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Isolation and functional characterization of proinflammatory acidic phospholipase A_2 from *Bothrops leucurus* snake venom

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ABSTRACT

In the present study, an acidic PLA_2, designated *Bl*-PLA_2, was isolated from *Bothrops leucurus* snake venom through two chromatographic steps: ion-exchange on CM-Sepharose and hydrophobic chromatography on Phenyl-Sepharose. *Bl*-PLA_2 was homogeneous on SDS-PAGE and when submitted to 2D electrophoresis the molecular mass was 15,000 Da and pI was 5.4. Its N-terminal sequence revealed a high homology with other Asp49 acidic PLA_2s from snake venoms. Its specific activity was 159.9 U/mg and the indirect hemolytic activity was also higher than that of the crude venom. *Bl*-PLA_2 induced low myotoxic and edema activities as compared to those of the crude venom. Moreover, the enzyme was able to induce increments in IL-12p40, TNF-α, IL-1β and IL-6 levels and no variation of IL-8 and IL-10 in human PBMC stimulated *in vitro*, suggesting that *Bl*-PLA_2 induces proinflammatory cytokine production by human mononuclear cells. *Bothrops leucurus* venom is still not extensively explored and knowledge of its components will contribute for a better understanding of its action mechanism.

1. Introduction

Phospholipase A_2—PLA_2 (EC 3.1.1.4)—is a class of enzymes that catalyze the hydrolysis of *sn*-2-acyl bond of *sn*-3-phospholipids, generating free fatty acids and lysophospholipids as products (van Deenen and de Haas, 1963) and are currently classified in 15 groups on the basis of disulphide bridge patterns, amino acid sequence similarity and catalytic mechanisms (Schaloske and Dennis, 2006). Snake venom PLA_2s are known to belong to groups I (Elapidae/Hydrophidae) and II (Viperidae/Crotalidae). The group II can be subdivided, subsequently, in two main subgroups, depending on the residue at position 49 in the primary structure: Asp49 (D49) are enzymatically active while Lys49 (K49) present low or no enzymatic activity (Lomonte et al., 2003). There are other subgroups, such as Asn49 (Tsai et al., 2004; Wei et al., 2006a), Ser49 (Krizaj et al., 1991; Polgar et al., 1996); Gln49 (Bao et al., 2005) and Arg49 (Chijiwa et al., 2006; Mebs et al., 2006; Wei et al., 2006b), but studies have found that catalytic activity is reduced or even abolished when an aspartic acid of native phospholipases A_2 is replaced by another amino acid (Li et al., 1994; Bao et al., 2005).

Snake venoms are rich sources of PLA_2s and these enzymes perform a variety of pharmacological and/or toxic effects, such as myotoxicity, hemolysis, edema, anticoagulation, effects on platelet aggregation, neurotoxicity, cardiotoxicity, hypotension, antitumoral and antimicrobial activities (Gutiérrez and Lomonte, 1997; Ketelhut et al., 2003; Stábeli et al., 2006; Rodrigues et al., 2007; Sami et al., 2008). Moreover, local inflammation is a prominent characteristic of snakebite envenomation by Viperidae and Crotalidae species (Dennis, 1994), due mainly to the presence of PLA_2s and metalloproteinases (Rucavado et al., 2002; Oliveira et al., 2009; Teixeira et al., 2009). The pathogenesis of systemic effects in *Bothrops* envenomation is complex, involving both the direct action of the venom components on the tissue and the release of various endogenous mediators (Petricevich et al., 2000). In literature, the action of both PLA_2s Asp49 and Lys 49 in skeletal muscle cells disruption, as well as its role in inflammatory reactions induction associated with edema, cellular infiltration and pain have been widely demonstrated (Barros et al., 1998; Petricevich et al., 2000; Carneiro et al., 2002; Chavez et al., 2005; Zamuner et al. 2005; Zuliani et al., 2005; Bonfim et al., 2008; Teixeira et al., 2009).

Abbreviations: *Bl*-PLA_2, acidic phospholipase A_2 from *Bothrops leucurus* venom; CK, creatine kinase; CV, crude venom; PBMC, peripheral blood mononuclear cell; PLA_2, phospholipase A_2; TFA, trifluoroacetic acid.

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Bothrops leucurus (white-tailed-jararaca) was described by Wagler in 1824 (BSH, 2010) and occupy the Northeast and Southeast regions of Brazil, including Ceará, Bahia and Espírito Santo States (Porto and Teixeira, 1995). This species has a large medical importance. An epidemiological survey, conducted in Bahia State in 2001, reported that in cases of confirmed bothropic envenoming with the identification of the snake, all of them were attributed to *B. leucurus* (Mise et al., 2007). Some components were isolated from this venom such as three non-hemorrhagic metalloproteinases, leucurolysin-a (leuc-a) (Bello et al., 2006), leucurolysin-B (leuc-B) (Sanchez et al., 2007) and BleucMP (Gomes et al., 2011), a coagulant thrombin-like enzyme, named leucurobin (leuc) (Magalhães et al., 2007), two L-amino acid oxidase (Torres et al., 2010; Naumann, et al., 2011), two basic phospholipases A_2 designed *bl*D-PLA_2 and *bl*K-PLA_2 (Higuchi et al., 2007) and a lectin (BIL) (Nunes et al., 2011).

The present article describes, for the first time, the isolation procedure and the biochemical characterization of a proinflammatory acidic phospholipase A_2 from *B. leucurus* venom, named *Bl*-PLA_2.

2. Materials and methods

2.1. Materials

B. leucurus snake venom was obtained from Bioagents Serpentarium (Batatais, SP, Brazil). CM-Sepharose, Phenyl-Sepharose CL-4B and C18 column were purchased from GE Healthcare. Ethylenediaminetetraacetic acid (EDTA), creatine kinase (CK-UV) kinetic kit, molecular weight protein standards and acrylamide were obtained from Sigma Chemical Co. The kits to cytokine measurement came from ELISA Set, BD OptEIA™—Human (BD Biosciences). All other chemical reagents were of analytical grade.

2.2. Animals and human blood

Swiss male mice were kindly provided by Vallée Institute (Minas Gerais, Brazil) and maintained under standard conditions (temperature 22 ± 1 °C, relative humidity 60 ± 5%, 12 h light/dark cycle) with diet and water *ad libitum*. The experimentation protocol was approved by the Committee of Ethics for the Use of Animals from Federal University of Uberlândia—Minas Gerais, Brazil (License number 08-2008) and is in agreement with ethical principles of animal experimentation adopted by the Brazilian Society of Science in laboratory animals. Human blood was collected from volunteers and approved by the Human Research Ethics Committees from Federal University of Triângulo Mineiro, Brazil (CEP-UFTM) (License number 1079).

2.3. Isolation of the acidic phospholipase A_2

Isolation of the acidic phospholipase A_2 was carried out according to Rodrigues et al. (2007), with some modifications. Samples of crude venom (86.3 mg) were dispersed into 2.0 mL of 0.05 M ammonium bicarbonate buffer, pH 7.8, cleared by centrifugation at 400 × *g* for 10 min and applied on a CM-Sepharose *fast flow* column (2.0 × 20 cm) which was previously equilibrated and initially eluted with the same buffer. A gradient was then applied up to 0.5 M buffer and fractions of 3 mL/tube were collected at a flow rate of 20 mL/h. The fraction showing PLA_2 activity was lyophilized and then applied on a Phenyl-Sepharose CL-4B column (1.0 × 10 cm) equilibrated with 0.01 M Tris–HCl buffer plus 4 M NaCl, pH 8.5. Elution occurred at room temperature, by decreasing concentrations of NaCl (4, 3, 2, 1 and 0.5 M) still in 0.01 M Tris–HCl buffer, pH 8.5, ending the elution process with water.

The active fraction was applied on a reverse phase HPLC using a C18 column of 2.0 × 2.5 cm (Shimadzu), which was equilibrated with solvent A (0.1% trifluoroacetic acid and 5% acetonitrile) and eluted under a concentration gradient of solvent B (60% acetonitrile, 0.1% trifluoroacetic acid) from 0 to 60% at a flow rate of 1 mL/min at room temperature. The protein contents were monitored at 280 nm and the single peak was separated and lyophilized to determine the N-terminal sequence and biochemical properties. Proteins were estimated by Bradford (1976).

2.4. Biochemical characterization

2.4.1. Electrophoresis

14% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Samples were heated at 100 °C for 5 min and then ran under reducing (SDS + 10% β-mercaptoethanol) conditions. The molecular mass markers used were: Bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa) (Amersham Biosciences).

2.4.2. Two-dimensional electrophoresis

In order to determine the molecular mass and pI, *Bl*-PLA_2 (25.0 μg) was precipitated with *2-D Clean-up* kit (GE Healthcare), according to manufacturer's instructions. After drying, the precipitated was solubilized in 200 μL of re-hydration buffer (6 M urea; 2 M thiourea; 2% ASB-14 (p/v); 15 mM DTT; 0.002% bromophenol blue 1%) plus 0.5% (v/v) of IPG buffer, pH 3–10 (GE Healthcare). Re-hydration was performed on IPGPhor III (GE Healthcare) system and took place under voltage of 40 V for 14 h. We used polyacrylamide strips with immobilized pH gradient (Immobiline DryStrip) at pH 3–10 range and 11 cm in length (GE Healthcare). After re-hydration, strips were submitted to isoeletric focusing (IEF) on IPGPhor III, 34636 V/h.

After IEF, focused strips were incubated for 15 min in 10 mL of equilibrium solution (1.5 M Tris–HCl pH 8.8; 6 M urea; 30% v/v glycerol; 2% w/v SDS; 0.002% bromophenol blue 1% w/v) plus 10 mg/mL of DTT (USB), followed by a second incubation in the same buffer, with the inclusion of 25 mg/mL iodoacetomide (Sigma). Finally, strips were subjected to electrophoresis on SDS-PAGE 8–18% (GE Healthcare) in the Multiphor II system according to the program suggested by the manufacturer's manual. Gels were stained with colloidal coomassie blue (2% (w/v) coomassie G-250) for 72 h, destained in distilled water and kept in ethanol 70% (v/v) until your digitalization in the scanner Image Scanner (GE Healthcare), using the Image Master LabScan™ v 5.0 program (with 300 dpi resolution). Analyses were done visually and also by the Image Master 2D Platinum v 6.0 (GE Healthcare) program, as recommended in the manual. The reference gel was obtained as a result of biological triplicate analyses.

2.5. N-terminal sequence

Bl-PLA_2 (1 mg) was dissolved in 0.25 M Tris–HCl buffer, pH 8.2, containing 0.1% (w/v) EDTA. For each mole of Cys, 30 moles of dithiothreitol were added. After being flushed with N_2, the solution was incubated for 4 h at 50 °C in the dark. Excess vinylpyridine was then added and incubation proceeded overnight. The reduced sample was then purified on a C18 RP-HPLC column and its N-terminal sequence was determined using a model PPSQ-23A Perkin-Elmer Procise Sequenator (Applied Biosystem Division, Foster City, CA, USA) with on-line identification of the corresponding phenylthiohydantoin derivatives. The N-terminal amino acid sequence of *Bl*-PLA_2 was aligned with sequences available in the Gene Bank and Swiss-Prot databases using the Clustal program.

2.6. Enzymatic activity

PLA_2 activity was assayed as described by de Haas and Postema (1968). It was determined upon egg-yolk emulsion, which contains

phosphatidylcholine as substrate, using samples with 10 μg of proteins. For the indirect hemolysis method, washed mice erythrocytes and hen's egg-yolk emulsion were used as substrate (Gutiérrez et al., 1988). Each experiment was performed in triplicate and independent assays were done.

2.7. Myotoxic activity

Groups of three Swiss male mice (18–22 g) were injected intramuscularly (i.m.) in the gastrocnemius muscle with 20 μg of crude venom and 20 μg or 50 μg of *Bl*-LA_2, dissolved in 50 μL of saline. The control group received only 50 μL of saline. After 3 h, the blood was collected by cardiac puncture in heparin-coated tubes and centrifuged at 320×*g* for 10 min at 4 °C for plasma separation. Activity of creatine kinase (CK) (EC 2.7.3.2) was then determined using 10 μL plasma, which were incubated for 3 min at 37 °C with 1 mL of the reagent, according to the Laborlab Kinetic CK–UV protocol. Activity was expressed in U/L, one unit resulting from the phosphorylation of 1 μmol of creatine/min at 25 °C. Independent assays were done.

2.8. Edema-inducing activity

Groups of five Swiss male mice (18–22 g) were injected in the subplantar region with the crude venom or *Bl*-PLA_2 (20 μg/50 μL saline). After 0.25, 0.5, 1, 2, 3, 6 and 24 h, the paw edema was measured with the aid of a low-pressure spring caliper (Mitutoyo-Japan) (Rodrigues et al., 2001). Zero time values were then subtracted and the differences reported as mean ± S.D. Independent assays were done.

2.9. PBMC and cell culture

Peripheral blood mononuclear cells (PBMC) were obtained from human whole blood donated by healthy volunteers, after centrifugation on a Ficoll-Paque Plus density gradient (GE Healthcare). PBMC were cultivated in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 0.002 M L-glutamine, 0.002 M sodium pyruvate, 0.001 M non-essential amino acids, 100 UI/mL penicillin, 100 μg/mL streptomycin, and incubated at 37 °C with 5% CO_2, under standard procedures. The cells were cultured at density of 1×10^6 cells/well in 24-well microplates. PBMC cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Mosmann (1983) after treatment with different doses of enzyme (2.5, 5 and 10 μg/well).

The PBMC were stimulated with crude venom or *Bl*-PLA_2 at different concentrations. Control PBMCs were cultured only in RPMI medium. The cells were incubated at 37 °C for 24 h. After stimulation, samples was centrifuged, the supernatants collected and stored at −70 °C before cytokine assays.

2.10. Cytokine measurement

TNF-α, IL-8, IL-12p40, IL-10, IL-1β and IL-6 levels in supernatants were measured by enzyme linked immunosorbent assay (ELISA), according to the manufacturer's instructions. Cytokine concentrations were then calculated by interpolation of the regression curve corresponding to known amounts of recombinant cytokines provided in the kit, and reported as pg/mL. Each experiment was done in triplicate and independent assays were done. Detection limits were: TNF-α, 1.4 pg/mL; IL-8, 0.6 pg/mL; IL-12p40, 1 pg/mL; IL-10, 1.2 pg/mL; IL-6, 4 pg/mL and IL-1β, 1.95 pg/mL.

2.11. Statistical analysis

The data were analyzed by Systat 10.2, BioStat5 and Statview softwares. The results were expressed as means ± standard deviation (S.D.). Statistic significance of differences between groups was evaluated by ANOVA or Kruskal–Wallis tests and confirmed by Tukey and Tukey-type tests. A p value<0.05 was considered to indicate statistically significant difference.

3. Results and discussion

Acidic PLA_2s have shown to be of high scientific interest due to their abundance in venom and their several functions acquired during evolution (Bonfim et al., 2008), such as bactericidal, antitumoral, parasiticidal and pro- or anti-inflammatory effects (Rucavado et al., 2002; Chacur et al., 2004; Rodrigues et al., 2004; Stábeli et al., 2006; Costa et al., 2008; Cruz et al., 2008; Nevalainen et al., 2008; Sami et al., 2008; Gebrim et al., 2009; Oliveira et al., 2009). Thus it becomes relevant to isolate and study the clinical potential of these enzymes.

Fractionations of *B. leucurus* venom by ion-exchange chromatography on CM-Sepharose column resulted in five major protein peaks (Fig. 1A). BL1 fraction, showing high phospholipase A_2 activity, was further fractionated on Phenyl Sepharose CL-4B and resolved into two new fractions, named BL1P1 and BL1P2 (Fig. 1B). BL1P2 was applied on a HPLC reverse-phase chromatographic column and presented one major peak, named *Bl*-PLA_2 (Fig. 1C).

The purification procedure according to Rodrigues et al. (2007) showed to be also efficient to isolate an acidic PLA_2 from *B. leucurus* snake venom, which corresponded to 5.4% of crude venom (Table 1), high percentage when compared to approximately 4% obtained for Bp-PLA_2, after Phenyl Sepharose (Rodrigues et al., 2007) and 1.8% for *bl*D-PLA_2, after all purification procedure (Higuchi et al., 2007). Homogeneity of *Bl*-PLA_2 was further demonstrated by SDS-PAGE (Fig. 1D). The purified protein consisted of a single polypeptide chain with apparent approximate molecular weight of 15,000 and its pI was 5.4 (Fig. 1E).

The acidic PLA_2 (*Bl*-PLA_2) has an estimated molecular weight fitting with literature data which establish that snake venom PLA_2s present molecular weight varying between 13,000 and 18,000 (Ward et al., 2001). Higuchi et al. (2007) isolated two basic phospholipases A_2 from *B. leucurus* snake venom with molecular weight for both of 14,000.

The first sixty amino acids at the N-terminal sequence of *Bl*-PLA_2 were determined (Fig. 2). As this protein contained Asp at position 48 (position 49 of the catalytic dyad, according to numbering of Renetseder et al. (1985)), it was determined as an Asp49 PLA_2. The N-terminal sequence analysis of *Bl*-PLA_2 showed that it contains all of phospholipase A_2 conserved residues involved in the calcium binding and in the catalytic network (Tyr28, Gly30, Gly32, His48 and Asp49) (Renetseder et al., 1985; Kudo and Murakami, 2002). N-terminal sequences alignments of *Bl*-PLA_2 with those of other bothropic Asp49-PLA_2s showed a high identity with acidic D49-PLA_2s, highlighting those from *B. jararacussu* (100%), *B. pirajai* (86%) and *B. insularis* (81%). The lowest identity observed between *Bl*-PLA_2 and acidic D49-PLA_2s was for *B. moojeni* (63%). On the other hand, alignments between *Bl*-PLA_2 and basic Asp49-PLA_2s showed identities around 60%, one more strong evidence of acidic character of *Bl*-PLA_2 (Fig. 2).

The specific PLA_2 activity of *Bl*-PLA_2 was 159.9 ± 11.9 U/mg, approximately two-folds higher than that of the crude venom (69.3 U/mg) (Table 1). Indirect hemolytic activity presented by *Bl*-PLA_2 was also higher than that of the crude venom (Table 1).

Bl-PLA_2 induced low myotoxicity when compared with crude venom (Table 2), regardless of enzyme concentration. Low or no myotoxicity was also observed for four isoforms (SIIISPIIA, SIIISPIIB, SIIISPIIIA and SIIISPIIIB) of acidic PLA_2s isolated from *B. jararacussu* (Ketelhut et al., 2003), BE-I-PLA_2, an acidic D49 phospholipase A_2 from *B. erythromelas* (Modesto et al., 2006), BaspPLA$_2$-II, a basic D49-PLA_2 from *B. asper* (Fernández et al., 2010) and for an acidic PLA_2, Ba SpII RP4, isolated from *B. alternatus* (Denegri et al., 2010). In general, acidic AspPLA_2s are less myotoxic than basic PLA_2s, although they are

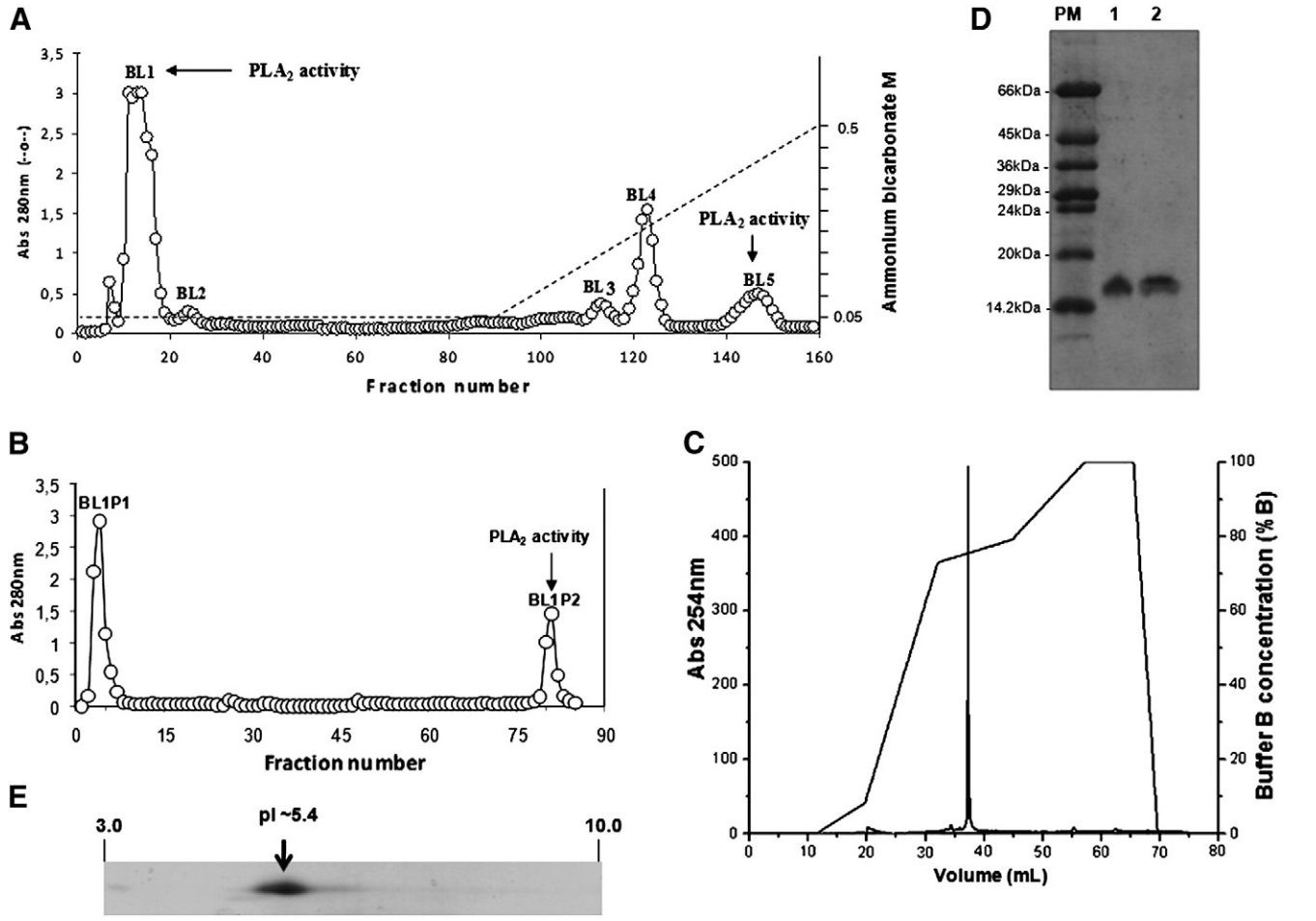

Fig. 1. Sequential purification steps of *Bl*-PLA$_2$. (A) *Bothrops leucurus* venom (86.3 mg) on CM-Sepharose *fast flow*, in 0.05 M ammonium bicarbonate buffer, pH 7.8. A gradient was then applied up to 0.5 M buffer and fractions of 3 mL/tube were collected at a flow rate of 20 mL/h. (B) Rechromatography of the active fraction, named BL1 (55 mg), on a column of Phenyl–Sepharose CL-4B previously equilibrated with 10 mM Tris–HCl buffer, pH 8.5 plus 4 M NaCl at room temperature and then eluted with a decreasing concentration gradient from 4 to 0 M NaCl plus 0.01 M Tris–HCl buffer, pH 8.5, finishing with water. (C) PLA$_2$ isolation (*Bl*-PLA$_2$) on a Shimadzu C18 reverse-phase high-performance liquid chromatography (RP-HPLC) column. (D) SDS-PAGE (14%) of the purified PLA$_2$. Lines: (PM) molecular weight marker, (1) reduced *Bl*-PLA$_2$ (10 μg), (2) non-reduced *Bl*-PLA$_2$ (10 μg). (E) Bidimensional electrophoresis: pI ~ 5.4 for *Bl*-PLA$_2$ was determined using the method previously described (see Methods section).

enzymatically more active (Ketelhut et al., 2003). Several studies have pointed out to a myotoxic site in Lys49-PLA2 enzymes (residues 115–116 to 129) constituted by combination of positively charged and hydrophobic residues in C-terminal region, while non-myotoxic D49 PLA$_2$s present, predominantly, neutral and negatively charged surfaces, which do not permit the approach to negatively charged membranes (Núñez et al., 2001; Chioato and Ward, 2003; Murakami and Arni, 2003). Dos Santos et al. (2009) suggest that following residues Lys20, Lys115, Arg118 and Tyr119, conserved in all bothropic Lys49-PLA$_2$s, are important to myotoxic action and they would be absent in Asp49-PLA$_2$s. Moreover, dimer formation in myotoxic PLA$_2$s results in increase in the area of the positively charged surface, which probably plays an important role in the recognition of the muscle membranes and, consequently, in the expression of myotoxic activity (Murakami and Arni, 2003). Finally, the lack of myotoxic activity may be explained by the access to the active site of the enzyme is through a

Table 1
Percent recoveries (% Rec.), phospholipase A$_2$ and hemolytic activities along the purification procedure of acidic PLA$_2$ from *B. leucurus* crude venom (*Bl*-PLA$_2$).

Sample	Protein[a]		Phospholipase A$_2$ Activity (U/mg)[b]	Hemolytic activity (mm)[c]				
	mg	% Rec		1 h	3 h	6 h	12 h	24 h
CV	86.3	100	69.3 ± 1.7	0	6.03 ± 0.57	10.31 ± 0.59	14.77 ± 0.42	22.06 ± 0.73
Step 1: CM-Sepharose (fraction BL1)	55	63.7	141.2 ± 11.5[d]	0	9,53 ± 0,5[d,e]	14,27 ± 0,05 [d,e]	19,12 ± 0,71[d,e]	28.68 ± 1.24[d,e]
Step 2: Phenyl Sepharose (fraction BL1P2)	4.7	5.4	158.7 ± 11.9[d]	0	9.96 ± 0.4[d]	14.94 ± 1.71[d]	20.77 ± 0.63[d]	34,52 ± 0.93[d]
Purity assay: RP-HPLC (*Bl*-PLA$_2$)	1.0[f]	–	159.9 ± 11.9[d]	0	10.95 ± 0.6[d,e]	16.35 ± 1.64[d,e]	21.03 ± 1.19[d,e]	35.47 ± 1.05[d,e]

[a] Bradford method (Bradford, 1976).
[b] PLA$_2$ activity (de Haas and Postema, 1968).
[c] Indirect hemolytic activity (Gutiérrez et al., 1988) expressed in mm at different time intervals ($n = 3$).
[d] Statistically significant difference ($p < 0.05$) in relation to CV. ([b]) Statistically significant difference between BL1 and *Bl*-PLA$_2$ ($p < 0.05$).
[e] Statistically significant difference between BL1 and *Bl*-PLA$_2$ ($p < 0.05$).
[f] 1.0 mg of BL1P2 was applied on Schimadzu C18 column, just to evaluate the purity assay.

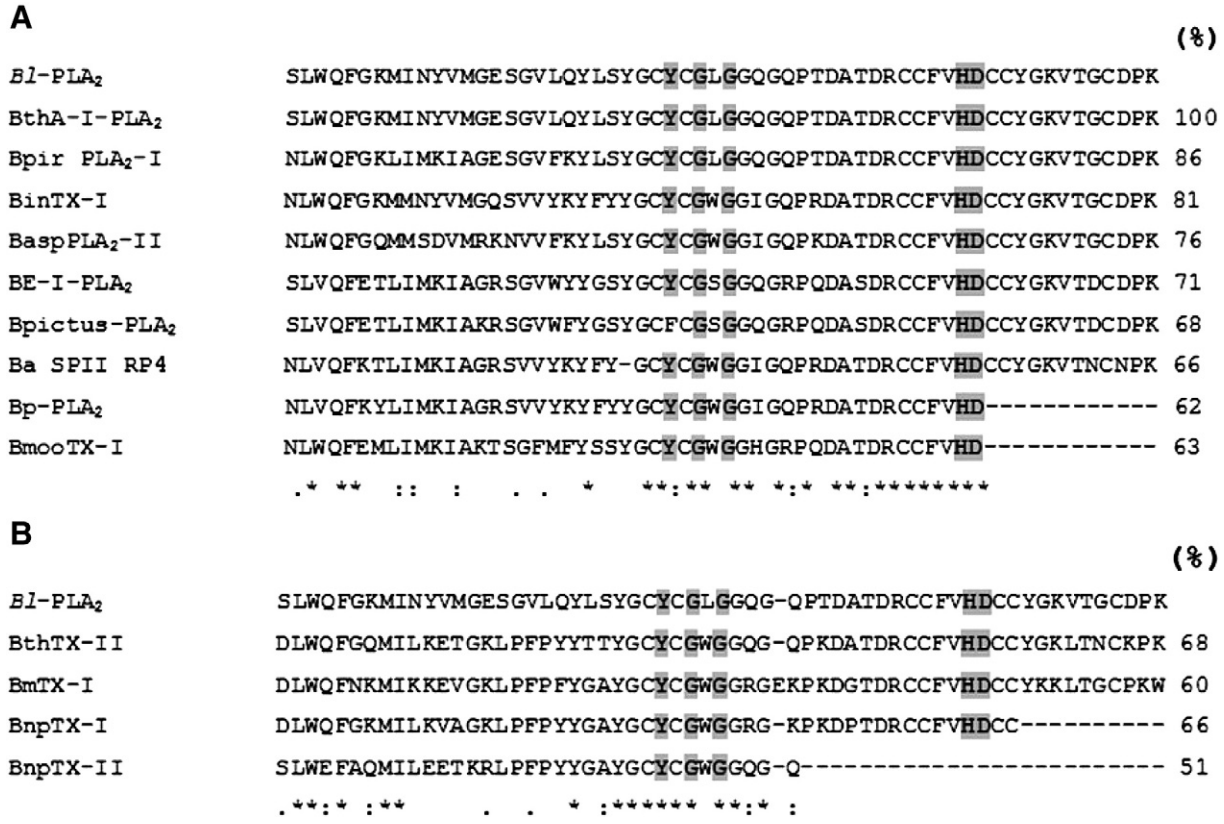

Fig. 2. Comparison of the N-terminal amino acid sequence of *B. leucurus* PLA_2 (*Bl*-PLA_2) with other bothropic Asp49-PLA_2s. (A) Acidic D49-PLA_2s: BthA-I-PLA_2 from *B. jararacussu* (GI: 56554156), Bpir PLA_2-I from *B. pirajai* (GI: 258676481), BinTX-I from *B. insulares* (GI: 26006832), BaspPLA$_2$-II from *B. asper* (GI: 292630844), BE-I-PLA_2 from *B. erythromelas* (GI: 86450426), Bpictus-PLA_2 from *B. pictus* (GI: 9652397), Ba SPII RP4 from *B. alternatus* (GI: 292630845), Bp-PLA_2 from *B. pauloensis* (Rodrigues et al., 2007), BmooTX-I from *B. moojeni* (Santos-Filho et al., 2008). (B) Basic D49-PLA_2s: BthTX-II from *B. jararacussu* (Pereira et al., 1998), BmTX-I from *B. moojeni* (GI:221272396), BnpTX-I e BnpTX-II from *B. neuwiedi* (Rodrigues et al., 2004). Conserved residues involved in calcium binding (Tyr28, Gly30 and Gly32) and in catalytic network (His48 and Asp49) are marked.

narrow channel in the non-myotoxic Asp49 PLA_2, meanwhile this channel is much wider in myotoxic Lys49PLA_2s (Murakami and Arni, 2003; Denegri et al., 2010).

Bl-PLA_2 was able to induce maximum edema in 30 min, compared with 1 h for the crude venom (Table 2). Several studies have demonstrated that the edema onset and the time required to achieve maximum levels vary considerably (Selistre et al., 1990), depending on changes in composition and origin of venoms, as well as differences in species of animals used as experimental model. The mechanism by which catalytically active PLA_2s induce edema may be explained by phospholipid hydrolysis, probably due to liberation of precursors of eicosanoids and platelet activator factors. Liberation of amines by mast cells was proposed as a possible mechanism of edema induction by PLA_2s (Landucci et al., 2000). It is also important to note that the catalytic activity has an important role, but it is not decisive in edematogenic effect, that is, the phospholipases A_2 may have a pharmacological domain independent of the catalytic site (Kini and Evans, 1989; Bonfim et al., 2008), since some Lys49 PLA_2s are able to induce edema (Soares et al., 2000; Oliveira et al., 2009).

Cell viability analysis was carried out to verify whether *Bl*-PLA_2 induces cytotoxic effects in the human PBMC. The cells were treated with different doses (2.5, 5 and 10 μg *Bl*-PLA_2/well) which were shown to be non-cytotoxic (Fig. 3). PBMC cells were used, whereas in previous studies it was found that PLA_2s could recruit leukocytes infiltration, mainly mononuclear cells, and that PLA_2s could induce such cells to release cytokines (Wei et al., 2006b). In this work, for the first time, we showed *in vitro*, the releasing profile of some pro and antiinflamatory cytokines by an acidic PLA_2 from *B. leucurus*. As result, we found that human PBMC stimulated *in vitro* with *Bl*-PLA_2 showed increments in IL-12p40, TNF-α, IL-1β and IL-6 levels in a dose-dependent manner, while no variation for IL-8 and IL-10 were observed (Fig. 4).

Table 2
Myotoxic and edematogenic activities of crude venom from *B. leucurus* and *Bl*-PLA_2.

Sample	CK (U/mL)[a]	Edema (%)[b]						
		0.25 h	0.5 h	1 h	2 h	3 h	6 h	24 h
Control	8.65 ± 0.97	5.79 ± 1.71	6.02 ± 2.79	6.66 ± 2.66	1.78 ± 0.9	1.1 ± 0.64	0.2 ± 0.1	0.2 ± 0.1
CV	55.65 ± 5.07	16.84 ± 4.65	23.16 ± 4.2	24.76 ± 2.96	20.96 ± 4.15	17.6 ± 5.26	15.34 ± 6.25	11.18 ± 4.22
Bl-PLA_2 (20 μg)	10.51 ± 1.79[c]	9.16 ± 2.26	11.92 ± 3.28	4.06 ± 1.22	2.85 ± 0.81	1.3 ± 0.36	0.96 ± 0.28	0.75 ± 0.24
Bl-PLA_2 (50 μg)	14.16 ± 2.69[c]	–	–	–	–	–	–	–

[a] 20 μg of CV and 20 or 50 μg of *Bl*-PLA_2 were injected in the right gastrocnemius muscle of Swiss male mice ($n = 5$). After 3 h, the animals were sacrificed and their blood was collected by cardiac puncture for later analysis of creatine kinase.
[b] 20 μg of CV or *Bl*-PLA_2 were injected in the subplantar region of Swiss male mice ($n = 5$). The increase of edema was measured after different intervals of time (Rodrigues et al., 2001).
[c] Statistically significant difference ($p < 0.05$) in relation to control and crude venom (CV).

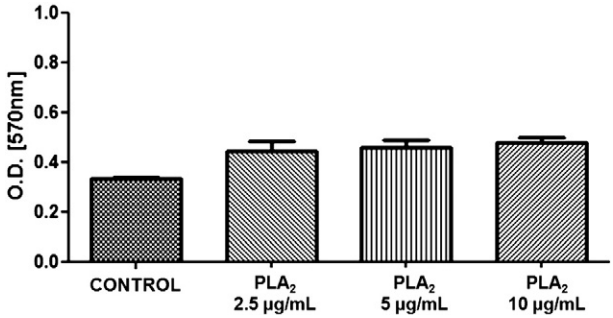

Fig. 3. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] metabolization by 2.0×10^5 peripheral blood mononuclear cells (PBMC) cultured in presence of different concentrations of BI-PLA_2 (2.5, 5.0 and 10.0 µg/mL) during 4 h at 37 °C/5% CO_2.

Inflammation is a major characteristic of envenomation by snakes from Viperinae and Crotalinae species (Rosenfeld, 1971; Sawai, 1980). Raised levels of proinflammatory cytokines have been detected in the serum of human victims or in experimental models following *Bothrops* and *Crotalus* envenoming (Barraviera et al., 1995; Barros et al., 1998; Petricevich et al., 2000; Cardoso et al., 2001; Carneiro et al., 2002; Rucavado et al., 2002; Chavez et al., 2005; Cruz et al., 2005; Zamuner et al., 2005; Zuliani et al., 2005; Cruz et al., 2008).

In their study, Wei et al. (2006b) showed that Promutoxin (Arg49), a PLA_2 isolated from *Protobothrops mucrosquamatus*, was also able to evoke TNF, IL-6 and IL-1β releasing in a dose-dependent manner and increased level of IL-12 in a concentration independent manner. We also observed that BnSP-7, a basic Lys49 myotoxin isolated from *Bothrops (neuwiedi) pauloensis*, was able to induce increments in proinflammatory cytokines, such as IL-6 and IL-1 β, by skeletal muscle cells (Oliveira et al., 2009). Another study testing two basic PLA_2s showed that both MT-II (catalytically inactive) and MT-III (catalytically active) induced increase in IL-1 and IL-6 levels in peritoneal fluid (Zuliani et al., 2005).

Mechanisms involved in the proinflammatory action of snake venoms are being actively investigated and most of this knowledge is based on studies using purified venom PLA_2s. This action can result by the different mechanisms such as enzymatic hydrolysis of phospholipids through the direct release of arachidonic acid from the plasma membrane; activation of intracellular processes in target cells (Zuliani et al., 2005) or interaction of distinct molecular regions in the catalytic site of Lys-49 PLA_2 with cell membranes which induce cell activation leading to an inflammatory reaction (Teixeira et al., 2003; Zuliani et al., 2005).

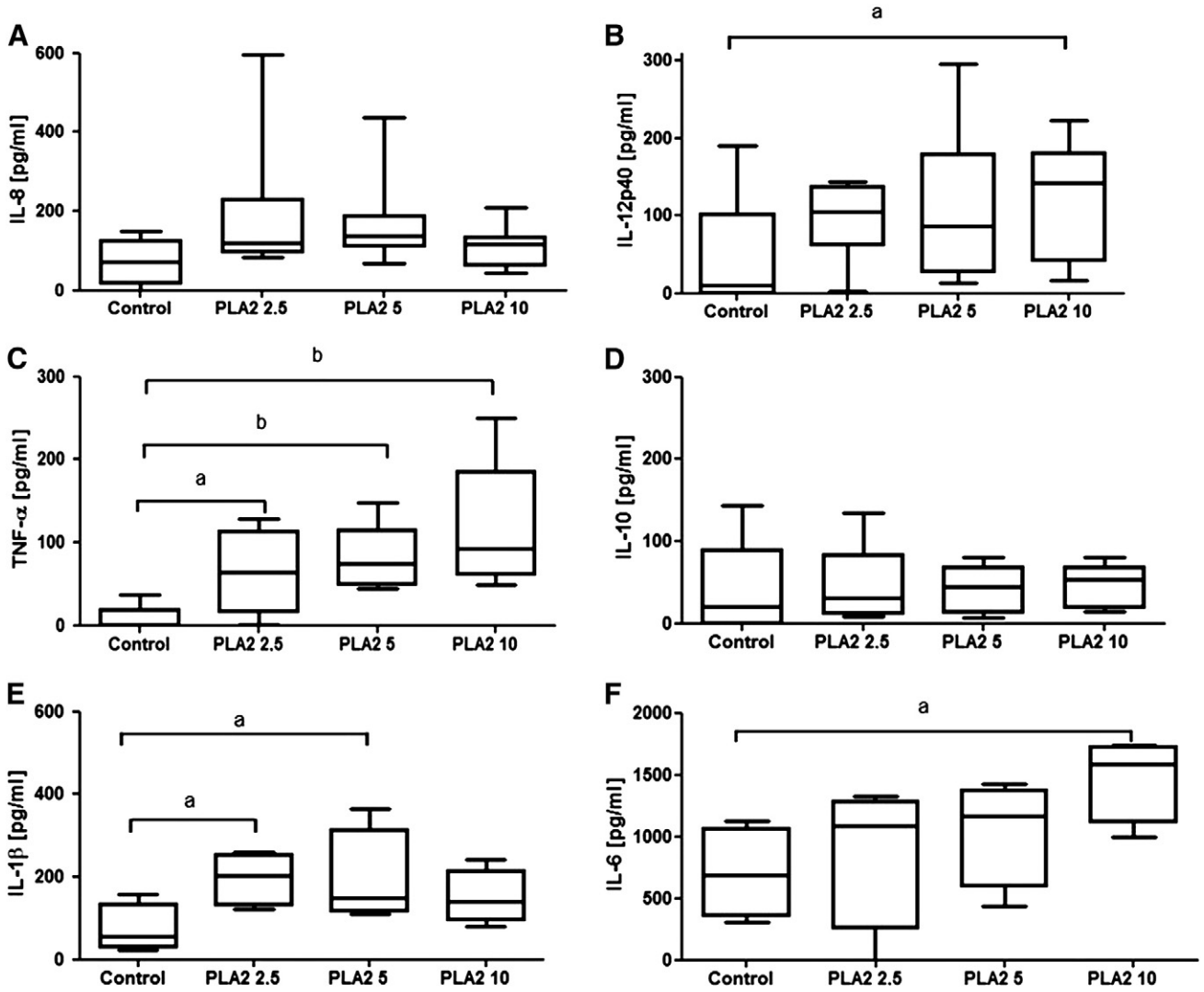

Fig. 4. Cytokines levels on PBMC culture. The production of IL-8 (A), IL-12p40 (B), TNF-α (C), IL-10 (D), IL-1β (E) and IL-6 (F) by PBMC were assayed in supernatants of cells stimulated with different concentrations (2.5, 5 and 10 µg/well) of *Bl*PLA_2. The experiments were performed in triplicate and analyzed statistically by ANOVA or Kruskal–Wallis tests and confirmed by Tukey and Tukey-type tests. ([a]) Corresponds to $p<0.05$ between the experimental groups and control group. ([b]) Corresponds to $p<0.01$ between the experimental groups and control group.

Thus, *Bl*-PLA_2, the newest acidic Asp 49 PLA_2 from *B. leucurus*, becomes a good target for study due to its inflammatory potential and low cytotoxicity. A better understanding of PLA_2 action mechanism in inflammatory process could provide important clues to comprehend the crude venom action and to develop novel therapeutic strategies to treat snakebite envenomation.

4. Conclusion

Bl-PLA_2, the acidic PLA_2 from *B. leucurus* snake venom, was isolated through two chromatographic steps, presenting an excellent purity degree. It possesses an Asp at the site 49, and therefore being classified as D49 PLA_2. *Bl*-PLA_2 did not show myotoxic activity, but induces edema and secretion of some proinflammatory cytokines from highly purified human monocytes, suggesting that it actively participates in the pathogenesis of snake bite.

Conflict of interest statement

No potential conflict of interest.

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