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Novel acidic phospholipase A_2 from *Porthidium hyoprora* causes inflammation with mast cell rich infiltrate

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ABSTRACT

Phospholipases A_2 (PLA_2) are a group of enzymes that hydrolyze phospholipids at the *sn*-2 position, being present in all nature. In venomous animals, these proteins assume a special role, being able to exert diverse pharmacological effects. In this work, authors identified a new isoform of PLA_2 in the venom of *Porthidium hyoprora*, which was isolated through sequential chromatographic steps and named PhTX-III. The enzyme was characterized biochemically and structurally. Structural studies using mass spectrometry confirmed an acidic secretory PLA_2, family IIA, with molecular mass of 13,620.9 Da and identification of 86% of its primary sequence. PhTX-III did not exhibit myotoxic, anticoagulant or antibacterial effects, often present in this class of enzymes. Although, it was capable of initiate inflammatory response, with local edema and release of cytokines IL-1α, IL-6 and TNF-α, probably due to mast cell degranulation.

1. Introduction

Phospholipases A_2 (EC 3.1.1.4), also called PLA_2, are a group of largely studied enzymes that hydrolyze phospholipids at the *sn*-2 position, releasing lysophospholipids and fatty acids. In snake venoms (as well as in other venomous animals) these proteins present an important hole not only in the digestion process, but can exhibit several pharmacological effects that will lead to or help in the capture of prey [20].

The PLA_2 are divided into 5 families (cytosolic, secretory, Ca++-independent, PAF acetylhydrolases, and the lysosomal) and 16 major groups. The snake venom PLA_2 are comprised into the secretory phospholipase A_2 family ($sPLA_2$), characterized by the small size (13–18 kDa), tertiary structure with 5–8 disulfide bridges, the presence of histidine in the active site and requirement of Ca^{++} for catalysis [30,8]. The secreted PLA_2 of New World Viperidae are classified into group IIA, with a molecular mass varying between 13 and 15 kDa, seven conserved disulfide bridges and a seven residues C-terminal tail [14,33].

Different isoforms of PLA_2 may exhibit one or more different pharmacological effects (which can happen through direct action or because of the product of its catalysis) like myotoxicity, cardiotoxicity, necrosis, anticoagulation, neurotoxicity, cytotoxicity and others [19,7]. One single species can contain different isoforms of these molecules, which can be, in some cases, acidic. Despite of its activity, the acidic PLA_2 usually shows weak neurotoxicity, myotoxicity and lethal potency in vivo, but induces other significant pharmacological effects such as inhibition of platelet aggregation, hypotension, inflammatory effects and cytotoxicity.

The different isoforms found in a snake venom perform different roles, like prey immobilization or digestion. The differences in the amino acid sequence and consequent structural differences will respond for such divergences in activities and biochemical properties.

Porthidium hyoprora is an endemic species from northwest region of South America, inside Amazon Forest [5]. Such geographical isolation may lead to significant differences in the biochemical composition of snake venom or specific proteins. The evolution process along geographical isolation might origin different variants of a protein, creating a diversity of pharmacological effects, and that would include PLA_2s with different characteristics [11].

The structure-function relationship in PLA_2 enzymes is not completely clear for all the effects already reported. It remains an

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unsolved question why some isoforms can perform specific roles while others, although very similar, cannot. The lack of some properties by some phospholipases A_2 and the slight differences in their structures are clues to understand how such toxins act [6].

In the present work, a new acidic PLA2, called PhTX-III, was isolated from *Porthidium hyoprora* venom and characterized in its structure, biochemistry and pharmacological properties, in an effort to comprehend more about this class of enzymes.

2. Material and methods

2.1. PhTX-III obtaining

Porthidium hyoprora crude venom (Sigma-Aldrich Chemical Co.; St. Louis, MO, USA) was purified through two chromatographic steps. First, 50 mg of crude venom was dissolved in 1 ml of Ammonium bicarbonate buffer (AMBIC) 1 M, pH 8.0 and then centrifuged at 9000*g* for 5 min. The supernatant was submitted to gel filtration chromatography using Sephadex G75 column (1.5 cm × 100 cm), previously equilibrated with AMBIC 50 mM, under a flow rate of 12 ml/h. The PLA_2 active pool was selected, lyophilized and 5 mg of it was dissolved in 120 μl of 0.1% (v/v) trifluoroacetic acid (solvent A) and 80 μl of AMBIC. The solution was again centrifuged at 9000*g* for 5 min and the supernatant was submitted to a reverse phase chromatography in a C5 Supelco (0.78 × 30 cm) column previously equilibrated with Solvent A. Samples were eluted with a linear gradient (0–100%, v/v) of acetonitrile 66% (solvent B) at a constant flow rate of 1.0 ml/min over 70 min. The elution profile was monitored at 280 nm, and the collected fractions were lyophilized and conserved at −20 °C (Fig. 1A). All chemicals and reagents were of analytical or sequencing grade.

2.2. PLA_2 activity

PLA_2 activity was measured using the assay described by Cho and Kezdy [4] and Holzer and Mackessy [15]. The standard assay mixture contained 200 μL of buffer (10 mM Tris–HCl, 10 mM CaCl2 and 100 mM NaCl, pH 8.0), 20 μL of substrate 4-nitro-3-(octanoyloxy) benzoic acid (3 mM), 20 μL of water and 20 μL of the testing sample in a final volume of 260 μL. The mixture was incubated for up to 40 min at 37 °C, and absorbance was monitored at every 10 min. The enzyme activity, expressed as the initial velocity of the reaction (V_0), was calculated based on the increase of absorbance after 20 min.

2.3. PhTX-III kinetic parameters

For evaluation of different conditions in PLA_2 activity, the above-described assay was performed with only punctual changes in the desired parameter. For temperature evaluation, the assay was performed varying temperature from 25 °C to 45 °C. The pH influence over activity was evaluated using different buffers: Sodium citrate buffer (pHs 4.0–5.0); Tris–HCl buffer (6.0–8.0); glicine buffer (9.0–10.0). The influence of divalent ions in reaction was performed either by substituting the Ca^{2+} by Ba^{2+}, Zn^{2+}, Mn^{2+} or Mg^{2+}, or adding these ions and reducing the amount of calcium from 10 mM to 1 mM. Influence of substrate concentration was measured by varying 4-nitro-3-(octanoyloxy) benzoic acid concentration from 0.05 to 10 mM.

2.4. Molecular mass determination by mass spectrometry

An aliquot (45 μL) of the PhTX-III was injected into a C18 column (100 μm × 100 μm) in a RP-UPLC (nanoAcquity UPLC, Waters). The UPLC was coupled with nanoelectrospray tandem mass spectrometry on a Q-T of Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nl/min. The gradient was 0–50% acetonitrile in 0.1% formic acid over 45 min. The instrument was operated in MS continuum mode and the data acquisition was from *m*/*z* 100 to 3000 at a scan rate of 1 s and an interscan delay of 0.1 s. The spectra were accumulated over about 300 scans and the multiple charged data produced by the mass spectrometer on the *m*/*z* scale were converted to the mass (molecular weight) scale using maximum-entropy-based software supplied with Masslynx 4.1 software package. The processing parameters were: output mass range 6000–20,000 Da at a "resolution" of 0.1 Da/channel; the simulated isotope pattern model was used with the spectrum blur width parameter set to 0.2 Da and the minimum intensity ratios between successive peaks were 20% (left and right). The deconvoluted spectrum was then smoothed (2 × 3 channels, Savitzky Golay smooth) and the mass centroid values obtained using 80% of the peak top and a minimum peak width at half height of four channels.

2.5. Tryptic digestion and analysis

The protein was reduced with DTT (in a final concentration of 5 mM) for 25 min at 56 °C and alkylated with Iodoacetamide (in a final concentration of 14 mM) for 30 min prior to the addition of trypsin (Promega's sequencing grade modifed). After trypsin addition (20 ng/μL in ammonium bicarbonate buffer 0.05 M), the

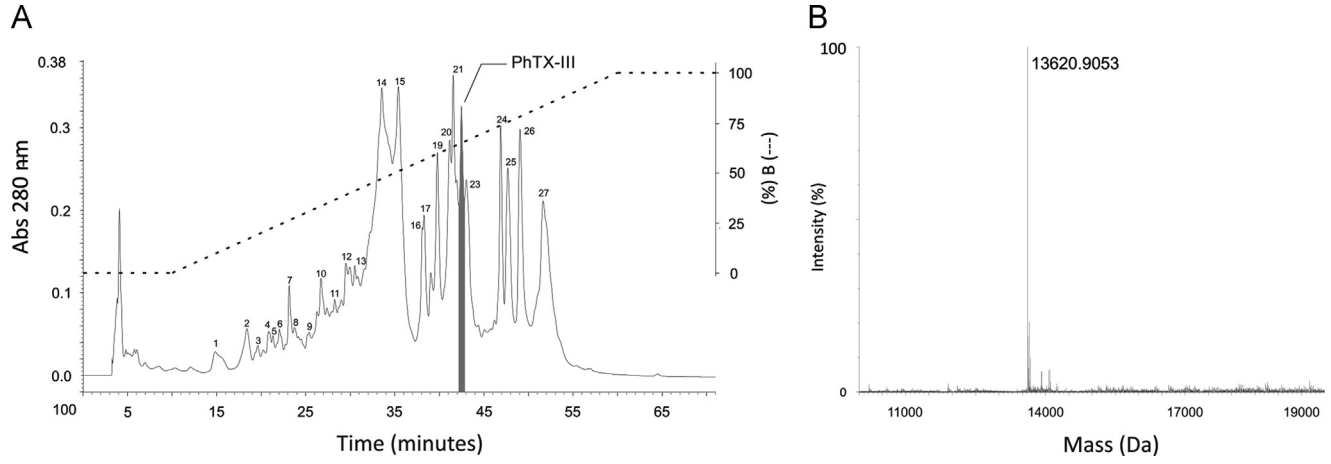

Fig. 1. (A) Elution profile of a reverse phase chromatography of the selected pool from gel filtration (pool II), using Supelco C5 column. In gray, the selected fraction, PhTX-III. (B) Deconvoluted Maldi-TOF mass spectrum of PhTX-III used to determine exact protein mass and attesting homogeneity of sample.

sample was incubated for 16 h at 37 °C. To stop the reaction, formic acid 0.4% was added and the sample centrifuged at 2500 rpm for 10 min. The pellet was discarded and the supernatant dried in a speed vac. The resulting peptides were separated by C18 (100 μm × 100 mm) RP-UPLC (nanoAcquity UPLC, Waters) coupled with nanoelectrospray tandem mass spectrometry on a QTof Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nl/min. Before performing a tandem mass spectrum, an ESI/MS mass spectrum (TOF MS mode) was acquired for each HPLC fraction over the mass range of 100–2000*m/z*, in order to select the ion of interest; subsequently, these ions were fragmented in the collision cell (TOF MS/MS mode). Raw data Files from LC–MS/MS runs were processed using Masslynx 4.1 software package (Waters) and analyzed using the MASCOT search engine version 2.3 (Matrix Science Ltd.) against the snakes database, using the following parameters: peptide mass tolerance of ± 0.1 Da, fragment mass tolerance of ± 0.1 Da, and oxidation as variable modifications in methionine and trypsin as enzyme.

2.6. Miotoxicity evaluation

Swiss male mice (18–20 g; $n=5$) received an intramuscular (i.m.) or an intravenous (i.v.) injection of 20 μg of PhTX-III in phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2) in a total volume of 50 μl. The i.m. group received the injection in the gastrocnemius muscle and the i.v. group in the tail vein. Control groups received only PBS. Blood was collected from the tail into heparinized capillary tubes after two hours and the plasmatic creatine kinase (CK; EC 2.7.3.2) activity was determined by a kinetic assay Ck-Nac, (creatine kinase, Beacon Diagnostics, Germany). The results were expressed as U/L according to the manufacturer.

2.7. Antibacterial activity

The antibacterial capacity of PhTX-III was tested using agar diffusion test, according to Bauer (1966). Six bacteria strains were used: *Escherichia coli* (*E. coli* ATCC 25922), *Pseudomonas aeruginosa* (*P. aeruginosa* ATCC 27853; *P. aeruginosa* 31NM) and *Staphylococcus aureus* (*S. aureus* ATCC 25923; *S. aureus* BEC9393; *S. aureus* Rib1). Commercially available antibiotics Ofloxacin and Imipinen were used as positive controls. The isolated PLA_2 was tested in crescent doses until 200 μg/ml.

2.8. Anticoagulant activity

Swiss male mice blood was used in the two different anticoagulant tests. Activated partial thromboplastin time (aPTT) and the prothrombin time (PT) tests. The blood was collected by cardiac puncture in 3.8% citrate-contained tubes in a volume ratio of 9:1. Blood was centrifuged and only plasma used in the tests. The volume of plasma used in the experiments was 45 μl with 5 μl of toxin solution. Control tests used 50 μl of plasma. Both tests used commercially available kits and automatic coagulometer.

2.9. Rat paw edema induction

Swiss male mice (18–20 g; $n=5$) received subplantar injections from 5 to 40 μg of PhTX-III in PBS; in a total volume of 50 μl. Control groups received only the PBS. The paw swelling was measured with an Electronic Caliper Series 1101 (INSIZE LTDA, SP, Brazil) at 0.5, 3, 6, and 24 h after administration. Edema was expressed as the percentual increase in the size of the treated group compared to the control group at each time. Minimal edematogenic dose was defined as the dose necessary to cause an increase of 30% in paw size.

2.10. Cytokines quantification

Swiss male mice (18–20 g; $n=5$) were injected a 10 μg dose of PhTX-III in the tibial muscle. Afterwards, blood samples were collected from the animals at different times using heparinized capillaries to check the plasma cytokines profile over time. Interleukin 1α (IL-1α), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) concentrations were determined using commercially available ELISA kits (BD OptEIA, BD Biosciences) following provided instructions.

2.11. Morphological analysis

Swiss male mice (18–20 g; $n=5$) gastrocnemius muscles were injected 10 μg of PhTX-III dissolved in PBS, in a total volume of 50 μl or only PBS buffer (control). After one hour, the animals were euthanized by cervical dislocation and had the muscles removed and fixed with formaldehyde 10% solution in Millonig's buffer (0.13 M sodium phosphate, 0.1 M NaOH, pH 7.4) for twelve hours at 4 °C. After that, the injected muscles were washed in water, ethanol dehydrated, diaphanized with xylene and paraffin-embedded. Longitudinal and transversal sections of 7 μm were stained by hematoxylin–eosin (HE) and Dominici staining and were analyzed under a Carl Zeiss Axio Scope.A1 light microscope. Photographs were obtained using Carl Zeiss AxioCam ICc3.

Modified Dominici staining was performed based on Litt [22]. Sections were stained in a mixture of Acid fucsin and Orange G for 30 min. After a quick rinse in ethanol 60%, they were stained in Toluidine blue 0.75% for 20 s. Solutions were prepared according to Tolosa [35].

3. Results

3.1. Enzyme structure studies

The mass spectrometry attested the toxin homogeneity after purification and revealed its exact molecular mass as 13,620.9 Da (Fig. 1B).

The peptides obtained by the tryptic digestion were submitted to MS/MS analysis, and the raw data were processed using Mascot MS/MS Ion Search software. It was possible to obtain 86% of the PhTX-III primary structure. The protein had its structure partially deduced by comparison with other proteins which data was already available in databases (NCBI and UniProt). Through such comparisons, it was possible to align the primary structure of PhTX-III with other similar PLA_2, revealing great similarity with other toxins from different snake venoms (Fig. 2). The alignment was performed using Clustal Omega [13,32,25].

Some notable residues are present in the sequence: The calcium binding loop, comprehended between residues 25 and 33, Y–G–C–Y–C–G–X–G–G (being X a serine substitution in PhTX-III); the fragment C–C–F–V–H–D–C–C–Y–G–K, highly conserved in the PLA_2, between residues 43 and 53, which contains part of the active site: H48, D49, Y52, D99.

The techniques applied revealed a protein with high identity with other already characterized PLA_2 (up to 75% identity), all of them isolated from snake venoms, acidic and containing 122 aminoacids. That is a strong indication that PhTX-III is an acidic PLA_2, which was later confirmed by prediction of the isoelectric point, estimated as 5.04, using the software EMBOSS Pepstats [13,25,28,32].

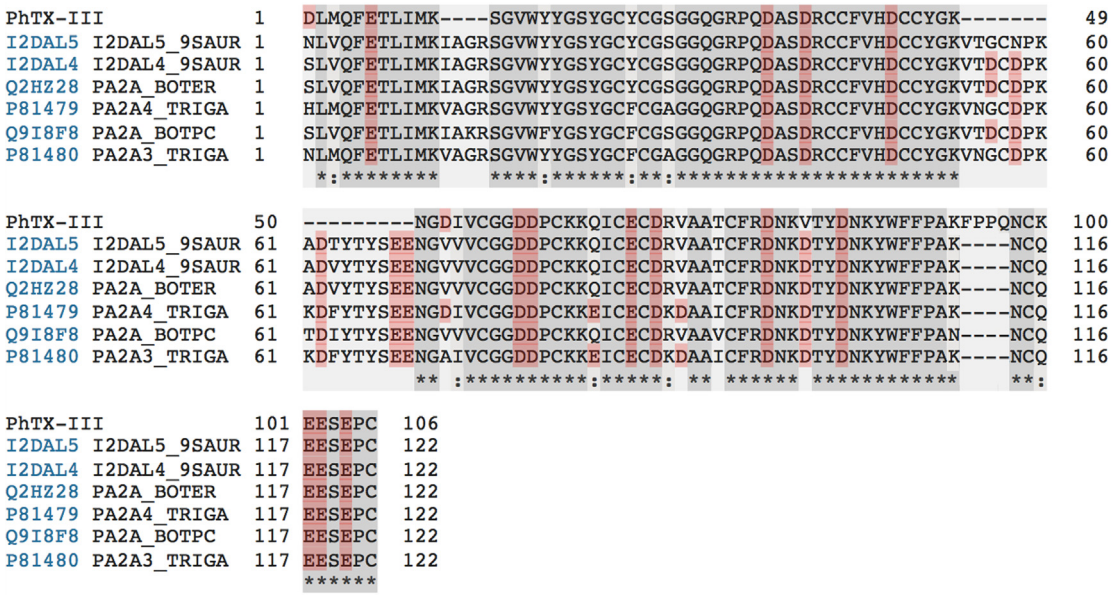

Fig. 2. (A) Aligning and comparison of PhTX-III obtained peptides with other acidic PLA_2 from snake venoms. All the molecules compared are acidic PLA_2, isolated from snakes: *Bothrops diporus* (UniProt I2DAL4; UniProt I2DAL5), *Bothrops erythromelas* (UniProt Q2HZ28), *Trimeresurus gramineus* (UniProt P81479; P81480) *Bothrops pictus* (UniProt Q9I8F8). The asterisk (*) means full consensus for that residue, colon (:) means partial consensus. The acidic residues are marked in red.

3.2. Enzymatic and biological activities

The PhTX-III presents phospholipolytic activity against the chromogenic substrate 4-nitro-3-(octanoyloxy) benzoic acid. The optimal temperature and pH are 37 °C (Fig. 3A) and 8.0 (Fig. 3B), respectively. The maximum velocity obtained by the substrate concentration studies was 10.81 U, with a Km of 4.43 mM. The enzyme is dependent of calcium ions to work properly and incubation with other divalent ions leads to loss of catalytic activity. Among the tested ions, magnesium-containing medium retained around 75% of the enzyme activity, and that is the best result. Zinc totally inhibits any trace of enzymatic activity (Fig. 3D).

The maximum tested dose of 20 μg of the PhTX-III, do not exhibit myotoxicity or anticoagulant activity. The intramuscular and intravenous injections do not lead to increases in the plasma CK content, which would indicate muscular lesion. The protein is also unable to exert any antibacterial activity even at very high doses, up to 200 μg/ml.

As stated, the studied toxin does not show anticoagulant effect. In the PT test, the clothing time was 20.97 ± 1.33 in the control group and 21.5 ± 2.69 in PhTX-III presence. For the aPTT test, the coagulation times were 33.17 ± 3.03 and 37.1 ± 1.65 in control group and with toxin added, respectively. In both cases, there was no statistical difference.

PhTX-III exhibited intense inflammatory effect evaluated by cytokine analysis and moderate edema formation subsequent to footpad injection. The edematogenic effect was monitored in the course of 24 h and revealed a dose-dependent relationship in the edema size and persistency. The minimal edematogenic dose found was 5 μg, which caused a 30% increase in the paw size after one hour. In this same time, the 20 and 40 μg doses caused 39% and 51% in size increase, respectively (Fig. 4). The lower doses do not show any signs of edema after 24 h, but in the higher one, the edema persisted, with paw size increased by 27%.

Thirty minutes after the toxin injection, the levels of inflammatory cytokines IL-1α, IL-6 and TNF-α started to exhibit a different profile compared with the control mice, injected with PBS. TNF-α peaked one hour after toxin injection (reaching 2496.35 ± 210.54 U/ml), but its concentration dropped quickly, standing close to basal levels after 3 h. IL-6 levels started to increase after 0.5 h, reaching its peak of 108.65 ± 2.41 pg/ml at 3 h. After that, its concentration started to decrease reaching basal levels after 12 h. IL-1α concentration rose one hour after toxin injection, and kept its behavior until 6 h, where it reached a maximum concentration that was persistent until 12 h (Fig. 5). The cytokines concentration increase is another evidence of the inflammatory properties of PhTX-III.

The histological sections in the muscles injected with PBS buffer or PhTX-III confirmed that the toxin do not cause muscle damage, confirming by the CK analysis. The muscle integrity was clear in both cases. The difference observed is the great amount of inflammatory infiltrate present in the toxin-injected muscles, with large number of leukocytes. Dominici staining made possible the identification of these cells as mastocytes (Fig. 6).

4. Discussion

In a constant effort to unveil the relationship between structure and function of the PLA_2, our group isolated a novel acidic isoform from the venom of *Porthidium hyoprora*, with high catalytic activity but unable to exert myotoxic, anticoagulant or antibacterial activities.

After the PhTX-III isolation, the primary structure studies identified it as a group IIA PLA_2 [30]. The comparison of the partial primary structure with other PLA_2 attested up to 75% of identity with acidic isoforms of that enzyme from different snake species venoms (Fig. 2). If observed properly in Fig. 2, the conserved residue sequences start and finish always one amino acid earlier than the usual by these enzymes. This happens due to a deletion of a residue, early in the chain, and it happens with frequency in the acidic group IIA PLA_2, nevertheless the usual numbering is used in order to describe the conserved regions [37,39,29]. This observation, along with the prediction of the isoelectric point, is another indication that the enzyme is acidic.

The phospholipolytic activity of PhTX-III was evaluated using the synthetic substrate 4-nitro-3-(octanoyloxy) benzoic acid and the values of optimal temperature and pH found were 37 °C and 8.0, respectively, which are very common and typical values for these enzymes [36,2,10,16,17,27]. Comparing PhTX-III activity in a

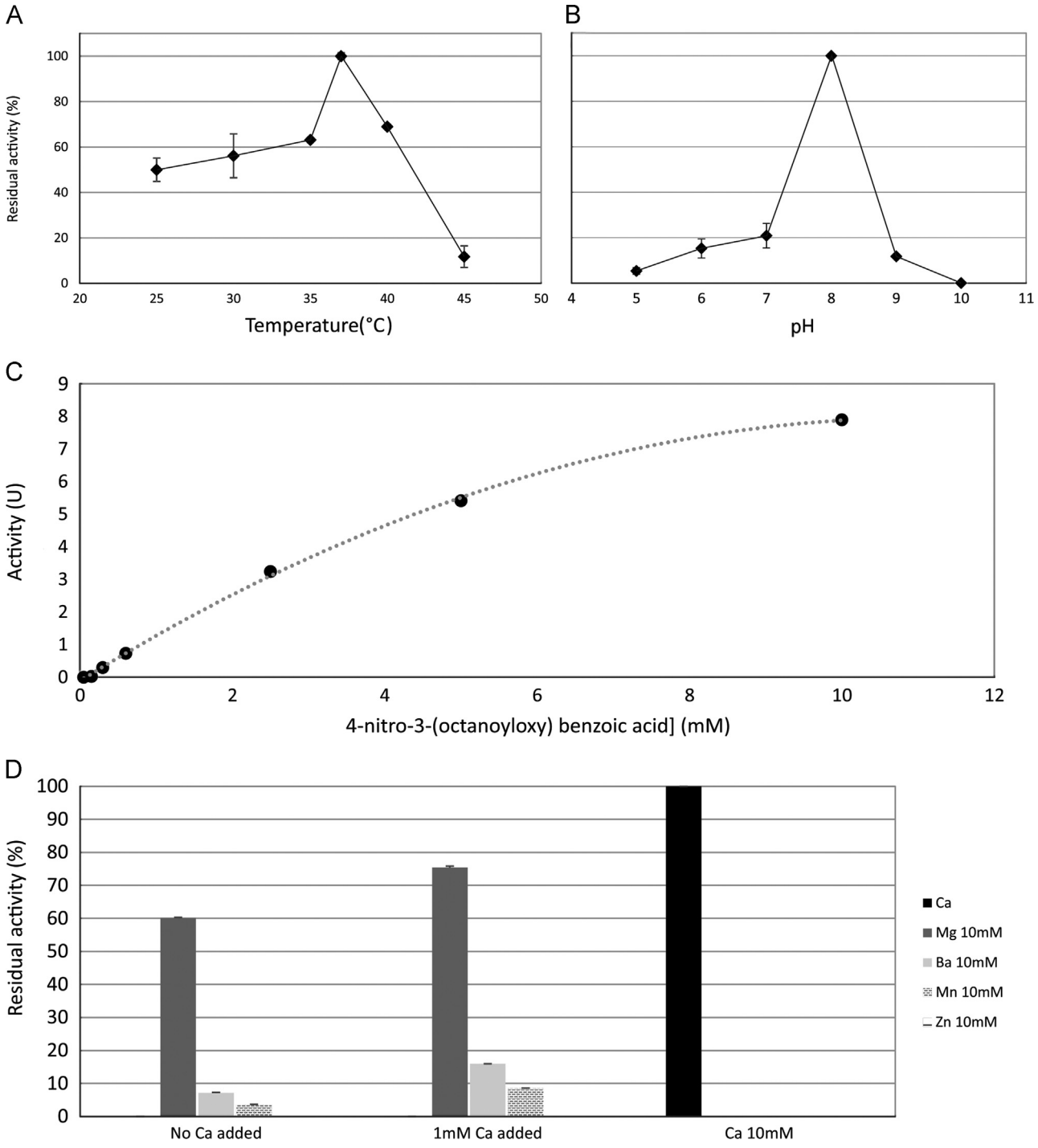

Fig. 3. PhTX-III activities under different circumstances. (A and B) Effect of temperature and pH over enzyme activity, respectively. (C) Effect of substrate concentration over PhTX-III using chromogenic substrate 4-nitro-3-(octanoyloxy)benzoic acid. (D) Effects of different divalent ions over PhTX-III activity, with or without addition of Ca^{+2}.

medium containing calcium, different divalent ions cannot support enzymatic activity properly. Other divalent ions seem to compete for the calcium binding site, and those with more similar ionic radii, like Zn^{2+} tend to cause more effective inhibition [40].

It is usual to find snake venom acidic PLA_2 with an only mild presence of pharmacological effects or totally missing them [18,9,38]. PhTX-III lacks some pharmacological properties tested, usually presented by its basic counterparts. Even at high doses, PhTX-III is not able to exert myotoxic, anticoagulant or antimicrobial activities.

The region predicted to be responsible for the myotoxic effects of PLA_2, comprehended between residues 79 and 87 contains negatively charged aminoacids and a very small amount of positive charges in PhTX-III. Kini and Evans [20] affirmed based on observation of several myotoxic phospholipases A_2, that this region must usually contain a great amount of positively charged residues in order to cause myotoxicity. Similarly, the enzyme site considered fundamental for the observation of antibacterial effects, must be rich in basic aminoacids. This area, the C-terminal region of the PLA_2, contains several negatively charged residues, which is probably the responsible for its lack of antibacterial activity.

Even with absence of some classical pharmacological effects, PhTX-III exhibits intense inflammatory properties, with formation

of edema and increase in plasma concentration of acute phase inflammatory cytokines (Figs. 4 and 5). Edemas are caused by microvascular permeability increase and appear after 1 h of PhTX-III injection in the mice paw. The edemas can be induced by arachidonic acid, resulting from the phospholipase A_2 catalysis, and by the release of IL-1 [1,31].

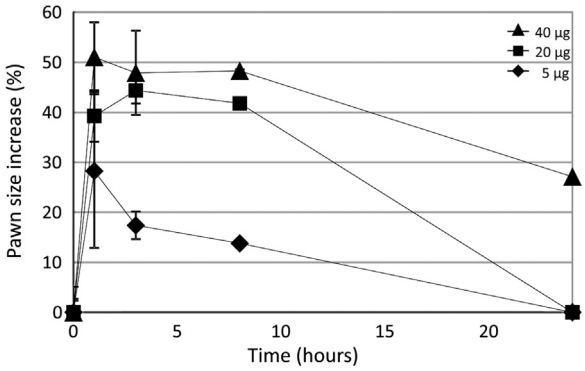

Fig. 4. Edema-forming activity in mice pawn after PhTX-III injection. Results are shown in percentage of increase of the hind pawn after application of different concentrations of toxin, from 0.5 to 24 h. The determined minimal edematogenic dose was 5 µg.

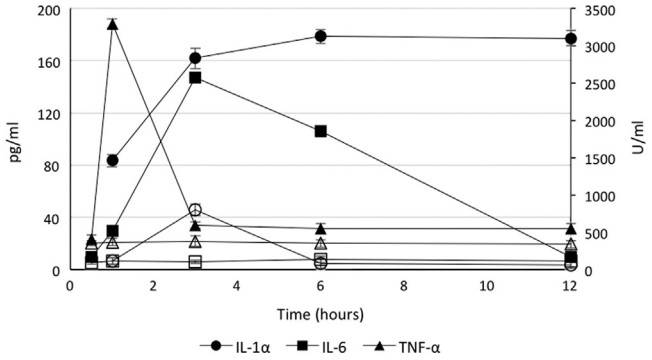

Fig. 5. Plasmatic concentration of cytokines IL-1α (pg/ml), IL-6 (pg/ml) and TNF-α (U/ml) along 12 h after toxin injection. Empty markers represent measurements made in the control group and filled markers, the group that received PhTX-III.

Huancahuire-Vega et al. [17] studying another PLA_2 of the same venom, PhTX-I, verified its capacity of elevating IL-6 plasma concentration, with a peak of 95 pg/ml after 3 h after injection. PhTX-III causes a peak of the cytokine at the same time after injection, but with more pronounced IL-6 release, of 147 pg/ml. Other authors obtained similar results with different inflammatory capable PLA_2 from different sources. In those, the cytokines serum concentrations peaked around three hours after toxin injection, same as in the present paper, but with less intensity [23,2].

Research using a PLA_2 isolated from *Bothrops roedingeri* revealed high inflammatory capabilities, with a similar behavior [12]. As stated by the researcher, TNF-α, is likely to be induced by PLA_2 action, which would induce the expression of selectins resulting in release of other interleukins, such as IL-1 and IL-6.

Histological observations in the toxin-injected tissues reveal no muscle damage, but great amount of inflammatory infiltrate, with large number of cells. Dominici staining made possible the identification of these as mast cells, as suspected by previous observations in sections stained with HE. Since there was no tissue damage or hemorrhage caused by PhTX-III, the source of cytokines can be explained by mast cell degranulation [12,34].

Some phospholipases A_2 can act on blood cells, including neutrophils [24], macrophages [41] and induction of mast cell degranulation [3,21]. As mentioned in Section 1, many pharmacological effects caused by PLA_2 requires catalytic activity or the presence of positive charges in its surface, in order to interact with the target, and mast cell degranulation is no exception to this rule [26].

Based on the stated results, at least part of the inflammatory manifestations can be due to mast cell degranulation, caused by the action of PhTX-III. Although PhTX-III is an acidic enzyme, it still contains positively charged residues, in a lower proportion. Further studies of activities and structure comparison with other PLA_2, basics and acids, can lead to future clues about what is the specific site of interaction between PLA_2 and mast cells.

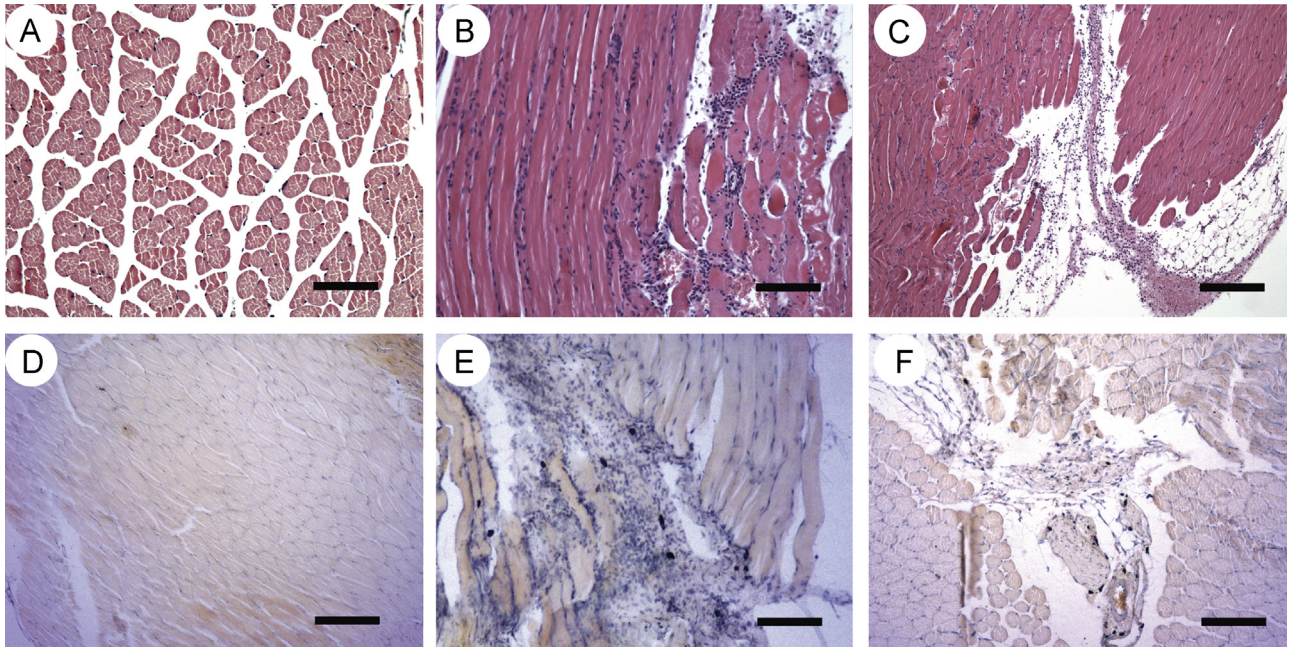

Fig. 6. Sections of the gastrocnemius muscles from mice injected with PhTX-III or phosphate buffer, where: A, B, C – HE stained muscle section, injected with phosphate buffer (A) and PhTX-III (B and C). D, E, F – Dominici stained muscle section, injected with phosphate buffer (D) and PhTX-III (E, F). Bar in A, B: 40 µm. Bar in C, D, E, F: 20 µm.

Acknowledgments

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.03.001.

References

[1] A.K. Abbas, A.H. Lichtman, S. Pillai, Imunobiologia Celular e Molecular, Elsevier Editora, 2008.
[2] A.K. Calgarotto, D.C.S. Damico, L.A. Ponce-Soto, P.A. Baldasso, S.L. Da Silva, G.H. M.F. Souza, M.N. Eberlin, S. Marangoni, Biological and biochemical characterization of new basic phospholipase A2 BmTX-I isolated from *Bothrops moojeni* snake venom, Toxicon 51 (8) (2008) 1509–1519.
[3] F. Chaves, G. León, V.c.H. Alvarado, J.M.a. Gutiérrez, Pharmacological modulation of edema induced by Lys-49 and Asp-49 myotoxic phospholipases A2 isolated from the venom of the snake *Bothrops asper* (terciopelo), Toxicon 36 (12) (1998) 1861–1869.
[4] W. Cho, F.J. Kezdy, Chromogenic substrates and assay of phospholipases A2, Methods Enzymol. 197 (1991) 75–79.
[5] D.F. Cisneros-Heredia, et al., Distribution and natural history of the Ecuadorian Toad-headed Pitvipers of the genus Bothrocophias, Herpetozoa 19 (2006) 10.
[6] F.F. Davidson, E.A. Dennis, Evolutionary relationships and implications for the regulation of phospholipase A2 from snake venom to human secreted forms, J. Mol. Evol. 31 (1990) 228–238.
[7] R. Doley, X. Zhou, R.M. Kini, Snake venom phospholipase A2 enzymes, in: S. P. Mackessy (Ed.), Handbook of Venoms and Toxins of Reptiles, CRC Press, Boca Raton, USA, 2010.
[8] R.E. Duncan, E. Sarkadi-Nagy, K. Jaworski, M. Ahmadian, H.S. Sul, Identification and functional characterization of adipose-specific phospholipase A2 (AdPLA), J. Biol. Chem. 283 (37) (2008) 25428–25436.
[9] J. Fernández, J.M. Gutiérrez, Y. Angulo, L. Sanz, P. Juárez, J.J. Calvete, B. Lomonte, Isolation of an acidic phospholipase A2 from the venom of the snake *Bothrops asper* of Costa Rica: biochemical and toxicological characterization, Biochimie 92 (3) (2010) 273–283.
[10] R.S. Floriano, V.C. Carregari, V.A. de Abreu, B. Kenzo-Kagawa, L.A. Ponce-Soto, M.A. da Cruz-Höfling, S. Hyslop, S. Marangoni, L. Rodrigues-Simioni, Pharmacological study of a new Asp49 phospholipase A2 (Bbil-TX) isolated from *Bothriopsis bilineata smargadina* (forest viper) venom in vertebrate neuromuscular preparations, Toxicon 69 (0) (2013) 191–199.
[11] J.L. Glenn, R.C. Straight, M.C. Wolfe, D.L. Hardy, Geographical variation in *Crotalus scutulatus* scutulatus (Mojave rattlesnake) venom properties, Toxicon 21 (1983) 119–130.
[12] M.A. Gomes Heleno, P.A. Baldasso, L.A. Ponce-Soto, S. Marangoni, Biochemical characterization and pharmacological properties of new basic PLA2 BrTX-I isolated from *Bothrops roedingeri* (Roedinger's Lancehead) Mertens, 1942, Snake Venom, BioMed. Res. Int. 2013 (2013) 13.
[13] M. Goujon, H. McWilliam, W. Li, F. Valentin, S. Squizzato, J. Paern, R. Lopez, A new bioinformatics analysis tools framework at EMBL-EBI, Nucleic Acids Res. 38 (Web Server issue) (2010) W695–699.
[14] R.L. Heinrikson, E.T. Krueger, P.S. Keim, Amino acid sequence of phospholipase A2-alpha from the venom of Crotalus adamanteus. A new classification of phospholipases A2 based upon structural determinants, J. Biol. Chem. 252 (14) (1977) 4913–4921.
[15] M. Holzer, S.P. Mackessy, An aqueous endpoint assay of snake venom phospholipase A2, Toxicon 34 (10) (1996) 1149–1155.
[16] S. Huancahuire-Vega, L.A. Ponce-Soto, D. Martins-de-Souza, S. Marangoni, Structural and functional characterization of brazilitoxins II and III (BbTX-II and -III), two myotoxins from the venom of *Bothrops brazili* snake, Toxicon 54 (6) (2009) 818–827.
[17] S. Huancahuire-Vega, L.A. Ponce-Soto, D. Martins-de-Souza, S. Marangoni, Biochemical and pharmacological characterization of PhTX-I a new myotoxic phospholipase A2 isolated from *Porthidium hyoprora* snake venom, Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 154 (2) (2011) 108–119.
[18] D.F.J. Ketelhut, M. Homem de Mello, E.L.G. Veronese, L.E. Esmeraldino, M. T. Murakami, R.K. Arni, J.R. Giglio, A.C.O. Cintra, S.V. Sampaio, Isolation, characterization and biological activity of acidic phospholipase A2 isoforms from *Bothrops jararacussu* snake venom, Biochimie 85 (10) (2003) 983–991.
[19] R.M. Kini, Phospholipase A2: a complex multifunctional protein puzzle, in: R. M. Kini (Ed.), Venom Phospholipase A2 Enzymes: Structure, Function and Mechanism, John Wiley & Sons, Chichester, England, 1997, pp. 1–28.
[20] R.M. Kini, H.J. Evans, A common cytolytic region in myotoxins, hemolysins, cardiotoxins and antibacterial peptides, Int. J. Peptide Protein Res. 34 (4) (1989) 277–286.
[21] E.C.T. Landucci, R.C. Castro, M.F. Pereira, A.C.O. Cintra, J.R. Giglio, S. Marangoni, B. Oliveira, G. Cirino, E. Antunes, G. De Nucci, Mast cell degranulation induced by two phospholipase A2 homologues: dissociation between enzymatic and biological activities, Eur. J. Pharmacol. 343 (2–3) (1998) 257–263.
[22] M. Litt, Studies in experimental eosinophilia. V. Eosinophils in lymph nodes of guinea pigs following primary antigenic stimulation, Am. J. Pathol. 42 (1963) 529.
[23] B. Lomonte, A. Tarkowski, L. Hanson, Host response to *Bothrops asper* snake venom, Inflammation 17 (2) (1993) 93–105.
[24] B. Lomonte, A. Tarkowski, L.Å. Hanson, Broad cytolytic specificity of myotoxin II, a lysine-49 phospholipase A2 of *Bothrops asper* snake venom, Toxicon 32 (11) (1994) 1359–1369.
[25] H. McWilliam, W. Li, M. Uludag, S. Squizzato, Y.M. Park, N. Buso, A.P. Cowley, R. Lopez, Analysis tool web services from the EMBL-EBI, Nucleic Acids Res. 41 (Web Server issue) (2013) W597–600.
[26] M. Murakami, N. Hara, I. Kudo, K. Inoue, Triggering of degranulation in mast cells by exogenous type II phospholipase A2, J. Immunol. 151 (10) (1993) 5675–5684.
[27] J.A. Pereanez, V. Nunez, S. Huancahuire-Vega, S. Marangoni, L.A. Ponce-Soto, Biochemical and biological characterization of a PLA(2) from crotoxin complex of *Crotalus durissus cumanensis*, Toxicon 53 (5) (2009) 534–542.
[28] P. Rice, I. Longden, A. Bleasby, EMBOSS: the European molecular biology open software suite, Trends Genet. 16 (6) (2000) 276–277.
[29] D.R. Rokyta, K.P. Wray, A.R. Lemmon, E.M. Lemmon, S.B. Caudle, A high-throughput venom-gland transcriptome for the Eastern Diamondback Rattlesnake (*Crotalus adamanteus*) and evidence for pervasive positive selection across toxin classes, Toxicon 57 (5) (2011) 657–671.
[30] R.H. Schaloske, E.A. Dennis, The phospholipase A2 superfamily and its group numbering system, Biochim. Biophys. Acta (BBA) – Mol. Cell Biol. Lipids 1761 (11) (2006) 1246–1259.
[31] F. Sebia-Amrane, F. Laraba-Djebari, Pharmaco-modulations of induced edema and vascular permeability changes by *Vipera lebetina* venom: inflammatory mechanisms, Inflammation 36 (2) (2013) 434–443.
[32] F. Sievers, A. Wilm, D. Dineen, T.J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Soding, J.D. Thompson, D.G. Higgins, Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega, Mol. Syst. Biol. 7 (2011) 539.
[33] D.A. Six, E.A. Dennis, The expanding superfamily of phospholipase A2 enzymes: classification and characterization, Biochim. Biophys. Acta (BBA) – Mol. Cell Biol. Lipids 1488 (1–2) (2000) 1–19.
[34] P. Starkl, T. Marichal, S.J. Galli, PLA2G3 promotes mast cell maturation and function, Nat. Immunol. 14 (6) (2013) 527–529.
[35] E.M.C.d. Tolosa, et al., Manual de técnicas para histologia normal e patológica, (2nd ed.), Manole, Barueri (2003) 341.
[36] M.H. Toyama, D.G. de Oliveira, L.O.S. Beriam, J.C. Novello, L. Rodrigues-Simioni, S. Marangoni, Structural, enzymatic and biological properties of new PLA2 isoform from *Crotalus durissus terrificus* venom, Toxicon 41 (8) (2003) 1033–1038.
[37] I.H. Tsai, Y.M. Wang, Y.H. Chen, A.T. Tu, Geographic variations, cloning, and functional analyses of the venom acidic phospholipases A2 of *Crotalus viridis viridis*, Arch. Biochem. Biophys. 411 (2) (2003) 289–296.
[38] M. Van der Laat, J. Fernández, J. Durban, E. Villalobos, E. Camacho, J.J. Calvete, B. Lomonte, Amino acid sequence and biological characterization of BlatPLA2, a non-toxic acidic phospholipase A2 from the venom of the arboreal snake *Bothriechis lateralis* from Costa Rica, Toxicon 73 (0) (2013) 71–80.
[39] Y.M. Wang, J. Parmelee, Y.W. Guo, I.H. Tsai, Absence of phospholipase A(2) in most Crotalus horridus venom due to translation blockage: comparison with *Crotalus horridus atricaudatus* venom, Toxicon 56 (1) (2010) 93–100.
[40] B.Z. Yu, O.G. Berg, M.K. Jain, The divalent cation is obligatory for the binding of ligands to the catalytic site of secreted phospholipase A2, Biochemistry 32 (25) (1993) 6485–6492.
[41] J.P. Zuliani, J.M. Gutiérrez, L.L.C. e. Silva, S.C. Sampaio, B. Lomonte, C. d. F. P. Teixeira, Activation of cellular functions in macrophages by venom secretory Asp-49 and Lys-49 phospholipases A2, Toxicon 46 (5) (2005) 523–532.