

ISOLATION AND FUNCTIONAL CHARACTERIZATION OF A BASIC PHOSPHOLIPASE A₂ FROM COLOMBIAN *Bothrops* *asper* VENOM

AISLAMIENTO Y CARACTERIZACIÓN FUNCIONAL DE UNA FOSFOLIPASA A₂ BÁSICA DEL VENENO DE *Bothrops asper* DE COLOMBIA

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ABSTRACT

Background: Snakebites represent a relevant public health issue in many regions of the world, particularly in tropical and subtropical countries of Africa, Asia, Latin America and Oceania. Snake venoms are complex mixtures of toxic enzymes and proteins, where the most important and abundant muscle-damaging components in snake venoms are phospholipases A₂ (PLA₂s). **Objective:** Isolate and characterize a phospholipase A₂ from Colombian *Bothrops asper* venom, in order to obtain information about venom composition of this species. **Materials and methods:** Cation-exchange chromatography followed by reverse phase HPLC were used to purify the protein. Mass spectrometry was used to determine its molecular mass. Biochemical characterization was performed using a synthetic substrate (4-nitro-3-octanoyloxy-benzoic acid). Myotoxic and edema-inducing activity of toxin were tested in mice, by measuring the plasma creatine kinase activity and footpad diameter, respectively. Moreover, cytotoxic activity was examined to murine skeletal muscle C2C12 myoblasts and myotubes. **Results:** A PLA₂ of *Bothrops asper* venom from Colombia (BaspCol-PLA₂) was purified. Its molecular mass was 13974.6 Da. The enzyme hydrolyzed a synthetic substrate with a K_M of 3.11 mM and a V_{Max} of 4.47 nmol/min, showing maximum activity at 40 °C and at pH 8.0. The PLA₂ required Ca²⁺ for activity. The addition of Mg²⁺, Cd²⁺, Mn²⁺ and Zn²⁺ (10mM) in the presence of low Ca²⁺ concentration (1mM) decreased the enzyme activity. The substitution of Ca²⁺ by mentioned divalent cations also reduced the activity to levels similar to those in the absence of Ca²⁺. Three internal fragments (CCFVHDCCY GK, AAAI/LCFRDNI/LNTYNDKK, DAAI/LCFR) identified by a mass spectrometry analysis showed similarity with previously reported *B. asper* PLA₂s. In mice, BaspCol-PLA₂ induced a conspicuous local myotoxic effect and moderate footpad edema. *In vitro*, this enzyme induced cytotoxic effect on both myoblasts and myotubes. Additionally, it was classified as weakly anticoagulant PLA₂, showing this effect at concentrations between 3 and 10 µg/mL when using human plasma. **Conclusions:** A PLA₂ was purified and named BaspCol-PLA₂, this enzyme displayed catalytic activity and molecular mass of 13974.6 Da. The toxin showed myotoxic, edema-forming, anticoagulant and cytotoxic activities.

Keywords: Snake venoms, snake bites, *Bothrops asper*, phospholipases A₂, necrosis, Colombia.

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RESUMEN

Antecedentes: Los accidentes ofídicos representan un grave problema de salud pública en muchas regiones del mundo, particularmente en países tropicales y subtropicales de África, Asia, América latina y Oceanía. Los venenos de serpiente son mezclas complejas de enzimas y proteínas tóxicas, donde las fosfolipasas A₂ (PLA₂s) son uno de los principales y más abundantes componentes que destruyen el tejido muscular. **Objetivo:** Aislar y caracterizar una fosfolipasa A₂ del veneno de *Bothrops asper* de Colombia, con el fin de obtener información acerca de la composición del veneno de esta especie. **Materiales y Métodos:** Se empleó cromatografía de intercambio catiónico seguida de HPLC en fase reversa para purificar la proteína. La masa molecular fue determinada por espectrometría de masas. La caracterización bioquímica fue llevada a cabo usando un sustrato sintético (**ácido** 4-nitro-3-octanoyloxi-benzoico). La actividad miotóxica y edematizante fue ensayada en ratones, al medir la actividad de la creatina kinasa en plasma y el aumento del diámetro de la almohadilla plantar, respectivamente. Además, la actividad citotóxica fue examinada en mioblastos y miotubos murinos C2C12. **Resultados:** Fue purificada una fosfolipasa A₂ básica (BaspCol-PLA₂) del veneno de *Bothrops asper* de Colombia. Su masa molecular fue 13974,6 Da. La enzima hidrolizó un sustrato sintético con un K_M de 3,11 mM y un V_{Max} de 4,47 nmol/min, mostrando actividad máxima a 40 °C y pH 8,0. La PLA₂ requirió Ca²⁺ para su actividad. La adición de Mg²⁺, Cd²⁺, Mn²⁺ y Zn²⁺ (10mM) en presencia de una baja concentración de Ca²⁺ (1mM) disminuyó la actividad enzimática. La sustitución de Ca²⁺ por otros cationes divalentes también redujo la actividad a niveles similares a aquellos presentados en ausencia de Ca²⁺. Tres péptidos internos (CCFVHDCCYGK, AAAI/LCFRDNI/LNTYNDKK, DAAI/LCFR) identificados por espectrometría de masas mostraron similitud con otras fosfolipasas A₂ de *B. asper* previamente descritas. Cuando BaspCol-PLA₂ fue inyectada en ratones indujo una miotoxicidad local considerable y un edema moderado. *In vitro*, esta enzima provocó efecto citotóxico sobre mioblastos y miotubos. Adicionalmente, esta proteína fue débilmente anticoagulante, mostrando este efecto sobre plasma humano en concentraciones entre 3 y 10 µg/mL. **Conclusiones:** Una PLA₂ fue purificada y llamada BaspCol-PLA₂, esta enzima presentó actividad catalítica y una masa molecular de 13974,6 Da. La toxina mostró actividad miotóxica, edematizante, anticoagulante y citotóxica.

Palabras clave: Venenos de serpiente, mordeduras de serpiente, *Bothrops asper*, fosfolipasas A₂, necrosis, Colombia.

INTRODUCTION

Snakebites represent a relevant public health issue in many regions of the world, particularly in tropical and subtropical countries of Africa, Asia, Latin America and Oceania (1). *Bothrops asper* is responsible for 50–80% of snakebites, and 60–90% of deaths attributable to snakebites in Central America and Northern South America (2). Envenoming by this species induces marked local tissue damage that includes pain, edema, hemorrhage, and myonecrosis (2). The most important and abundant muscle-damaging components in snake venoms are phospholipases A₂ (PLA₂; EC 3.1.1.4). These enzymes hydrolyze sn-2 ester bond of glycerophospholipids, releasing a fatty acid and a lysophospholipid (3). In addition, PLA₂s can induce several pharmacological effects such as edema, modulation of platelet aggregation, as well as neurotoxicity and anticoagulation (3, 4). Snake venom PLA₂s are classified into groups I or II, based on their sequence and mode of disulphide pairings. Group I PLA₂s are found in

the venoms of Elapidae snakes, whereas group II PLA₂s are present in the venoms of Viperidae snakes (4). The group II is further divided into two main subgroups: Asp49 and Lys49 (PLA₂ homologues) variants. In the latter, the aspartic acid residue at position 49, critically involved in calcium binding and essential for catalytic activity, is replaced by lysine. Due to this and other critical substitutions, the Lys49 PLA₂s cannot bind calcium efficiently and are considered enzymatically inactive (5, 6). Although catalytic activity has shown to play a role in the toxic actions of some venom PLA₂s, it is not essential in the case of Lys49 PLA₂s, which use non-enzymatic mechanisms to alter membrane homeostasis (6).

Several PLA₂s have been identified from *B. asper* venom including acidic and basic phospholipases A₂. Ferlan and Gubensek (7) purified an acidic enzyme (PLA₂ I) from the venom of Costa Rica's specimens. Alagón et al (8) characterized three acidic isoforms from the venom of *B. asper* from Mexico, named

PLA₂ 1, PLA₂ 2 and PLA₂ 3. Recently, Fernández et al. (9) isolated and characterized an acidic enzyme (BaspPLA₂-II) of *B. asper* from the Pacific region of Costa Rica. All of these isoforms are Asp49. The basic isoforms known as myotoxins I (Asp49) (10), II (Lys49) (11), III (Asp49) (12) and IV (Lys49) (13) have been isolated from the venom collected in Costa Rica, while another basic PLA₂ isolated from this species of unspecified origin was reported by Mebs and Samejima (14). In addition, a cDNA coding for an Lys49 isoform of Costa Rican *B. asper* was cloned and its sequence deposited in GenBank (AAF14241; = UniProtKB Q9PVE3, unpublished). This diversity of PLA₂ isoforms found in *B. asper* venom is in agreement with recent proteomic studies that evidenced marked geographical, ontogenetic, and individual venom variations (15).

On the other hand, the issue of intra-species venom variability has relevant implications for antivenom production, especially in species with wide geographic distribution as *B. asper*, which is distributed from southern Mexico to northern regions in South America (2). In the same way, despite that antivenoms could be effective in neutralize venoms from snake of geographically separated populations, it has been demonstrated that antivenoms tend to be more effective in the neutralization of homologous venoms (16). Additionally, differences in the immune response of horse to various types of venom components have been observed, especially to some P-I SVMPS and PLA₂s (17). Thus, is important to know the composition of regional venom. In the present work, a basic PLA₂ (BaspCol-PLA2) from the venom of Colombia's *B. asper* has been isolated and characterized, in order to obtain insights into its possible biological roles and its relevance to the pathophysiology of envenoming by this species in the Northwest region of the country. In addition, this work looks for new information about composition of *B. asper* venom from Colombia, the biochemical and toxicological profile of an isolated PLA₂ and its comparison with other PLA₂s isolated from *B. asper* venom and other snakes from *Bothrops* genus.

MATERIAL AND METHODS

Venom and animals

The venom was obtained by manual extraction of 18 specimens from Antioquia, northwest region of Colombia, maintained in captivity at the Serpentarium of the Universidad de Antioquia (Medellín,

Colombia). Venoms were centrifuged at 3000 rpm for 10 min, and supernatants were lyophilized and stored at -20°C until used. For *in vivo* assays, Swiss Webster mice, 18–20 g body weight, were used. All experiments were conducted in accordance with guidelines of the Universidad de Antioquia Ethics Committee.

Isolation of PLA₂

Two hundred and fifty mg of *B. asper* venom were diluted in 0.05 M Tris, 0.1 M KCl (pH 7.0) and applied to a Carboxymethyl - Sephadex C 25 column (1.8 cm x 30 cm), which had been pre-equilibrated with the same buffer. Proteins were eluted at a flow rate of 1.0 mL/min with a KCl gradient from 0.1 to 0.75 M (10), and elution profile was monitored at 280 nm. The fractions corresponding to main peaks were pooled, lyophilized, evaluated by PLA₂ activity and sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, five milligrams of basic fraction containing PLA₂ activity were dissolved in 0.25 M ammonium bicarbonate at pH 8.0, and applied to a C-18 column (Shimadzu) for RP-HPLC. Proteins were eluted with a linear gradient from 0 to 66.0% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid, at a flow rate of 1.0 ml/min. The elution profile was monitored at 280 nm in a UV/VIS photodiode array detector (Shimadzu) and fractions were manually collected, lyophilized and stored at -20°C.

Electrophoresis and molecular mass determination

SDS-PAGE was performed on 15 % gels, under non-reducing or reducing (2-mercaptoethanol, 5%, v/v) conditions (18). Proteins were stained with Coomassie blue R-250. The molecular mass of BaspCol-PLA₂ was determined by electrospray ionization mass spectrometry (ESI-MS) on an Ion Trap LC/MS 1200 series (Agilent Technology) operated in Enhanced Multiple Charge positive mode in the range *m/z* 200–4000.

PLA₂ activity

PLA₂ activity was measured using the assay described by Cho and Kézdy (19) and Holzer and Mackessy (20), modified for 96-well plates. The standard assay mixture contained 200 μL of buffer (10 mM Tris-HCl, 10 mM CaCl₂, 100 mM NaCl, pH 8.0), 20 μL of substrate at different concentrations (4-nitro-3-octanoyloxy-benzoic acid), 20 μL of water and 20 μL of PLA₂ (at 1 μg/μL) in a final

volume of 260 μL . After the addition of PLA₂ (20 μg), the mixture was incubated 40 min at 37 °C, and the absorbance was read at 10 min intervals. The optimum pH and temperature of the PLA₂ were determined by incubating the enzyme in buffers (10 mM citrate, 10 mM phosphate, 10 mM Tris, and glycine 10 mM) of different pH (4.0–9.0), and in 10 mM Tris–HCl, pH 8.0, at different temperatures (25–45 °C). The effect of substrate concentration on enzyme activity was determined by measuring the absorbance increase after 20 min of incubation in 10 mM Tris–HCl, pH 8.0, at 37 °C. The enzyme activity, expressed as the initial velocity of the reaction (V_0), was calculated based on the increase in absorbance after 20 min. All assays were conducted in triplicate, and the absorbances read at 425 nm (Awareness, Stat Fax 3200).

Myotoxic activity

Groups of four mice received an intramuscular (i.m.) injection of 50 μg of toxin diluted in 100 μL of PBS (0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2), in the gastrocnemius. A control group received 100 μL of PBS. At different time intervals (1, 2, 3, 6, 12, and 24 hr) the blood was collected from the tail into heparinized capillary tubes, and the plasma creatine kinase (CK; EC 2.7.3.2) activity was determined by a kinetic assay (Wiener Lab, CK-NAC UV-AA). Activity was expressed in U/L, one unit defined as the phosphorylation of 1 μmol of creatine/min at 25 °C.

Edema-forming activity

Groups of four mice received a subcutaneous (s.c.) injection of 10, 20, 40 and 80 μg of PLA₂ in 50 μL of PBS, into the right footpad. The left footpad received 50 μL of PBS, as a control. After 2 h, footpad thickness was measured with a caliper, in millimeters. Edema was expressed as the increase percentage in thickness of the right foot, as compared to the left one, and the minimum edema-forming dose (MED) was defined as the toxin dose inducing 30% edema. Experiments were carried out in duplicate. The time-course analysis of edema was performed by injecting one MED in the right footpad of mice. The left footpad received 50 μL of PBS, as a control. Then, the edema was measured at 1, 2, 3, 6 and 24 h as described.

Cytotoxic activity

Cytotoxic activity was assayed on murine skeletal muscle C2C12 myoblasts and myotubes

(ATCC CRL-1772) as described Lomonte et al. (21). Several amounts of toxin (5, 10, 20, 40 μg) were diluted in 150 μL of assay medium (Dulbecco's Modified Eagle's Medium supplemented with 1% fetal calf serum) and added to cells in 96-well plates. Controls for 0 and 100% toxicity consisted of assay medium and 0.1% Triton X-100, respectively. After 3 h at 37 °C, a supernatant aliquot was collected for determination of lactic dehydrogenase (LDH; EC 1.1.1.27) activity released from damaged cells, using a kinetic assay (Wiener LDH-P UV).

Anticoagulant activity

Different PLA₂ amounts (0.15–10 μg) diluted in 100 μL of PBS (3.1–100 $\mu\text{g}/\text{mL}$) were added to 0.5 mL of human plasma, and incubated for 10 min at 37 °C. Plasma aliquots incubated with PBS were used as control. Then, coagulation times were recorded after adding 0.1 mL of 0.25 M CaCl₂, in several assays (n= 5) (22). These doses were selected in order to determine whether the PLA₂ is a strong, weak or non-anticoagulant enzyme, as defined by Kini (23).

Protein identification by HPLC-nESI-MS/MS.

Purified lyophilized protein was diluted in 8 M urea containing 10 mM DTT at pH 8.0, and the disulfide bridges were then reduced by incubation at 37 °C for 2 h. Iodoacetamide was used for alkylating the free thiols of cysteine residues, a 25% molar excess of iodoacetamide, relative to the total number of thiols, was eventually chosen and the mixture was incubated for 1.5 h at 37 °C in the darkness. The reaction was stopped by injecting the mixture onto a RP-HPLC column, followed by lyophilization of the collected peak. Afterwards, ten micrograms of isolated PLA₂ was hydrolyzed with sequencing grade bovine pancreatic trypsin in 0.4% ammonium bicarbonate, pH 8.5, for 4 h at 37 °C, at an enzyme:substrate ratio of 1:100 (w/w). Then, the digestion product was subjected to nano HPLC column C-18 in a Mass Spectrometer LC/MSD IonTrap 1200 series (Agilent Technology). The results of the mass spectra of peptides were run in the program Spectrum Mill (Agilent Technology) and Mascot (MatrixScience) in the NCBI nr protein databases. Peptide sequences were searched for similarity using BLAST, and the sequences of PLA₂s isolated from *B. asper* venom were obtained from Uni-Prot and aligned with identified peptides using the program ClustalW (24).

Statistical analysis

Significance of the differences recorded in enzymatic assays were analyzed by one-way ANOVA, followed by Bonferroni's test. In cytotoxic and myotoxic activities, two-way ANOVA followed by Bonferroni's test was applied. One-way ANOVA followed by Dunnett's test was carried out in anticoagulant activity. In all cases, $p < 0.05$ was considered significant, and the results are shown as mean \pm SEM of n indicated in each case. It was used the software SPSS 14.0 (<http://www.spss.com>; SPSS Inc. 233 South Wacker Drive, 11th Floor, Chicago, IL 60606-6412).

RESULTS

Isolation of PLA₂

Figure 1A shows the Cation-exchange chromatography on CM-Sephadex C-25 of *B. asper* venom, which separated them into six fractions (I - VI). Myotoxic and PLA₂ activities were restricted only to fraction V, which was separated by RP-HPLC into fractions V-1 through V-3, as shown in figure 1B. These fractions showed myotoxic activity when they were injected in mice. However, PLA₂ activity was displayed only by fraction V-3. This suggests that V-1 and V-2 could be Lys49 PLA₂s, while V-3 could be an Asp49 PLA₂. Fraction V-3 was named BaspCol-PLA₂. The molecular mass of PLA₂ estimated by SDS-PAGE was ~ 14 KDa as displayed figure 1C. This was confirmed by ESI-MS, where molecular mass obtained was 13974.6 Da, as indicates figure 2.

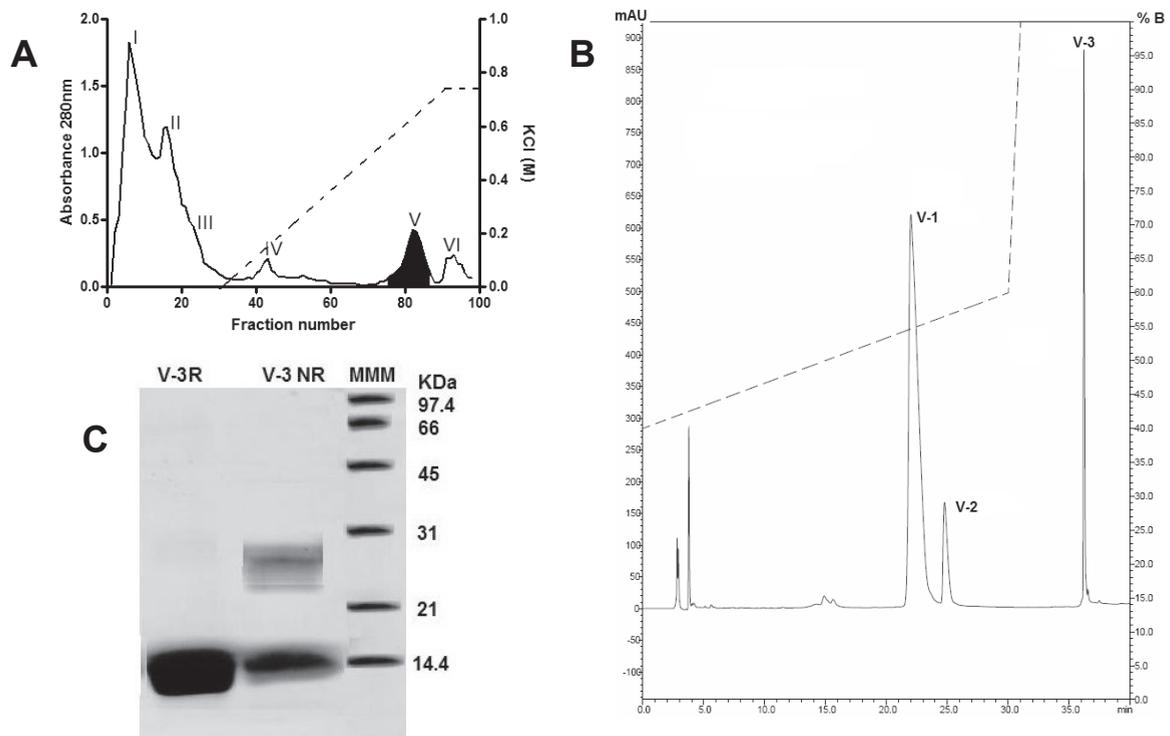


Figure 1. (A) Cation-exchange chromatography of *B. asper* venom from Colombia on Carboxymethyl-Sephadex C 25. Main peaks were identified as I through VI. (B) Fractionation of peak V by RP-HPLC on C-18 column. V-I and V-II presents lack of PLA₂ activity, thus suggesting that they are Lys49 PLA₂s, while V-III has PLA₂ activity, corresponding to Asp49 PLA₂ and named BaspCol-PLA₂. (C) Electrophoresis of BaspCol-PLA₂, V-3 NR: BaspCol-PLA₂ in non-reducing conditions; V-3R: BaspCol-PLA₂ in reducing conditions. The low range molecular mass standards were used.

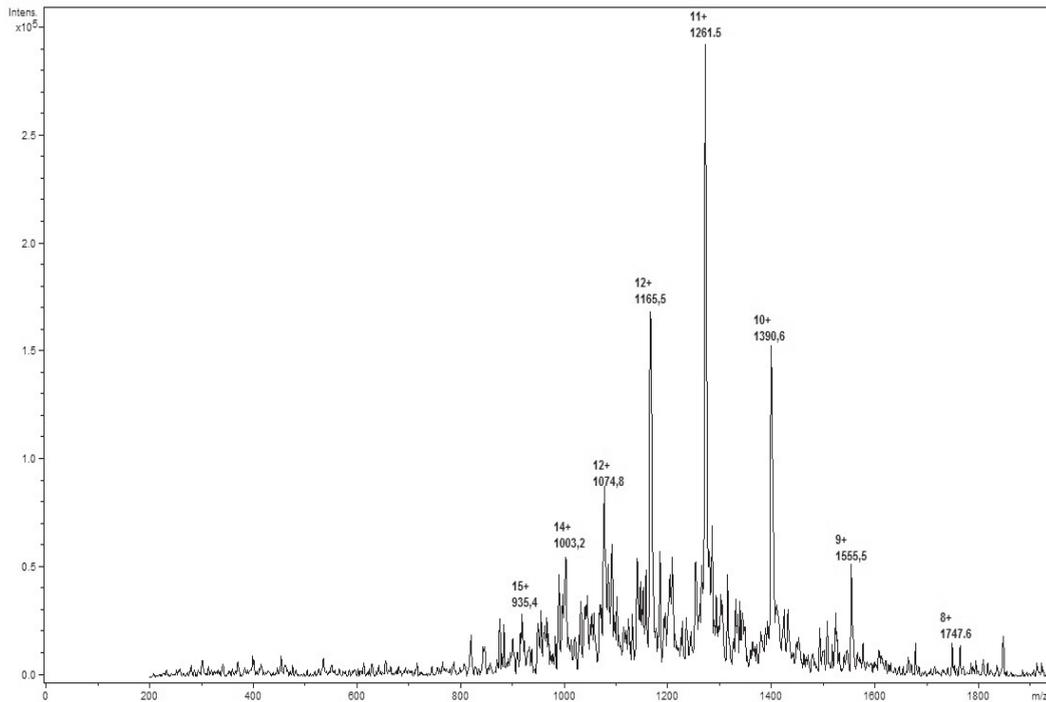


Figure 2. Electrospray ionization mass spectrum (ESI-MS) of the isolated PLA₂. The isotope-averaged molecular mass of the protein calculated from the series of multiple-charged ions (15+ – 8+) was 13,974.6 Da.

Enzymatic characterization

The PLA₂ activity of BaspCol-PLA₂ was studied using the monodisperse substrate 4-nitro-3-(octanoyloxy) benzoic acid. Under the conditions used, PLA₂ showed a Michealian behavior indicated in figure 3A. The V_{Max} was estimated to be 4.47 nmol/ min and the K_M was 3.11 mM. figures 3B and 3C displayed that maximum enzyme activity occurred at 40 °C and the optimum pH was 8.0, respectively. In addition, in figure 3D is evidenced that BaspCol-PLA₂ showed a strict dependence on calcium ions and it was active in concentrations of 1 and 10 mM Ca²⁺, with highest activity obtained with 10 mM ($p < 0.05$). On the other hand, the addition of Mg²⁺, Cd²⁺, Mn²⁺ and Zn²⁺ (10 mM) in the presence of low Ca²⁺ concentration (1mM) decreased the enzyme activity. The substitution of Ca²⁺ by these other divalent cations also reduced the activity to levels similar to those in the absence of Ca²⁺.

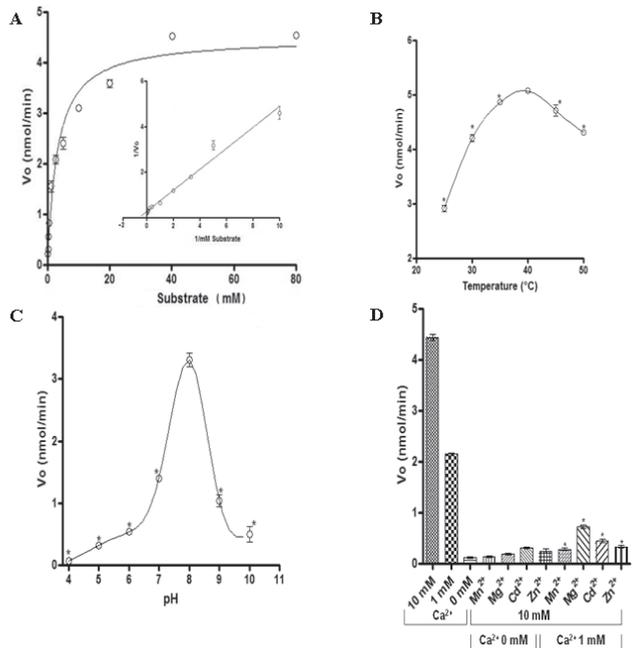


Figure 3. (A) Effect of substrate concentration on kinetics of BaspCol-PLA₂. Insert: Lineweaver-Burk plot. (B) Optimum temperature of PLA₂ activity, * represents a significant difference in comparison to 40 °C. (C) Optimum pH of PLA₂ activity. * represents a significant difference in comparison to pH: 8. (D) Influence of metal ions on PLA₂ activity, * represents a significant difference in comparison to Ca²⁺ 1 mM; n = 6

Biological activities

As displayed in figure 4, BaspCol-PLA₂ induced a conspicuous myotoxic effect, evidenced by the rapid elevation of plasma CK activity, which reached a maximum 2 h after injection, and returned to normal by 24 h. In addition, in figure 5 is evidenced that this enzyme also induced moderate footpad edema, with a MED of $45.2 \pm 2.4 \mu\text{g}$, at 2 h, evidencing the local increase in vascular permeability. Time-course analysis of edema showed that this effect reached its highest point after 2 h and returned to normal by 24 h.

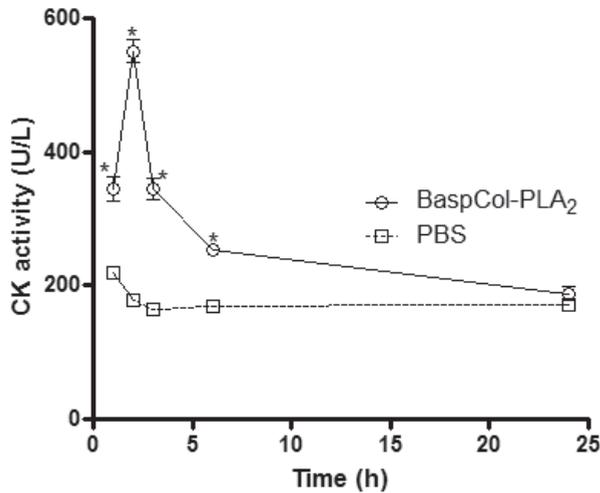


Figure 4. Time-course of the increase plasma CK activity after intramuscular injection of $50 \mu\text{g}/\text{mouse}$ of BaspCol-PLA₂. * represents a significant difference in comparison to control; $n = 8$.

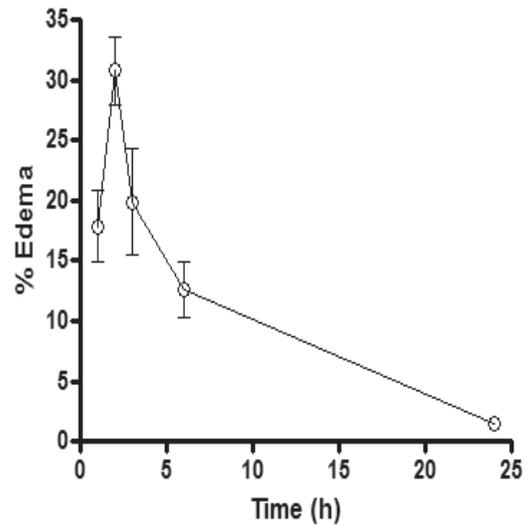


Figure 5. Time-course of the edema-inducing activity (MED, $45 \mu\text{g}/\text{mouse}$), which was expressed as the increased percentage in the diameter of the treated group to that of the control group at each time interval; $n = 8$.

Figure 6A shows that the PLA₂ induced cytotoxicity on myoblast as well as on myotubes, with a significantly higher effect recorded with the latter at doses of 10, 20 and $40 \mu\text{g}/\text{well}$ ($p < 0.05$). As shown in figure 6B, BaspCol-PLA₂ had anticoagulant effect on human platelet-poor plasma. Doses of $0.31 \mu\text{g}/100 \mu\text{L}$ ($3.1 \mu\text{g}/\text{mL}$) or higher caused a delay in clot formation (8–30 min), whereas $0.15 \mu\text{g}/100 \mu\text{L}$ ($1.5 \mu\text{g}/\text{mL}$) of toxin did not delay coagulation time ($p > 0.05$). Clot formation in control samples occurred at $6.48 \pm 0.22 \text{ min}$.

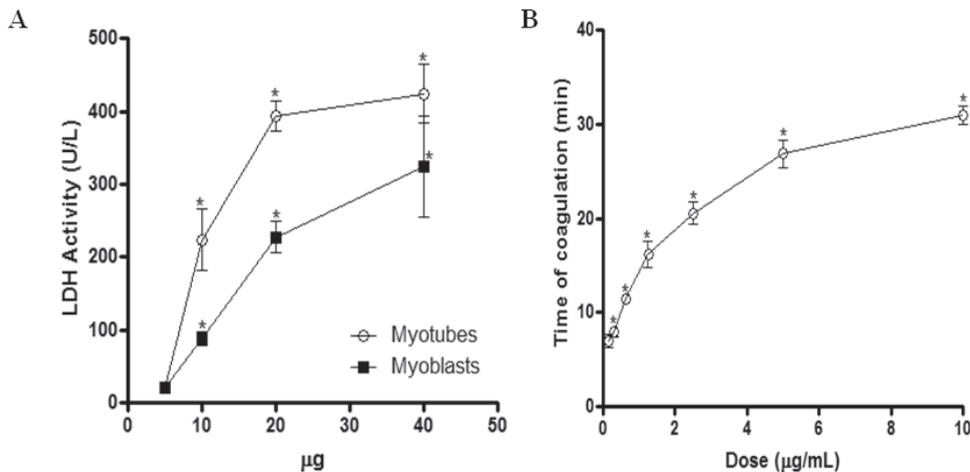


Figure 6. (A) *In vitro* cytotoxic activity of BaspCol-PLA₂ on murine C2C12 skeletal muscle myotubes and myoblasts. Cell lysis was estimated by the release of lactic dehydrogenase (LDH) to supernatants, after 3h of exposure to the toxin, in a volume of $150 \mu\text{L}/\text{well}$. Each point represents mean \pm SD. * represents a significant difference between specific doses; $n = 6$. (B) Anticoagulant activity of BaspCol-PLA₂ in different doses (0.15 – $10 \mu\text{g}/\text{mL}$). * represents a significant difference in comparison to control, which showed coagulation time of $6.48 \pm 0.22 \text{ min}$; $n = 5$.

Protein identification

After protein digestion and peptide separation in a nano C-18 HPLC column, some peptides were identified: CCFVHDCCYGK, AAAI/LCFRDNI/LNTYNDKK, DAAI/LCFR

by HPLC-nESI-MS/MS. The mono-isotopic masses of peptides were: 1505.5 Da, 2014.1 Da and 852.4 Da. Lysine residues shown in bold were deduced on the cleavage and missed cleavage by trypsin, and Cystein residues are shown as alkylated

cysteine (C). These peptides matched with a number of viperid snake venom PLA₂s as determined by a BLAST search. The active site of BaspCol-PLA₂ was identified in the first peptide. Figure 7 display an alignment of the peptides identified in BaspCol-PLA₂ with other PLA₂s isolated from *B. asper* venom. The toxin from Colombian *B. asper* venom showed some identity (48-60%) with corresponding segments of other PLA₂s isolated from Costa Rica's *B. asper*. 10-13)

MyotoxinII	SLFELGKMILQETGKNPAKSYGAYGCNCGVLGRGPKDATDRCCYVHKCCYKCLTGCNPK	60
M1-3-3	SLVELGKMILQETGKNPVTSYGAYGCNCGVLGRGPKDATDRCCYVHKCCYKCLTGCNPK	60
MyotoxinIV	SLVELGKMILQETGKNPLTSYGVYGCNCGVGRHHPKPDGTRCCYVHKCCYKCLTDCDPK	60
MyotoxinIII	SLIEFAKMILEETKRLPPFPYTTYGCYCGWGGQGPDKATDRCCFVHDCCYKLSNCKPK	60
BaspPLA ₂ -II	NLWQFGQMMSDVMRKNVVFYKLSYGCYCGWGGIGQPKDATDRCCFVHDCCYKVTGCDPK	60
BaspCol-PLA ₂	-----CCFVHDCCYGK-----	11
	* * * * *	
MyotoxinII	KD--RYSYSWKDKTIVCGENNSCLKELCECDKAVAICLRENLTYNKKYR--YYLKPLCKK	117
M1-3-3	KD--RYSYSWKDKTIVCGENNSCLKELCECDKAVAICLRKRLDTYNKKYKNNYLKPFCKK	118
MyotoxinIV	KD--RYSYSWKDKTIVCDENNPCLKELCECDKAVAICLRENLDTYNKKYK--IYPKFFCKK	117
MyotoxinIII	TD--RYSYSRKSQVVICGEGTPECKQICECDKAAAVCFRENLRTYKKRYM--AYPDLLCKK	117
BaspPLA ₂ -II	MDIYTYTYSKENGDVVCGGDDPCKKQICECDRVAAICFRDNKDTYDSKYW--FYGAKNCQE	119
BaspCol-PLA ₂	-----AAAXCFRDNXNTYNDKK-----DAAXCFR	35
	* * * * *	*
MyotoxinII	-ADAC	121
M1-3-3	-ADPC	122
MyotoxinIV	-AEPC	121
MyotoxinIII	PAEKC	122
BaspPLA ₂ -II	DSEPC	124
BaspCol-PLA ₂	-----	

Figure 7. Sequence alignment of peptides identified from BaspCol-PLA₂, with PLA₂ sequences previously reported from *B. asper* venom, were performed with the program Clustal W. Myotoxin II from *B. asper*, accession number P24605 (Identity 51%). Myotoxin III from *B. asper*, accession number P20474 (Identity 57%). Myotoxin IV from *B. asper*, accession number P0C616 (Identity 48%). BaspPLA₂-II, acidic PLA₂ from *B. asper*, accession number P86389 (Identity 60%). M1-3-3 basic PLA₂ cDNA cloned from the venom gland of *B. asper* from Costa Rica (GenBank entry AAF14241; unpublished) (Identity 48%). * represents conserved amino acids. X represents Iso or Leu.

DISCUSSION

A myotoxic PLA₂, named BaspCol-PLA₂, was purified from the venom of *B. asper* of the northwestern region from Colombia. The elution profile was comparable to other that used the similar conditions for isolating myotoxins from *Bothrops* venoms (10, 11, 25). This basic PLA₂ showed enzymatic activity on monodisperse substrate (V_{max} : 4.47 nmol/min and K_M : 3.11 mM), with a strict requirement of Ca²⁺, and maximum activity at pH 8.0 and 40 °C. These characteristics are common to other bothropic and crotalic PLA₂s (26-28), and

strongly suggest that position 49 of BaspCol-PLA₂ corresponds to Asp49, as was demonstrated in one of the identified peptides (CCFVHDDCCYGK). The K_M value of BaspCol-PLA₂ is higher in comparison with those calculated for other PLA₂s by using non-monodispersed substrates (i.e. using aggregated substrate). This is explained by the preference of PLA₂s for aggregated substrates, such as glycerophospholipids membrane. In addition, these enzymes suffer an interfacial activation when aggregated substrates (3-5). It is known that snake venom PLA₂s are enzymes that resist extreme values of pH and temperature (3), which is in agreement with the

values of pH and temperature of maximum activity of BaspCol-PLA₂.

Injection of *B. asper* venom induces local myonecrosis in mice (29-31), in agreement with clinical observations (2). The most important and abundant muscle-damaging components in this venom are basic PLA₂s and PLA₂ homologues, as demonstrated by neutralization experiments (32). These toxins disrupt the integrity of muscle cell plasma membrane, and although details of this mechanism remain unknown at molecular level, it is clear that catalytically-dependent and independent events are involved (6, 33). Plasma CK activity reached a maximum at 2 h after injection of BaspCol-PLA₂. Similar results were obtained with D49 PLA₂s BbTx-III from *B. brazili* (26) and Cdcum6 from Colombian *Crotalus durrisus cumanensis* (27). However, slightly different results were obtained with other D49 PLA₂s from *Bothrops* venoms, such as myotoxin I from Costa Rican *B. asper* venom, whose maximum myotoxic activity occurred three hours after its injection (10). Likewise, D49 PLA₂s BmjeTx-I and II from *B. marajoensis* caused maximum of plasma CK activity six hours after their injection (28). Phospholipid hydrolysis by Asp49 PLA₂s plays a critical role in muscle fiber plasma-membrane destabilization, since alkylation of His48 decreases myotoxic effect (34). The partial inhibition after catalytic inactivation suggests that molecular regions distinct from the catalytic site are able to interact and disrupt the integrity of muscle fiber plasma-membrane. The identity of these molecular regions remains largely unknown. Observations with myotoxic Asp49 PLA₂s agree with the general hypothesis proposed by Kini and Evans (35) to explain the pharmacological profile of venom PLA₂s, i.e. that these enzymes have in addition to the active site some molecular regions that determine their toxicity and tissue specificity. Small differences in these regions and in the catalytic properties could explain the differences mentioned above to induce myotoxic effect. In the same way, the use of cell cultures, such as rodent lines of skeletal muscle myoblasts/myotubes, appears to correlate with their *in vivo* myotoxicity (36). This study analyzed in which stage of cellular differentiation of C2C12 myoblasts into myotubes BaspCol-PLA₂ was more cytotoxic. Higher susceptibility of myotubes was clearly observed, as originally described by Angulo and Lomonte (36), using a number of myotoxic PLA₂s and PLA₂

homologues from crotalid venoms. Similar findings were obtained with other bothropic PLA₂s such as BmjeTx-I and II (28), and Bj-V from *B. jararacussu* (37). This fact is probably not only due to the ability of these enzymes to disturb the membrane, but it also seems to be involved with the expression acceptors/receptors with high affinity for the PLA₂s on plasmatic membrane of myotubes. This process could take place when differentiation of myoblast in myotubes is performed. In addition, it is still not possible to state which among the acceptors/receptors or the specific region of PLA₂s are involved, or even the type of interactions implicated in the process (3, 33).

BaspCol-PLA₂ also induced a moderate edema with highest effect two hours after its injection. Similar results were obtained with other D49 from *Bothrops* venoms, such as BmjeTx-I and II from *B. marajoensis* (28) On the other hand, different results were obtained with Myotoxin I, BmTx-I, and BbTx-III from Costa Rican *B. asper*, *B. moojeni*, and *B. brazili* venoms, respectively, whose maximum edema-inducing activity was one hour after their injection (22, 26, 38). Edema caused by Asp49 PLA₂s may be due to their combined effect to hydrolyze phospholipid membrane (resulting in the loss of membrane integrity), as well as their metabolic activity generating pro-inflammatory products such as eicosanoids, whose function is to amplify the inflammatory event (39). However, it has been shown that enzymatic inhibition of PLA₂s do not completely abolish their edema-forming activity (34), similarly to the myotoxic effect, and as proposed Kini and Evans (35). This suggests the presence of molecular regions that are also responsible for inducing this effect.

Depending on their anticoagulant potency, PLA₂ enzymes have been classified into strong, weak and non-anticoagulant enzymes. Strong anticoagulant PLA₂ enzymes inhibit blood coagulation at low concentrations (<2 µg/mL). Weak anticoagulant PLA₂ enzymes showed anticoagulant effects between 3 and 10 µg/mL (23). BaspCol-PLA₂ caused delay of clot formation at doses of 3.1 µg/mL or higher. Therefore, this enzyme was classified as weak anticoagulant PLA₂. Early PLA₂ studies suggested that catalytic activity is essential for anticoagulant effect (40); however, recent studies have proposed that strong anticoagulant PLA₂s inhibit by both enzymatic and non-enzymatic mechanisms. The latter are mediated by an "anticoagulant site", which

would be located in a region between residues 54 and 77, considering that this region is positively charged in the PLA₂s with high anticoagulant activity. In PLA₂s with moderate or low anticoagulant activity, there is a predominance of negative or neutral charges in this region (23, 41).

The main limitation of this study was the lack of total sequence of BaspCol-PLA₂. Thus, we need to perform further studies to obtain total sequence in order to find the regions with highest and lowest identity regard to other snake venom PLA₂s.

CONCLUSION

A basic PLA₂ was isolated from Colombian *B. asper* venom; it has catalytic activity and molecular mass of 13974.6 Da. The toxin showed myotoxic, edema-forming, anticoagulant and cytotoxic activities. The activities displayed by this toxin are involved in the local effects observed in Colombian *B. asper* snakebites. However, molecular mechanisms involved in these effects remain unknown, and further studies of this myotoxin may provide more elements to obtain a better understanding of toxic effects induced by *B. asper* venom. In addition, this work gave insights about the composition of *B. asper* venom from Colombia that contains toxins with similar toxic and biochemical profile of those previously reported for toxins isolated from *B. asper* in other countries.

Conflicts of interest

The authors declare no conflicts of interest.

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REFERENCES

- Williams D, Gutiérrez JM, Harrison R, Warrell DA, White J, Winkler KD, *et al.* The Global Snake Bite Initiative: an antidote for snake bite. *Lancet*. 2010 Aug 24; 74 (9): 1735-1767.
- Otero-Patiño R. Epidemiological, clinical and therapeutic aspects of *Bothrops asper* bites. *Toxicon*. 2009; Dec 1; 54 (7): 998-1011.
- Kini RM. Excitement ahead: structure, function and mechanism of snake venom phospholipase A₂ enzymes. *Toxicon*. 2003; Dec 15; 42 (8): 827-840.
- Six DA, Dennis EA. The expanding superfamily of phospholipase A₂ enzymes: classification and characterization. *Biochim Biophys Acta*. 2000; Oct 31; 1488 (1-2): 1-19.
- Arni RK, Ward RJ. Phospholipase A₂-a structural review. *Toxicon*. 1996; Aug; 34 (8): 827-841.
- Lomonte B, Angulo Y, Calderón, L. An overview of Lysine-49 phospholipase A₂ myotoxins from crotalid snake venoms and their structural determinants of myotoxic action. *Toxicon*. 2003; Dec 15; 42 (8): 885-901.
- Ferlan I, Gubensek F. Phospholipases of *Bothrops asper* venom. *Period Biol*. 1978; 80: 31-36.
- Alagón AC, Molinar RR, Possani LD, Fletcher PL Jr, Cronan JE Jr, Julia JZ. Venom from the snake *Bothrops asper* Garman. Purification and characterization of three phospholipases A₂. *Biochem J*. 1980; Mar 1; 185 (3): 695-704.
- Fernández J, Gutiérrez JM, Angulo Y, Sanz L, Juárez P, Calvete JJ, *et al.* Isolation of an acidic phospholipase A₂ from the venom of the snake *Bothrops asper* of Costa Rica: biochemical and toxicological characterization. *Biochimic*. 2010; 2010 Mar 1; 92 (3): 273-283.
- Gutiérrez JM, Ownby CL, Odell GV. Isolation of a myotoxin from *Bothrops asper* venom: partial characterization and action on skeletal muscle. *Toxicon*. 1984 Jan 1; 22 (1): 115-128.
- Lomonte B, Gutiérrez JM. A new muscle-damaging toxin, myotoxin II, from the venom of the snake *Bothrops asper* (terciopelo). *Toxicon*. 1989 Jul 1; 27 (7): 725-733.
- Kaiser II, Gutierrez JM, Plummer D, Aird SD, Odell GV. The amino acid sequence of a myotoxic phospholipase from the venom of *Bothrops asper*. *Arch Biochem Biophys*. 1990 May 1; 278 (2): 319-325.
- Díaz C, Lomonte B, Zamudio F, Gutiérrez JM. Purification and characterization of myotoxin IV, a phospholipase A₂ variant, from *Bothrops asper* snake venom. *Nat Toxins*. 1995 May 1; 3 (1): 26-31.
- Mebs D, Samejima Y. Isolation and characterization of myotoxic phospholipases A₂ from crotalid venoms. *Toxicon*. 1986 Feb 1; 24 (2): 161-168.
- Alape-Girón A, Sanz L, Escolano J, Flores-Díaz M, Madrigal M, Sasa M, *et al.* Snake venomomics of the lance head pitviper *Bothrops asper*: geographic, individual, and ontogenetic variations. *J Proteome Res*. 2008 Aug; 7 (8): 3556-3571.
- Segura A, Herrera M, Villalta M, Vargas M, Uscanga-Reynell A, de León-Rosales SP, *et al.* Venom of *Bothrops asper* from Mexico and Costa Rica: intraspecific variation and cross-neutralization by antivenoms. *Toxicon*. 2012 Jan; 59 (1): 158-162.
- Gutiérrez JM, Escalante T, Rucavado A. Experimental pathophysiology of systemic alterations induced by *Bothrops asper* snake venom. *Toxicon*. 2009 Dec 1; 54 (7): 976-987.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970 Aug 15; 227 (5259): 680-685.
- Cho W, Kézdy FJ. Chromogenic phospholipase A₂ substrates and assays. *Methods Enzymol*. 1991 Sept; 197: 75-79.
- Holzer M, Mackessy SP. An aqueous endpoint assay of snake venom phospholipase A₂. *Toxicon*. 1996 Oct; 34 (10): 1149-1155.
- Lomonte B, Angulo Y, Rufini S, Cho W, Giglio JR, Ohno M, *et al.* Comparative study of the cytolytic activity of myotoxic phospholipases A₂ on mouse endothelial (tEnd) and skeletal muscle (C2C12) cells in vitro. *Toxicon*. 1999 Jan; 37 (1): 145-158.
- Gutiérrez JM, Lomonte B, Chaves F, Moreno E, Cerdas L. Pharmacological activities of a toxic phospholipase A₂ isolated from the venom of the snake *Bothrops asper*. *Comp Biochem Physiol C*. 1986 Jan; 84 (1): 159-164.
- Kini RM. Structure-function relationships and mechanism of anticoagulant phospholipase A₂ enzymes from snake venoms. *Toxicon*. 2005 Jun 15; 45 (8): 1147-1161.
- Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and

- weight matrix choice. *Nucleic Acids Res.* 1994 Nov 11; 22 (22): 4673-4680.
25. Núñez V, Arce V, Gutiérrez JM, Lomonte B. Structural and functional characterization of myotoxin I, a Lys49 phospholipase A₂ homologue from the venom of the snake *Bothrops atrox*. *Toxicon.* 2004 Jul; 44 (1): 91-101.
 26. Huancahuire-Vega S, Ponce-Soto LA, Martins-de-Souza D, Marangoni S. Structural and functional characterization of brazilitoxins II and III (BbTX-II and -III), two myotoxins from the venom of *Bothrops brazili* snake. *Toxicon.* 2009 Nov; 54 (6): 818-827.
 27. Pereañez JA, Núñez V, Huancahuire-Vega S, Marangoni S, Ponce-Soto LA. Biochemical and biological characterization of a PLA₂ from crotoxin complex of *Crotalus durissus cumanensis*. *Toxicon.* 2009 Apr; 53 (5): 534-542.
 28. Ponce-Soto LA, Martins-de-Souza D, Marangoni S. Neurotoxic, myotoxic and cytolytic activities of the new basic PLA₂ isoforms Bmj_cTX-I and Bmj_cTX-II isolated from the *Bothrops marajoensis* (Marajó Lancehead) snake venom. *Protein J.* 2010 Feb; 29 (2): 103-113.
 29. Arce V, Brenes F, Gutiérrez JM. Degenerative and regenerative changes in murine skeletal muscle after injection of venom from the snake *Bothrops asper*: a histochemical and immunocytochemical study. *Int. J. Exp. Pathol.* 1991 Apr; 72 (2): 211-226.
 30. Gutiérrez JM, Arroyo O, Bolaños R. Mionecrosis, hemorragia y edema inducidos por el veneno de *Bothrops asper* en ratón blanco. *Toxicon.* 1980 Sept; 18 (5-6): 603-610.
 31. Otero R, Osorio RG, Valderrama R, Giraldo AC. Efectos farmacológicos y enzimáticos de venenos de serpientes de Antioquia y Chocó (Colombia). *Toxicon.* 1992 May; 30 (5-6): 611-620.
 32. Lomonte B, León G, Angulo Y, Rucavado A, Núñez V. Neutralization of *Bothrops asper* venom by antibodies, natural products and synthetic drugs: contributions to understanding snakebite envenomings and their treatment. *Toxicon.* 2009 Dec 1; 54 (7): 1012-1028.
 33. Gutiérrez JM, Ownby CL. Skeletal muscle degeneration induced by venom phospholipases A₂: insights into the mechanisms of local and systemic myotoxicity. *Toxicon.* 2003 Dec 15; 42 (8): 915-931.
 34. Soares AM, Giglio JR. Chemical modifications of phospholipases A₂ from snake venoms: effects on catalytic and pharmacological properties. *Toxicon.* 2003 Dec 15; 42 (8): 855-868.
 35. Kini RM, Evans HJ. A model to explain the pharmacological effects of snake venom phospholipases A₂. *Toxicon.* 1989 Jun; 27(6): 613-635.
 36. Angulo Y, Lomonte B. Differential susceptibility of C2C12 myoblasts and myotubes to group II phospholipase A₂ myotoxins from crotalid snake venoms. *Cell Biochem Funct.* 2005 Sept; 23 (5): 307-313.
 37. Bonfim VL, Ponce-Soto L, Novello JC, Marangoni S. Cytotoxic Action in Myoblasts and Myotubes (C2C12) and Enzymatic Characterization of a New Phospholipase A₂ Isoform (Bj-V) from *Bothrops jararacussu* Venom. *Protein Pept Lett.* 2006 Jul; 13 (7): 707-713.
 38. Calgarotto AK, Damico DC, Ponce-Soto LA, Baldasso PA, Da Silva SL, Souza GH, et al. Biological and biochemical characterization of new basic phospholipase A₂ BmTX-I isolated from *Bothrops moojeni* snake venom. *Toxicon.* 2008 Jun 15; 51 (8): 1509-1519.
 39. Teixeira CF, Landucci EC, Antunes E, Chacur M, Cury Y. Inflammatory effects of snake venom myotoxic phospholipases A₂. *Toxicon.* 2003 Dec 15; 42 (8): 947-962.
 40. Verheij HM, Boffa MC, Rothen C, Bryskaert MC, Verger R, De Haas G. Correlation of enzymatic activity and anticoagulant properties of phospholipase A₂. *Eur J Biochem.* 1980 Nov; 112 (1): 25-32.
 41. Kini RM, Evans HJ. Structure-function relationships of phospholipases. The anticoagulant region of phospholipases A₂. *J Biol Chem.* 1987 Oct 25; 262-(30): 14402-14407.