures of pit viper type II PLA_2s (PDB codes IMG6 and 1PPA) snakes from the New World [*14*, *15*], disulfide

pairings have been classified as Cys26-115, Cys28-44, Cys43-95, Cys49-122, Cys50-88, Cys57-81 and Cys75-86 (**Table 2, Figure 5A**). However, as mentioned previously, it is important to note that a heterologously expressed PLA_2, which contains 14 cysteines could theoretically form up to 135,135 isoforms, if only the disulfide pairing possibility is considered. This means that the number of possible structural forms for a protein rich in cysteines increases with the number of cysteines in the molecule; consequently, in order to obtain a structural, long-standing and *in vivo* functional recombinant protein with *n* number of half-cysteines, the correct structure must be one of the N! / PD! A^{PD} structural forms, where N is the number of half-cystines, PD is the number of cystines, and A is 2 (a disulfide arrangement). To show that the three rBamPLA2_1 obtained were different oxidized cystines having different disulfide pairing, the native PLA_2 and the rBamPLA2_1 isoform 3, were enzymatically digested with trypsin (Fig. 5B and 5C). The digested fractions were separated by RP-HPLC, and the most prominent protein fractions were analyzed using mass spectrometry. The HPLC elution profiles display different digested fraction patterns for both the native PLA2 and the rBamPLA2_1 isoform 3, which may be a consequence of their different disulfide pairings. Figure 5B shows a HPLC fraction of the enzymatically digested native PLA_2 that elutes after an extended retention time (31 min). This fraction had a molecular mass of 9,048.8 Da, which probably corresponds to the theoretical molecular mass of 9,048.0 Da of all viable covalently linked peptide fragments, connected by their disulfide bridges (see amino acid sequences inside **Figure 5B**). These linked peptide fragments concur with the proposed disulfide pairing for pit viper PLA_2s. In contrast, figure 5C shows a HPLC fraction at 17.5 min, of the enzymatically digested rBamPLA2_1 isoform 3, with an experimental molecular mass of 1,454.3 Da, which may correspond to the theoretical molecular mass of 1,453.7 Da of peptide fragments with amino acid sequences, covalently linked by Cys57-Cys95. Moreover, figure 5C also shows a HPLC fraction at 26.9 min, of the same enzymatically digested rBamPLA2_1 isoform 3, with an experimental molecular mass of 2,264.6 Da, which may correspond to the covalently linked peptide fragments Cys44-Cys86, Cys50-Cys88 and Cys43-Cys49, with a theoretical molecular mass of 2,265.4 Da (Figure 5C, inset). Therefore, the molecular masses obtained for the digested rBamPLA2_1 isoform 3 differ from those of the proposed

disulfide arrangement for pit viper PLA_2s, indicating that rBamPLA2_1 isoform 3 has a different disulfide arrangement, when compared to that of native PLA_2.

Biological analysis of rBamPLA2_1

Toxicity and phospholipid binding

The toxicity of rBamPLA2_1 was compared to the native PLA_2 (LD_{50}=117 μg/20 g in mice); however, rBamPLA2_1 was not toxic to mice up to 300 μg/20 g mouse (n=3), when injected intravenously. In a protein-lipid overlay assay, it was observed that rBamPLA2_1 interacts with different biologically important lipids found in cell membranes (**Figure 6**). rBamPLA2_1 binds slightly better to phosphatidic acid (PA), phosphatidylserine (PS) and phosphatidylinositol 3,4-bisphosphate (PI3,4) (**Figure 6**). PA, PS and PI3,4 are known to comprise some of the principal acidic phospholipids in mammalian cell membranes [*16*]. Similar results were observed in an identical protein-lipid overlay assay, using native PLA_2.

Horse immunization, antibody recognition and antibody titers

A horse was immunized with 7 mg of a mixture of recombinant **rBamPLA2_1 isoforms.** After 3 months of immunization, the horse was bled, and the capacity of its serum to recognize **rBamPLA2_1** and native PLA_2s from bothrops venoms was observed. Figure 7A shows the protein composition of venoms from several species of *Bothrops*, where phospholipases, as is evident from their molecular weights, may have between one tenth and one third of their respective venom composition (see red triangle mark in Figs. 7A and 7B). The apparent molecular mass of **rBamPLA2_1** (Figure 7A, lane 1) appears to be larger than other natural PLA_2s; possibly the result of extra basic residues (6His-tag) at the N-terminal of **rBamPLA2_1**, which may retard protein migration, as previously observed [*12*]. Figure 7B illustrates how horse anti-**rBamPLA2_1** recognizes venom proteins from *Bothrops* species. Antibody-recognition was observed for proteins in the range of 10 to 15 kDa, which represent the native PLA_2s from *Bothrops* venoms. Furthermore, figure 8A shows the horse antibody titers against the venom of the *Bothrops* species; *B. jararacussu, B. diporus, B. moojeni, B. alternatus, B. ammodytoides* and *B. asper,* compared to that of rBamPLA2_1 titer. The venoms of *B. jararacussu, B. diporus,*

B. moojeni and *B. alternatus* were less recognized by anti-**rBamPLA2_1** than the venoms of *B. ammodytoides* and *B. asper*. Although antibody recognition is evident, the half maximal effective concentration (EC_{50}) was quite low for *B. ammodytoides* and *B. asper* (803 and 2012, respectively, see **Table 3**). Additionally, anti-**rBamPLA2_1** was capable of reducing the indirect hemolytic activity of *B. ammodytoides* venom, activity that was closely related to phospholipase activity. Figure 8B shows the complete and partial indirect hemolytic neutralization of *B. ammodytoides* venom in the presence of a bivalent *Bothrops* antivenom (*B. diporus* and *B. alternatus* venoms used as immunogens), and in the presence of anti-**rBamPLA2_1**. This data proves that **rBamPLA2_1** may be a suitable immunogen for producing anti-PLA_2 antibodies to decrease PLA_2 activity of *Bothrops* phospholipases, related to several toxic activities, such as myotoxicity or interference with platelet function; at least in *B. ammodytoides* venom.

In order to assess the capacity of anti-**rBamPLA2_1** for recognizing PLA_2s, in contrast to other viper venoms, it was tested against crotoxin (P62022.1), a rattlesnake PLA_2 from *Crotalus durissus terrificus.* Although **rBamPLA2_1** and crotoxin are 57% similar (**Table 4**), anti-**rBamPLA2_1** was unable to recognize crotoxin (**Figure 9A**). Correspondingly, anti-crotoxin antibodies were unable to recognize rBamPLA2_1 (**Figure 9B**).

This low reactivity reinforces evidence that indicates the redundancy of anti-*Bothrops* phospholipase antibodies for recognizing and neutralizing crotoxin, corroborating the specificity of this enzyme. However, the reactivity of the anti-**rBamPLA2_1** on other *Bothrops* venoms heightens interest in the study on the possible neutralization of toxic activities related to these enzymes, elicited from a recombinant enzyme as immunogen. Although at present anti-snake venom production for therapeutic purposes consists of polyclonal antibodies produced in animals, mostly using whole venom as immunogens, new methodologies are being studied [*17*] and the use of recombinant proteins for the generation of polyclonal or monoclonal antibodies or neutralizing fragments through molecular biology methods may indicate future strategies for anti-venom production [*18*].

Concluding remarks

For the first time, this work presents the use of recombinant nontoxic PLA_2

(rBamPLA2_1) isoforms to produce horse serum antibodies against native PLA_2s from homologous bothrops venoms. One major drawback to this work refers to the three identified isoforms of rBamPLA2_1, which may overlap the activity of a possible well-structured rBamPLA2_1. The phospholipid binding activities, the activities on egg-yolk phospholipids and the circular dichroism spectra of rBamPLA2_1 isoforms suggest that some basic secondary structure is maintained, even though different disulphide pairings may be presented. Even though rBamPLA2_1 had low phospholipase activity and was not lethal to mice, it behaves as an immunogen for producing antibodies against PLA_2s from related *Bothrops* species. The use of recombinant toxins as immunogens to produce neutralizing antibodies against animal venoms such as scorpions (*Centruroides, Androctonus*), elapids (*Micrurus, Dendroapsis, Naja*) and spiders (*Loxoceles*) has been already reported [*17-19*]. In the same way, recombinant PLA_2s could be used to generate anti-PLA_2 antibodies to reduce the toxic activities related to phospholipases, such as myotoxicity and platelet alterations, caused by *Bothrops* viper envenomation.

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest

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Tables

Table 1. Amino acid sequence of rBamPLA2_1 compared to the native PLA2

Protein	Amino acid sequence*	ID (%)
PLA2 native	HLMQFETLIKKIAGRSGVWFYGFYG**C**Y**C**GSGGRG**K**PKDATDR**CC**FV**HDCCY**GKV**T**G**C**DPKM	100
rBamPLA2_1	HLMQFETLIKKIAGRSGVWFYGFYG**C**Y**C**GSGGRG**Q**PKDATDR**CC**FV**HDCCY**GKV**A**G**C**DPKM	95
	********************************:********************:******	
PLA2 native	DFYTYSEENGVVV**C**GGDDP**C**KKQI**C**E**C**DRVAAT**C**FR**D**NK**TYD**NNKYWFYPA**K**N**C**QEESEP**C**	100
rBamPLA2_1	DFYTYSEENGVVV**C**GGDDP**C**KKQI**C**E**C**DRVAAT**C**FR**D**NK**DTY**NNKYWFYPA**E**N**C**QEESEP**C**	95
	************************************ *********:*********	

*The residues in bold black differ in both native PLA2 and rBamPLA2_1. The four highly conserved residues H48, D49, Y52 and D99 in blue represent the active site of elapid PLA_2s. The underlined C49 and C122 are distinctive for Type II phospholipases. ID means percentage of identity.

Table 2. Amino acid sequence of rBamPLA2_1 and two PLA_2s from New World pit viper venoms

PLA2	Sequence
	1--------10--------20--------30--------40-------50---------61
rBamPLA2_1	HLMQFETLIKKIAGRSGVWFYGFYG***C***Y***C***GSGGRGQPKDATDR***CC***FVHD***CC***YGKVAG***C***DPKM
1MG6*	SLFELGKMIWQETGKNPVKNYGLYG***C***N***C***GVGGRGEPLDATDR***CC***FVHK***CC***YKKLAD***C***DSKK
1PPA*	SVLELGKMILQETGKNAITSYGSYG***C***N***C***GWGHRGQPKDATDR***CC***FVHK***CC***YKKLTD***C***NHKT
	:::: .:* : :*:. : ** *** ** * **:* **********.*** *:*.*: *
	62------70--------80--------90-------100------110---------122
rBamPLA2_1	DFYTYSEENGVVV***C***GGDDP***C***KKQI***C***E***C***DRVAAT***C***FRDNKDTYNNKYWFYPAEN***C***QEESEP***C***
1MG6	DRYSYKWKNKAIV***C***GKNQP***C***MQEM***C***E***C***DKAFAI***C***LRENLDTYNKSFRYHLKPS***C***KKTSEQ***C***
1PPA	DRYSYSWKNKAII***C***EEKNP***C***LKEM***C***E***C***DKAVAI***C***LRENLDTYNKKYKAYFKLK***C***KKPDT-***C***
	* *:*. :* .::* .:** :::****:. * *:*:* *:.: : .*:: . *

*1MG6 and 1PPA are the PDB codes of the crystal structures of PLA_2s from the snake venoms of *Agkistrodon acutus* and *Agkistrodon piscivorus piscivorus.* Their disulfide pairing has to be Cys26-115, Cys28-44, Cys43-95, Cys49-122, Cys50-88, Cys57-81 and Cys75-86.

Table 3. Titers of anti- rBamPLA2_1 against rBamPLA2_1, bothrops venoms and crotoxin, and titers of anti-crotoxin against rBamPLA2_1

Antibody	Protein	Titers	CI*
Horse anti-rBamPLA2_1	rBamPLA2_1	28,077	21,748 - 36,247
	Native PLA2	1,572	1,325 - 1,865
	B. ammodytoides	803	641 - 1,006
	B. jararacussu	184	162 - 207
	B. diporus	20	17 - 23
	B. moojeni	34	31 - 35
	B. alternatus	18	15 - 21
	B. asper	2,012	1,669 - 2,426
	Crotoxin	0	
Rabbit anti-crotoxin	rBamPLA2_1	0	

*Confidence intervals (95%)

Table 4. Amino acid sequence of rBamPLA2_1 compared to crotoxin

Protein	Amino acid sequence	ID (%)*
Crotoxin	HLLQFNKMIKFETRKNAIPFYAFYG**C**Y**C**GWGGRGRPKDATDR**CC**FV**HDCCY**GKLAK**C**NTKW	100
rBamPLA2_1	HLMQFETLIKKIAGRSGVWFYGFYG**C**Y**C**GSGGRGQPKDATDR**CC**FV**HDCCY**GKVAG**C**DPKM	57
	::.:** : :..: **.**********:******************:** *: *	
Crotoxin	DIYPYSLKSGYIT**C**GKGTW**C**EEQI**C**E**C**DRVAAE**C**LR**R**SLSTYKYGYMFYPDSR**C**RGPSET**C**	100
rBamPLA2_1	DFYTYSEENGVVV**C**GGDDP**C**KKQI**C**E**C**DRVAAT**C**FR**D**NKDTYNNKYWFYPAEN**C**QEESEP**C**	57
	* *:* ** :.* :.** . *::********** *:* . .**:* *** ..*: ** *	

*ID means identity

Figure legends

Figure 1. Representation of the gene construction for the heterologous expression of rBamPLA2_1. (**A**) The primary structure of rBamPLA2_1. (**B**) The genetic construction used for the expression of the recombinant rBamPLA2_1. The 6His-coding sequence is part of the pQE30 vector and is located upstream of the *Bam*HI/*Pst*I-cloned gene, so the recombinant protein gets 6His-tagged at the amino terminus. Downstream of the *Bam*HI site, the sequence coding for the FXa recognition site is introduced (IEGR is highlighted) right before the mature toxin's sequence. Two stop codons (asterisks) are included at the end of the sequence coding for the mature toxin, upstream of the *Pst*I cloning site. (**C**) The first forward and the last reverse oligonucleotides used for rBamPLA2_1 assembly. The structural elements added to the sequence of the recombinant toxin are shown (the *Bam*HI and *Pst*I sites as well as the stop codons are underlined).

Figure 2. SDS-PAGE and Western-blot of rBamPLA2_1 expressed in *E. coli* cells. **A**) SDS-PAGE; **B**) Western-blot. Right lane shows the molecular weight markers in kDa; Lane number 1 cells without IPTG induction; lane number 2, cells with IPTG induction; lane number 3, inclusion bodies; lane 4, supernatant; lane 5, recirculating; lanes 6 and 7, first and second wash with 30 mM Imidazol; lanes 8 to 14 corresponds to the expressed proteins after purification by affinity column the elution with 400 mM imidazol rBamPLA2_1.

Figure 3. Chromatographic separation of rBamPLA2_1. **A**) Chromatographic separation of rBamPLA2_1 from 400 mM imidazol fractions. **B**) The SDS-PAGE inside shows at the right lane the molecular weight markers in kDa; lane number 1, the complete venom from *B. ammodytoides*; lane number 2, inclusion bodies; lane 3, fraction 1 from HPLC after protein folding; lane 4, fraction 2 from HPLC after protein folding; lane 5 fraction 3 from HPLC after protein folding. **C**) Phospholipase assay on agarose egg yolk phospholipases.

Figure 4. Circular dichroism of native PLA_2 and rBamPLA2_1. The secondary structure

analysis revealed higher content of α-helix than that of β-strands.

Figure 5. RP-HPLC profiles of the enzymatically digested $nPLA_2$ and rBamPLA2_1. A) proposed arrangement of disulfide bridges in native PLA_2. Both nPLA2 (B) and rBamPLA2_1 isoform 3 (C) were enzymatically digested with trypsin. The peptide digested fractions were separated using an analytic C_{18} reverse-phase column (Vydac 214 TP 4.6x250mm, USA) using 0.1% trifluoroacetic acid, TFA, in water, as solvent A, and 0.1% TFA in acetonitrile as solvent B. The gradient was run from 20 to 60% solvent B, during 40 min at 1 mL/min, the peptide fragments were detected at 280 nm. The HPLC fractions collected were analyzed using mass spectrometry.

Figure 6. Interaction of phospholipases to membrane lipids. **A**) Binding of recombinant rBamPLA2_1 and **B**) native PLA2 from *B. ammodytoides*. The binding of phospholipases to membrane lipids were revealed using anti IgG rBamPLA2_1 (0.1 mg/mL) as the first antibody and anti-horse coupled to alkaline phosphatase as the second antibody.

Figure 7. Protein content of venoms from different species of *Bothrops* revealed by SDS-Gel and Western-blot. **A** and **B**. lanes, 1) rBamPLA2_1; 2) *B. ammodytoides*; 3) *B. jararacussu*; 4) *B. diporus*; 5) *B. moojeni*; 6) *B. alternatus*; 7) *B. asper*. The SDS-Gel had 50 µg per line, and the Western-blot had 10 µg per line (first antibody was horse IgG anti-rBamPLA2_1, and the second antibody was horse IgG coupled to alkaline-phosphatase).

Figure 8. Antibody recognition of horse anti-**rBamPLA2_1** to venoms from *Bothrops* species. **A**) Anti-**rBamPLA2_1** recognition to venoms from different species of *Bothrops*. **B**) Inhibition of the hemolytic activity of *B. ammodytoides* venom by a bivalent snake antivenom and anti-**rBamPLA2_1**.

Figure 9. Antibody recognition between rBamPLA2_1 and crotoxin. **A**) Recognition of anti-**rBamPLA2_1** to crotoxin. **B**) Recognition of anti-crotoxin to **rBamPLA2_1**.

Figure 1

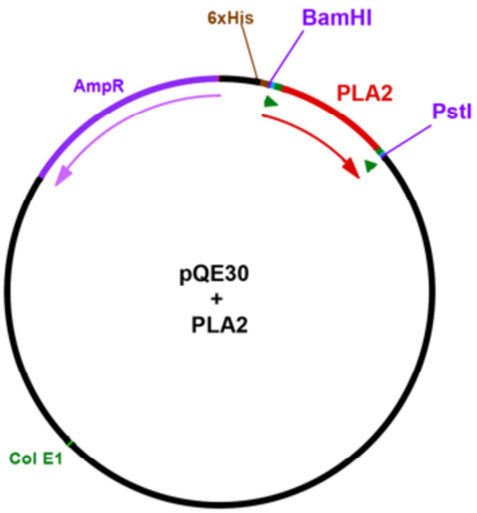
A)

BamH1

-*PT5*-MRGSHHHHHHGSIEGRHLMQFETLIKKIAGRSGVWFYGFYGCYCGSGGRGQPKDATDRCCFVHDCCYGK
VAGCDPKMDFYTYSEENGVVVCGGDDPCKKQICECDRVAATCFRDNKDTYNNKYWFYPAENCQEESEPC••L Q

Pst1

B)



C)

Oligo FW BamH1-FXa-PLA

GGA TCC ATC GAG GGA AGG CAC CTG ATG CAA TTT GAG Tm= 52 °C
G S I E G R H L M Q F E

Oligo Rv Pst1-Stop-Stop-PLA

CTG CAG TTA CTA GCA TGG CTC TGA CTC CTC Tm= 58 °C
Q L * * C P E S E E

Figure 2

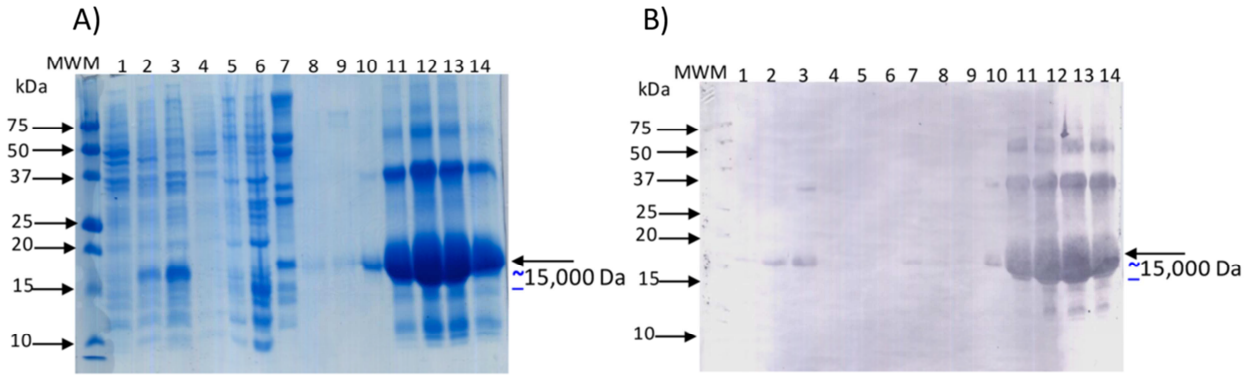


Figure 3

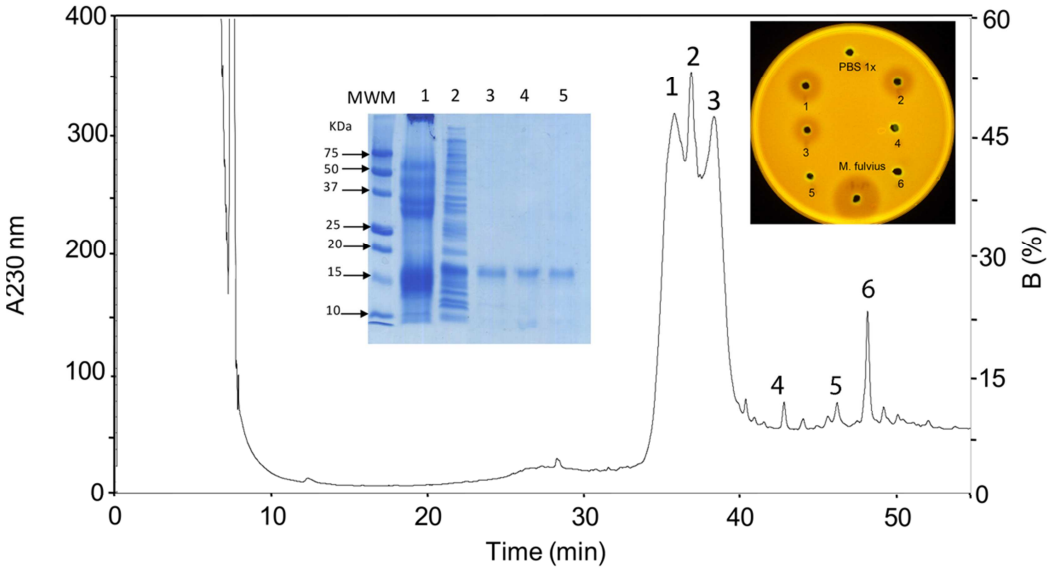


Figure 4

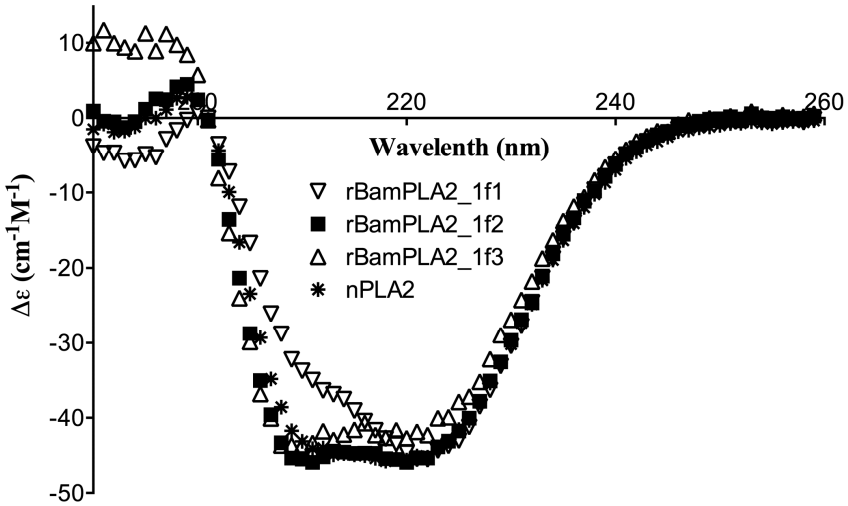


Figure 5

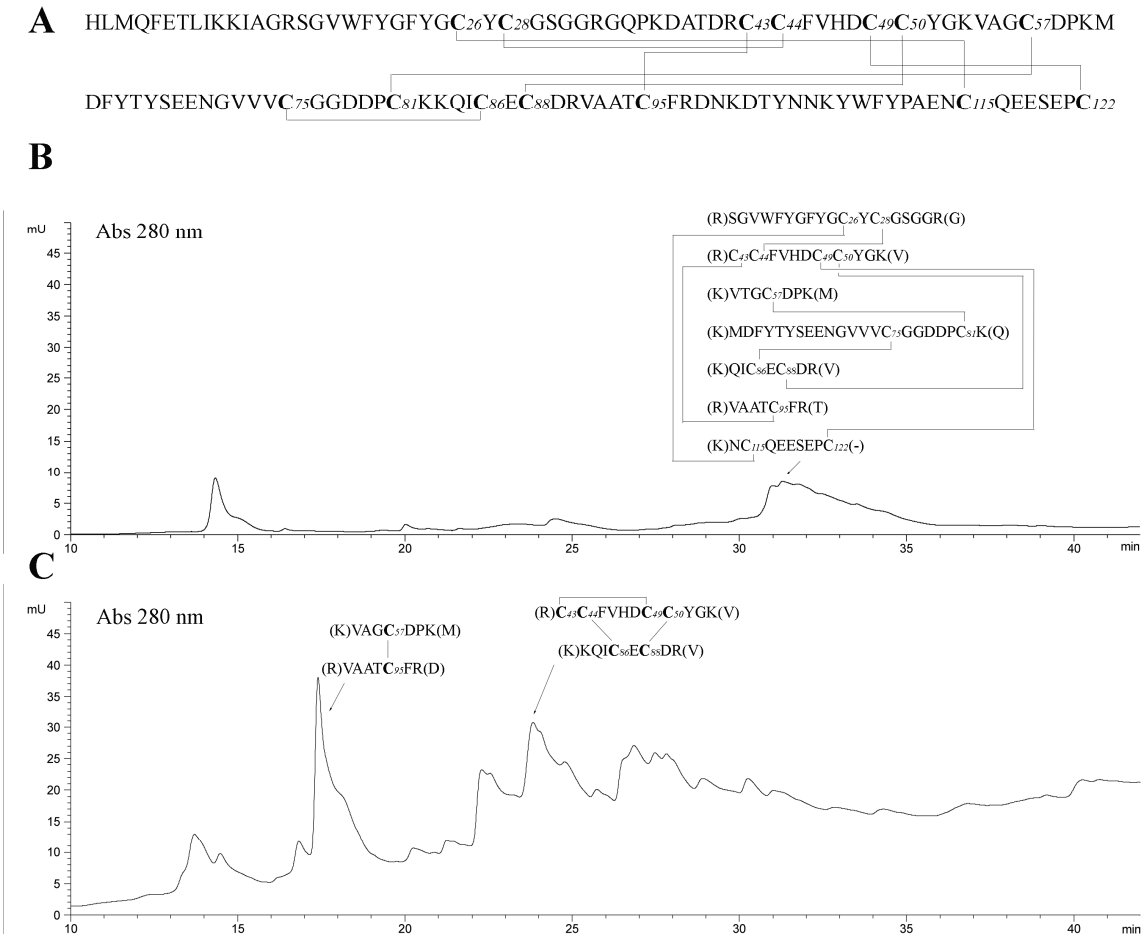

A
HLMQFETLIKKIAGRSGVWFYGFYGC26YC28GSGGRGQPKDATDRC43C44FVHDC49C50YGKVAGC57DPKM
DFYTYSEENGVVVC75GGDDPC81KKQIC86EC88DRVAATC95FRDNKDTYNNKYWFYPAENC115QEESEPC122
B
Abs 280 nm
mU
(R)SGVWFYGFYGC26YC28GSGGR(G)
(R)C43C44FVHDC49C50YGK(V)
(K)VTGC57DPK(M)
(K)MDFYTYSEENGVVVC75GGDDPC81K(Q)
(K)QIC86EC88DR(V)
(R)VAATC95FR(T)
(K)NC115QEESEPC122(-)
min
C
Abs 280 nm
mU
(K)VAGC57DPK(M)
(R)VAATC95FR(D)
(R)C43C44FVHDC49C50YGK(V)
(K)KQIC86EC88DR(V)
min

Figure 6

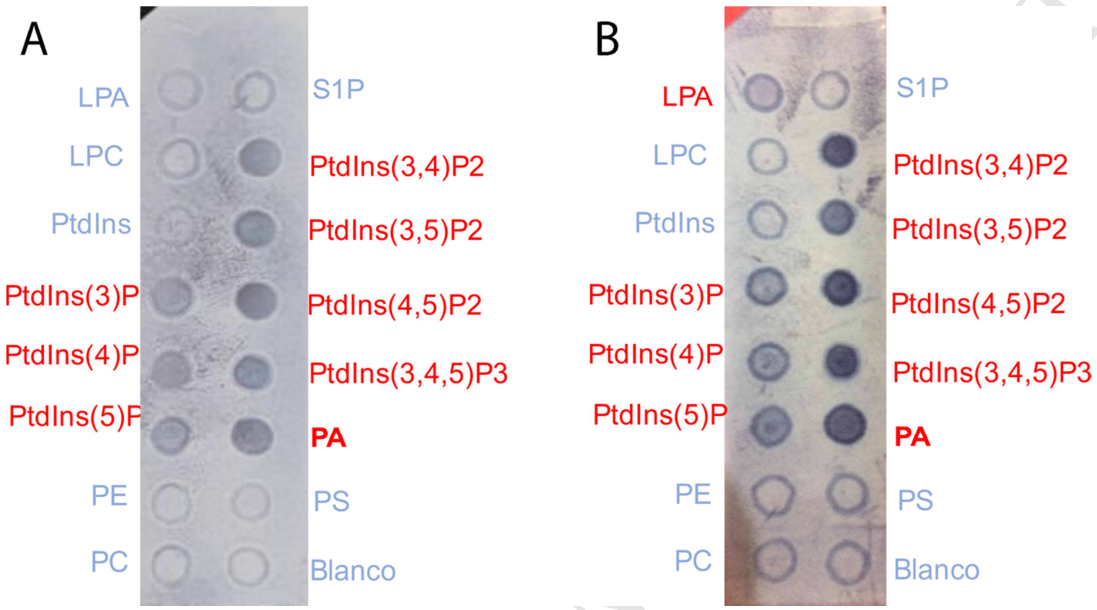


Figure 7

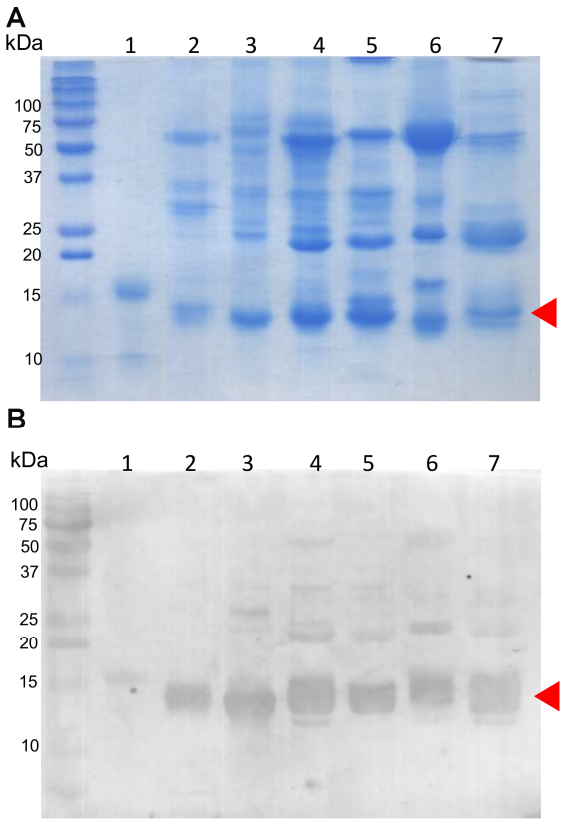


Figure 8

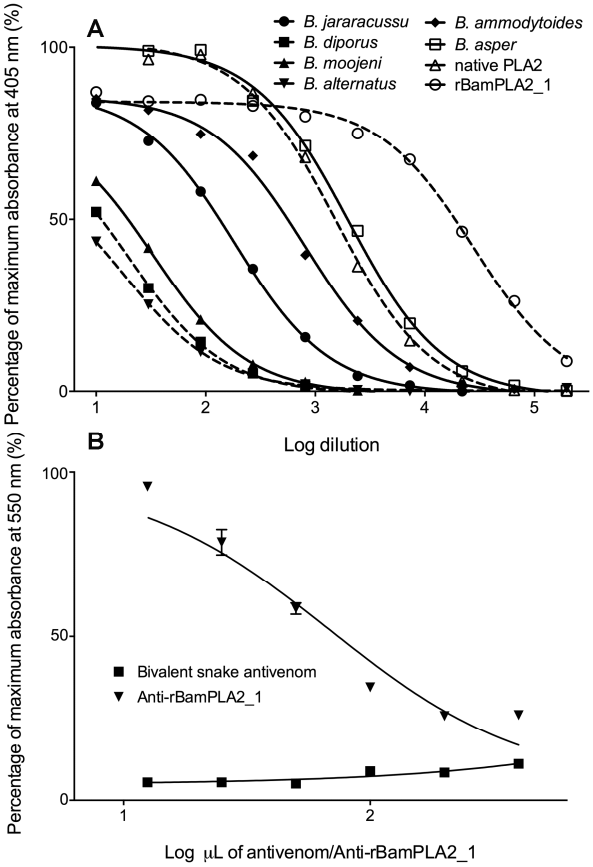
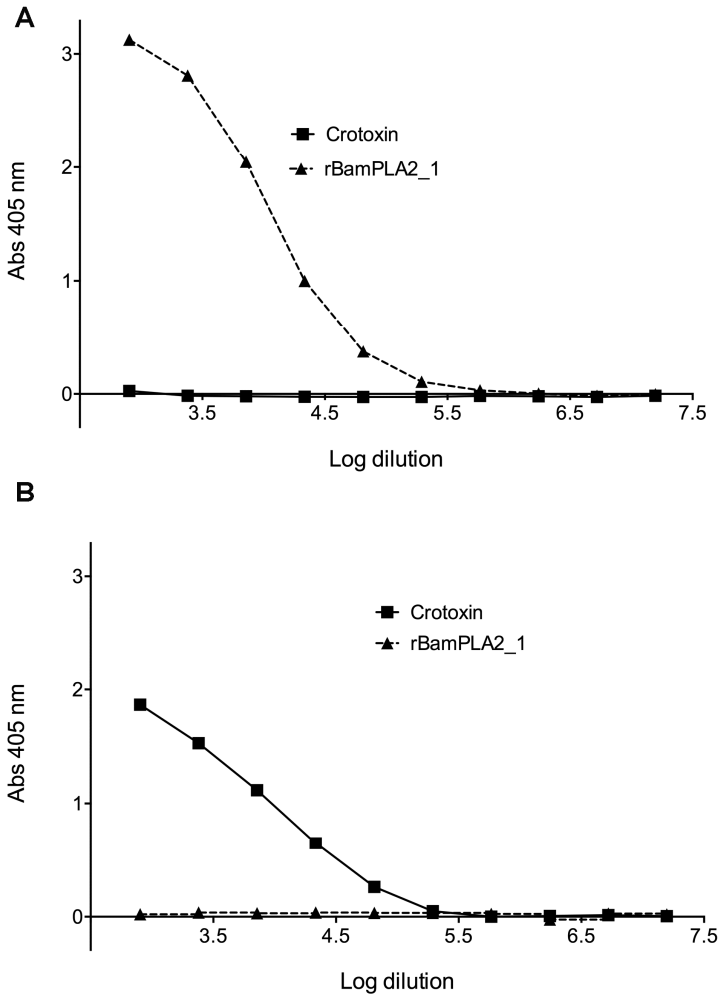
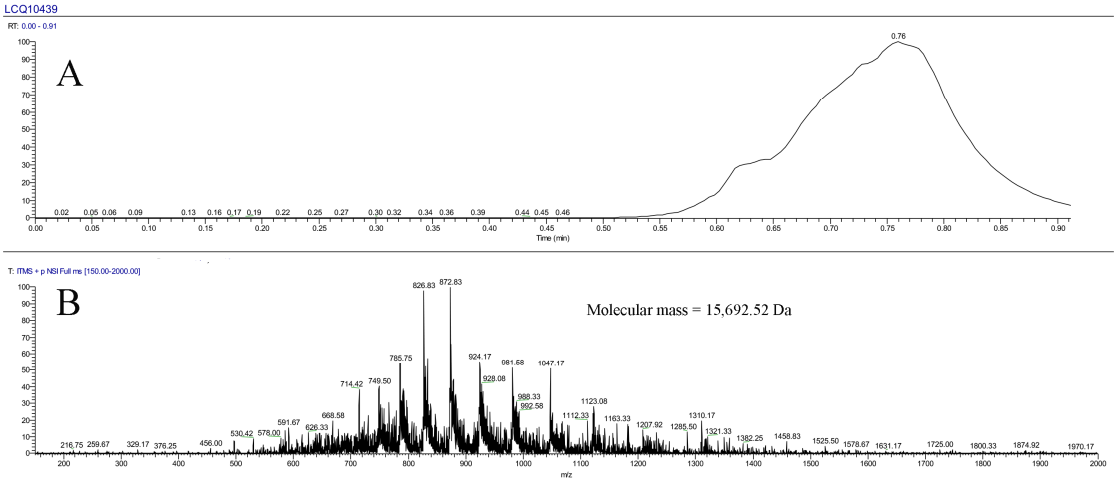

A
B. jararacussu
B. diporus
B. moojeni
B. alternatus
B. ammodytoides
B. asper
native PLA2
rBamPLA2_1
Percentage of maximum absorbance at 405 nm (%)
Log dilution
B
Bivalent snake antivenom
Anti-rBamPLA2_1
Percentage of maximum absorbance at 550 nm (%)
Log μL of antivenom/Anti-rBamPLA2_1

Figure 9


A
Abs 405 nm
3
2
1
0
3.5
4.5
5.5
6.5
7.5
Log dilution
Crotoxin
rBamPLA2_1
B
Abs 405 nm
3
2
1
0
3.5
4.5
5.5
6.5
7.5
Log dilution
Crotoxin
rBamPLA2_1

Supplementary Figure 1. A) Total ion current of the LC-MS analysis of rBamPLA2_1, and B) High-resolution electrospray mass spectrum of rBamPLA2_1. Multiply charged species were detected at high resolution, and then using deconvolution software the experimental molecular mass was discerned.



Highlights

- A type II phospholipase from the venom gland of *B. ammodytoides* was cloned and heterologously expressed.
- The recombinant phospholipase was active on egg yolk phospholipids and recognize membrane phospholipids.
- The recombinant phospholipase generated horse antibodies that inhibited partially the hemolytic activity of *B. ammodytoides* venom.
- Recombinant phospholipases may be used to enhance regional bothropic antivenoms.