



Purification, sequencing and structural characterization of the phospholipase A₁ from the venom of the social wasp *Polybia paulista* (Hymenoptera, Vespidae)[☆]

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Abstract

The biochemical and functional characterization of wasp venom toxins is an important prerequisite for the development of new tools both for the therapy of the toxic reactions due to envenomation caused by multiple stinging accidents and also for the diagnosis and therapy of allergic reactions caused by this type of venom. PLA₁ was purified from the venom of the neotropical social wasp *Polybia paulista* by using molecular exclusion and cation exchange chromatographies; its amino acid sequence was determined by using automated Edman degradation and compared to the sequences of other vespidae venom PLA₁'s. The enzyme exists as a 33,961.40 Da protein, which was identified as a lipase of the GX class, liprotein lipase superfamily, pancreatic lipases (ab20.3) homologous family and RP2 sub-group of phospholipase. *P. paulista* PLA₁ is 53–82% identical to the phospholipases from wasp species from Northern Hemisphere. The use restrained-based modeling permitted to describe the 3-D structure of the enzyme, revealing that its molecule presents 23% α -helix, 28% β -sheet and 49% coil. The protein structure has the α/β fold common to many lipases; the core consists of a tightly packed β -sheet constituted of six-stranded parallel and one anti-parallel β -strand, surrounded by four α -helices. *P. paulista* PLA₁ exhibits direct hemolytic action against washed red blood cells with activity similar to the Cobra cardiotoxin from *Naja naja atra*. In addition to this, PLA₁ was immunoreactive to specific IgE from the sera of *P. paulista*-sensitive patients.

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Keywords: Wasp venom; Phospholipase; Immunoreactivity; IgE; Molecular modeling; Hymenoptera; Hemolysin; Allergen

[☆]**Ethical statement:** The authors certify that the protocol no. 006/06 about “Investigations of structural and functional activities of proteic toxins social wasp venoms” agrees with ETHICAL PRINCIPLES IN ANIMAL RESEARCH adopted by Brazilian College of Animal Experimentation (COBEA) and was approved *Ad referendum* in that date by the BIOSCIENCE INSTITUTE/UNESP ETHICAL COMMITTEE FOR ANIMAL RESEARCH (CEEa).

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1. Introduction

Vespid venoms are known to cause sting allergy and even death in humans and animals (Müller, 2002). Ants, bees and wasps present unique as well as homologous venom allergens when compared to each other (King and Spangfort, 2000). Immunological studies on vespid venoms have shown that the most important allergenic toxins from these venoms are phospholipases, hyaluronidases, antigens 5 and acid phosphatases (Hoffman, 1978; King, 1980).

PLAs are relatively common in Hymenopteran venoms, occurring mainly as -A2 and -B types (Abe et al., 2000). The PLA₁ and PLA₂ catalyze the specific hydrolysis of ester bonds of 1,2-diacyl-3-*sn*-glycerophospholipids, at the positions *sn*-1 and *sn*-2, respectively, converting these substrates into their corresponding lyso compounds with the release of fatty acids. Thus, the PLAs are able to disrupt the phospholipid packings from several types of biological membranes, leading to pore formation and/or cell lysis (Dotimas and Hider, 1987).

In contrast to the PLA₂ isozymes, the physiological function (s) of PLA₁ molecules remain unknown. Presently extra- and intracellular PLA₁'s from different organisms are known; these enzymes share no sequence homologies and probably must have distinct functions (Aoki et al., 2007).

Apparently, some PLA₁'s have a broad substrate specificity and hydrolyze both phospholipids and triacylglycerols (TG) as well as galactolipids. In contrast, other PLA₁'s show strict substrate specificity and act specifically on phosphatidylserine (PS) and phosphatidic acid (Aoki et al., 2007).

When compared with each other, PLA₁'s and PLA₂'s also present no sequence homologies and apparently have distinct functions. PLA₂'s were characterized in honeybees (Kuchler et al., 1989; Müller et al., 1995) and in some neotropical social wasps (Oliveira and Palma, 1998; Costa and Palma, 2000), while PLA₁ have been partially characterized in some wasp species from northern hemisphere (King et al., 1984, 2003) and ants (Hoffman et al., 2005). The PLA₂ from social wasp venoms seems to be glycoproteins, occurring in many different oligomeric forms and presenting high hemolytic activities (Oliveira and Palma, 1998; Costa and Palma, 2000). Meanwhile, the PLA₁ are generally associated to allergic and inflammatory processes, but are not well characterized biochemically (King et al., 2003; Moawad et al., 2005).

The Brazilian fauna of social wasps is constituted of almost 500 species, from whom very little is known about their venom biochemistry, pharmacology and immunology. *Polybia paulista* is a neotropical social wasp endemic from Southeast Brazil, causing many stinging accidents every year in this region of country. The future development of a vaccine against venom allergy and/or an antivenom to prevent toxic reactions caused by wasp stings will require as a prerequisite a complete structural information about the most important proteins of these venoms. In this study, we have purified, sequenced, modeled and characterized biochemically a PLA₁ from the venom of the neotropical social wasp *P. paulista*.

2. Methods

2.1. Venom collection

Workers of *P. paulista* were captured in the University Campus, at Rio Claro, SP, southeast Brazil. The freshly collected wasps were immediately frozen and dissected. The venom reservoirs were removed from the sting apparatus by pulling them out of bodies with forceps. The venom reservoirs were then carefully washed and suspended in small volumes of a solution of protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 2 μ M Leupeptine), thawed, punctured, washed several times with the protease inhibitors solution to extract the venom and centrifuged at 10,000g for 10 min at 4 °C. The supernatants were collected, lyophilized and maintained at -80 °C until use.

2.2. Blood sera

Sera from five *P. paulista*-sensitized patients were obtained in the Division of Clinical Immunology and Allergy from the Hospital of General Clinics of University of São Paulo Medical School, São Paulo, Brazil, with the approval of the São Paulo University Ethics Board. The sera were collected before starting the specific immunotherapy treatment of each patient and stored at -20 °C until used.

2.3. Protein assay

Protein was determined by the method of Bradford (BioRad), using bovine serum albumin as standard (Bradford, 1976).

2.4. Protein purification

The freeze-dried venom (40 mg) from approximately 1500 wasps was solubilized in 5 mM ammonium acetate pH 6.8 and submitted to a size-exclusion chromatography in a Sephacryl S100 column (60 cm × 26 mm, 320 mL—Amersham Biosciences) coupled to an ÄKTA-FPLC system. The column was previously equilibrated and eluted with the same solubilization solution. Fractions of 4 mL were collected at a flow rate of 1 mL/min; the elution was monitored by measuring the absorbance at 280 nm and PLA activity was assayed as described below. The fractions presenting PLA activity were pooled, lyophilized, dialyzed against distilled water and analyzed for protein homogeneity.

Thus, the recovered protein content (4.3 mg) from the size-exclusion chromatography was solubilized in 50 mM sodium acetate buffer pH 5.2 and submitted to cation exchange chromatography in a HiPrep FF CM column (160 mm × 10 mm, 20 mL—Amersham Biosciences) coupled to an ÄKTA-FPLC system. The column was previously equilibrated with the same solubilization buffer and the elution was performed under isocratic elution with 50 mM sodium acetate buffer pH 5.2 for the first 20 min, and then under a linear gradient from 0 to 1 N sodium chloride in the same buffer, from 20.01 to 122.00 min at a flow rate of 1 mL/min; fractions of 1 mL were collected. Elution was monitored by measuring the absorbance at 280 nm and PLA activity was assayed as described below. The acquisition and treatment of the chromatographic data were carried out by using UNICORN software. The fractions presenting PLA activity were pooled, lyophilized, dialyzed against distilled water and submitted to SDS-PAGE, ESI-MS analysis and to automated amino acid sequencing by Edman Degradation.

2.5. Phospholipase activity

The assays were carried out by using a spectrophotometric method based on pH change due to the delivering of fatty acids, as described elsewhere (Araujo and Radvanyi, 1987). The reaction medium contained 15 μmoles phosphatidylcholine, 18 μmoles Triton-X, 5 μmoles calcium chloride, 80 nmoles phenol red and 7.5 μmoles Tris in a final volume of 2.5 mL, at pH 7.9. The absorbance was measured at 558 nm. The reaction was initiated by the addition of protein fractions/crude venom and the

decrease in the absorbance of phenol red, caused by the acidification of medium, was measured after 5 min of incubation at 37 °C. The absorbance was proportional to the liberation of fatty acids in the assay conditions. One unit of PLA activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol phosphatidylcholine/h in 1 mL of the reaction medium at pH 7.9 and 37 °C.

2.6. Determination of phospholipase specificity

In order to determine the type of phospholipase activity, the crude venom of *P. paulista* and/or its fractions were incubated at 37 °C in presence of 1-stearoyl-2-oleoyl-3-*sn*-glycerophosphorylcholine and 1-oleoyl-2-stearoyl-3-*sn*-glycerophosphorylcholine (Sigma Chem. Co.). Fine suspensions of phospholipids were prepared by sonication in a medium constituted of: 1 mM Tris-HCl (pH 7.9), containing 10 mM sodium chloride, 20 mM potassium chloride, 10 mM calcium chloride and 0.5% (v/v) Triton X-100. For the purpose of identification of products formed by phospholipase digestion, the same buffer described above was used, but the concentration of Tris-HCl was increased to 50 mM. The digests were examined by thin-layer chromatography (TLC) on silica gel plates (Whatman LK6DF) as described by King et al. (1984): 35 μL phospholipids suspension (1.5 mg/mL) was applied to the preadsorbent zone and the plate was developed in chloroform/methanol/0.1 N HCl (60:35:5).

The spots were visualized by exposure to iodine vapor for detection of monoacyl phospholipids. The digests were also examined directly for the presence of saturated fatty acids by chromatography on freshly prepared silica gel plates which had been dipped in 5% (w/v) silver nitrate and then dried at 110 °C for 1 h. After developing in presence of hexane/diethylether/acetic acid (70:30:1), the spots were visualized under UV light after spraying with 0.2% (w/v) dichlorofluorescein in ethanol.

2.7. Electrophoresis

To compare the protein profiles of the enzyme purified in each chromatographic step, SDS-gel electrophoresis was performed by using 12.5% (w/v) Tris-Glycine gel. Thirty micrograms of protein were loaded per lane. The molecular weight markers kit used was BenchMarkT Protein Ladder (Invitrogen); it consists of recombinant proteins ranging in MW

from 10 to 190 kDa. The gel was stained with Coomassie Brilliant Blue (CBB) (Sigma Chem. Co.) and destained by using a solution containing 10% (v/v) methanol and 10% (v/v) acetic acid. Two-dimensional (2-D) SDS-PAGE was performed as briefly described: IEF was run on an IPG-phor system (Amersham Pharmacia Biotech). Liophilized venom protein samples (400 µg) were mixed with a rehydration solution containing 8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Trisbase, 0.5% V/V IPG buffer, 18 mM DTT and traces of bromophenol blue to give a total volume of 350 µL and applied to IPG dry strips (pH 3–10; GE Healthcare). After rehydration for 16 h, IEF was carried out by applying 500 V for 1 h; 1000 V for 1 h; and 8000 V for 6 h at 50 mA/strip. After focusing, the strips were soaked for 20 min in reduction solution (6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 125 mM dithiothreitol) followed by 20 min in alkylation solution (6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 125 mM iodoacetamide). The SDS-PAGE step was performed in 12.5% (w/v) polyacrylamide gels run on a Ruby system (GE Healthcare) at 15 °C. After 2-DE, gels were stained with CBB G250 and scanned using Image Master System (GE Healthcare).

2.8. Western blotting

After electrophoresis, the gel was soaked in transfer buffer Towbin (189 mM glycine, 10 mM SDS, 10% (v/v) methanol and 20 mM Tris pH 11.0) for 5 min at 25 °C. During this time a polyvinylidene difluoride (PVDF) membrane was rinsed with 100% methanol and stored in the transfer buffer. The gel was assembled into a blotting apparatus (Semi-Dry TE-77-GE Healthcare) and electroeluted for 1 h with a current of 100 mA. The PVDF membrane was then washed in deionized water for 5 min, stained with 0.1% (w/v) CBB in 40% (v/v) methanol for 1 min and destained with 50% (v/v) methanol for 5 min. The membrane was finally rinsed in deionized water for 5 min and air dried.

2.9. Immunoblotting

After the electrotransference, the intact PVDF membranes were incubated with 5% (w/v) nonfat dry milk in PBS buffer under shaking overnight. The membranes were then incubated for 2 h with individual samples of sera from *P. paulista* venom-sensitive patients. After this step, they were incu-

bated with a 1/4000 dilution of primary antibody Biotin (Zymed) in PBS buffer with 5% (w/v) nonfat milk for 2 h. The PVDF membranes containing the immunoblots were incubated for 2 h with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (1/8000 in PBS buffer with 5% nonfat milk, Zymed). The blotted protein spots were detected by chemiluminescence using ECL Advanced Western blotting kits (GE Healthcare). The images were acquired by autoradiography enclosing the membranes with Hyperfilm ECL (10 × 10 cm²; GE Healthcare) into Hypercassettes (GE Healthcare), which were subjected to multiple exposure times (from 30 s to 3 min), according to the instructions of manufacturer.

2.10. Mass spectrometry

Mass spectra were acquired on a triple quadrupole (Quatro II) mass spectrometer instrument (Micromass, UK), equipped with a standard electrospray probe, adjusted to ca. 5 µL/min. During all experiments, the source temperature was maintained at 80 °C and the needle voltage at 3.6 kV, applying a drying gas flow (nitrogen) of 200 L/h and a nebulizer gas flow (nitrogen) of 20 L/h. The mass spectrometer was calibrated with intact horse heart myoglobin and its typical cone-voltage induced fragments. The cone sample to skimmer lens voltage, controlling the ion transfer to the mass analyzer, was maintained at 30 V. About 50 pmol of each sample was injected into electrospray transport solvent. The ESI mass spectra were obtained in the continuous acquisition mode, scanning from *m/z* 100 to 2000 with a scan time of 7 s.

2.11. Amino acid sequencing by Edman degradation

N-terminal sequence analysis of intact PLA₁ and its proteolytic fragments were carried out with pulse liquid-phase microsequencer (Shimadzu, mod, PPSQ-21) operating standard programs. PLA₁ was reduced in presence of 8 mol/L urea, 0.15 mol/L Tris-HCl and 2.5 mmol/L 1,4-dithiothreitol, pH 8.7 and alkylated (when necessary) by using 7.0 mmol/L iodoacetate and then desalted on a C8 reversed-phase column. *P. paulista* PLA₁ was incubated with trypsin (proteomics grade, Promega) or α-chymotrypsin (Sigma Chem. Co., USA) for 18 h at 37 °C in presence of 25 mmol/L ammonium bicarbonate pH 7.9; proteolytic fragments were separated by means of HPLC (C₈ column, 5 µm, 4.6 × 150 mm²;

Wakosil, Japan); individual fractions were collected, lyophilized, solubilized in bidistilled water and submitted to amino acid sequencing by using automated Edman degradation chemistry.

2.12. Molecular modeling

For molecular modeling, the PLA₁ from *P. paulista* was used for the restrained-based modeling approach as implemented in the program MODELLER, which is an automated approach for comparative modeling by satisfaction of spatial restraints (Sali, 1995). The modeling procedure began with an alignment of the protein sequence to be modeled (target) with related known three-dimensional (3-D) protein structure used as template (guinea pig lipoprotein lipase-1GPL (Carriere et al., 1997)). This alignment is the input to the program. The output is a 3-D model for the target sequence containing all main-chain and side-chain nonhydrogen atoms. Several slightly different models can be calculated by varying the initial structure. A total of 1000 models were generated by the program Parmodel (Uchôa et al., 2004); the final model was selected based on stereochemical quality. The overall stereochemical quality of the final models was assessed by the program PROCHECK (Laskowski et al., 1993). All optimization processes were performed on a Beowulf cluster with 16 nodes (Bio-Comp, AMD Athlon XP 2100+). The overall stereochemical quality of the final models of *P. paulista* PLA₁ (Poly p1) was assessed by the program PROCHECK (Laskowski et al., 1993).

The Root Mean Square Deviation (RMSD) differences from ideal geometries for bond lengths and bond angles were calculated with X-PLOR (Schwieters et al., 2003). The G-factor values were calculated using PROCHECK (Laskowski et al., 1993). The Verify-3D measures the compatibility of a protein model with its sequence, using a 3D profile (Kabsch and Sander, 1983).

2.13. Hemolysis

Washed rats red blood cells (WRBC) were used to evaluate the hemolytic activity of the enzyme. WRBC were prepared by washing 50 mL of Wistar rats red blood cells suspension 3 times with physiological saline solution [0.85% (w/v) sodium chloride and 10 mM calcium chloride] and suspending the cells in 50 mL of the same solution. Aliquots of WRBC were then incubated at 37°C in the

presence of *P. paulista* PLA₁ (final concentration of 100 µg/mL) for 120 min, with gentle mixing. Samples were then centrifuged, and the absorbance of the supernatants was measured at 540 nm. The absorbance measured from lysed WRBC in presence of 1% (v/v) Triton X-100 was considered as 100%. Results are expressed as means ± SD of five experiments.

3. Results

3.1. Purification of the phospholipase A₁

In order to purify this protein, the crude venom of *P. paulista* was initially submitted to a size-exclusion chromatography in Sephacryl S-100 column; eight peaks of proteins were eluted and assigned from I to VIII as shown in Fig. 1. The PLA activity was assayed all over the chromatographic profile and it was concentrated in peak III (Table 1); fractions 45–55 which contained about 89% of the PLA activity originally present in the crude venom were pooled from peak III, lyophilized and submitted to refractionation through cation exchange chromatography in a Hiprep FF CM column at pH 5.2. The chromatographic profile (Fig. 2) showed eight peaks, which were assigned from A to H and the PLA activity was assayed through all the protein profiles; the activity was associated to the peaks A, B and E, which contained about 29%, 24% and 27%, respectively, of the total PLA activity present in the crude venom.

The fractions corresponding to each one of these peaks were pooled, dialyzed against distilled water, lyophilized and submitted to an SDS-PAGE. Peak III of size-exclusion chromatography (Fig. 1) was further purified by cation exchange chromatography (Fig. 2); apparently, the molecular masses of the proteins in peak III would be expected to be nearly the same, since they are from the same fraction of size-exclusion chromatography. However, after the cation exchange chromatography (Fig. 2) the migration distances of the bands in SDS-PAGE (Fig. 3) indicate that these proteins are largely distributed through a wide series of MW values from 10 to 30 kDa. These results suggest that these proteins form large aggregates in solution which cannot be separated by size-exclusion chromatography.

Despite containing a significant amount of PLA activity recovered from the crude venom, the peaks A and E are far from being pure, as may be

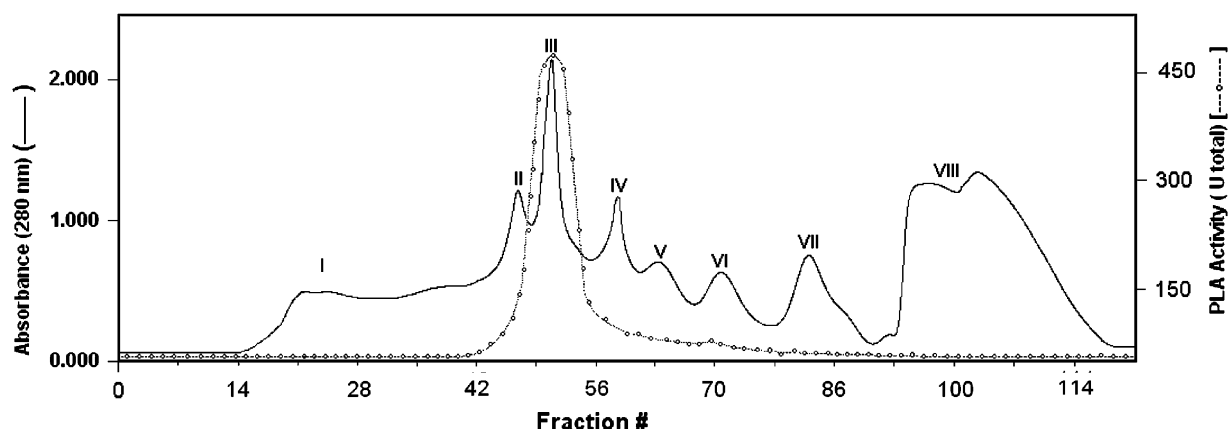


Fig. 1. Size-exclusion chromatography profile of the crude venom of the wasp *P. paulista* carried out in a Sephacryl S-100 column (60 cm × 26 mm, 320 mL—Amersham Biosciences). Elution was performed with 5 mM ammonium acetate pH 6.8 and fractions of 4 mL were collected at a flow rate of 1 mL/min at 4 °C. Protein elution was monitored at 280 nm (—) and PLA activity (—○—) through the color shifting of the phenol red.

Table 1

Records of protein and PLA activity recovery at the different steps of purification protocol

Step	Vol (mL)	Total protein (mg)	Phospholipase activity (U/mL)	Total activity (U_{Total}) ^a	Recovery of activity (%)	Specific activity (U/mg)	Purification
Crude venom	2	40	2250	5500	100	137	—
Molecular exclusion chromatography (Sephacryl S-100)	40	4.3	123	4920	89	1144	8.3
Cation exchange chromatography (Hiprep FF CM)							
Fraction A	14	0.94	114	1600	29	1702	12.4
Fraction B	8	0.45	163	1304	24	2898	21.0
Fraction E	20	1.10	75	1500	27	1363	9.9

^a U_{Total} = nmol/mL.

observed in Fig. 3; however, this figure also shows that peak B seems to be highly homogeneous, since it presents a single protein band, corresponding to MW of 34 kDa (Fig. 3). In order to confirm the homogeneity of this preparation, the purified protein was analyzed through ESI-MS analysis, revealing a single protein component with a molecular mass of 33,961.40 Da (not shown result). An analysis of the purification records of Table 1 reveals that taking into account the peaks A, B and E a total of 80% of the PLA activity was recovered after the two chromatographic steps. However, only peak B may be considered pure and it contains 24% of the total PLA activity; the enzyme present in this fraction was purified 21-fold in relation to the crude venom. Taking into account the protein recovery after the two chromatographic steps, the purified

PLA must correspond to 1.1% of *P. paulista* venom.

3.2. PLA specificity

The assay of PLA activity used in the present investigation, based on the spectrophotometric monitoring of the pH change through the color shifting of the phenol red, due to the delivering of fatty acids during the reaction (Araujo and Radvanyi, 1987), does not consider the identification of the end products formed by the enzyme digestion. Thus, despite detecting the PLA activity, this method alone does not permit to distinguish between -A1 and -A2 types of hydrolysis. This way, at least theoretically, the purified PLA would be related to PLA₁, to PLA₂ or would even correspond to a

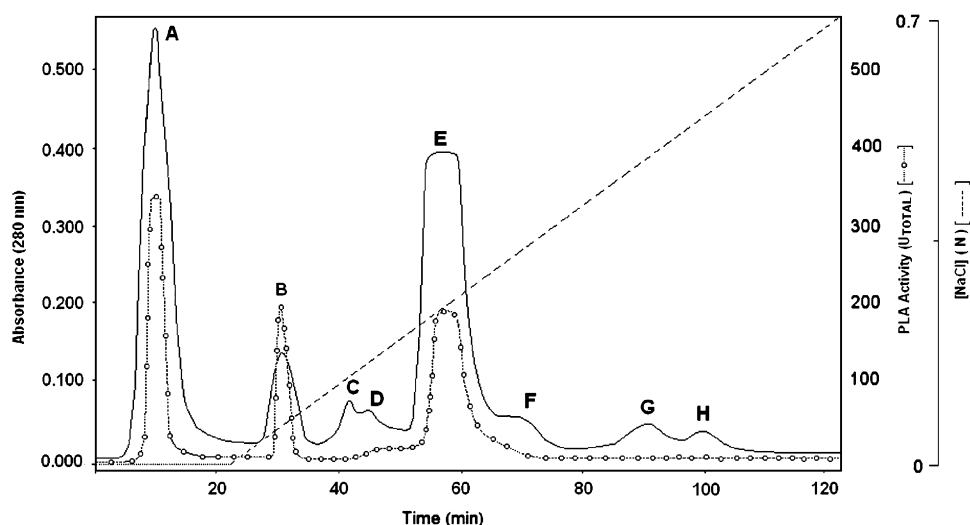


Fig. 2. Cation exchange chromatography profile of the fraction containing PLA activity (peak III) obtained in the size-exclusion chromatography in Sephacryl S100. The fractionation was carried out in a Hiprep column FF CM (160 mm × 10 mm, 20 mL—GE Healthcare) previously equilibrated and eluted until the first 20 min with 50 mM sodium acetate buffer pH 5.2, and then under a linear gradient from 0 to 1 N NaCl in the same buffer from 20.01 to 122.00 min; fractions of 2 mL were collected at a flow rate of 1 mL/min at 4 °C. Protein elution was monitored at 280 nm (—) and PLA activity (—○—) through the color shifting of the phenol red; (-----) represents the NaCl gradient.

mixture of both enzymes. In order to solve this problem, the purified enzyme was incubated with two different PLA substrates, 1-stearoyl-2-oleoyl-3-*sn*-glycerophosphorylcholine and 1-oleoyl-2-stearoyl-3-*sn*-glycerophosphorylcholine. The qualitative estimation of the products resulting from these incubations through TLC, after 4 h of digestion of 1-stearoyl-2-oleoyl-3-*sn*-glycerophosphorylcholine with the purified enzyme, revealed only the presence of stearic acid as product of enzyme hydrolysis; no oleic acid was detected by using this substrate. Meanwhile, the incubation of the enzyme with 1-oleoyl-2-stearoyl-3-*sn*-glycerophosphorylcholine as substrate resulted only in the production of oleic acid as product of reaction; no stearic acid was detected as product of this digestion (results not shown). Apparently, these results indicate that the cleavages of both substrates occurred at the positions *sn*-1, suggesting that the enzyme purified as described above must correspond to a PLA1-type (E.C. 3.1.1.32).

Since this protein was pure enough to be sequenced, it was reduced, alkylated and submitted to Edman degradation sequencing, resulting in an N-terminal sequence of 23 amino acid residues (LIPECPFNEYDILFFVYTRQQRD), which in turn was analyzed by the protein engine search tool BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>),

indicating a potential PLA₁ from vespid venoms. This result provided an additional evidence of PLA identity.

3.3. Amino acid sequencing

The unequivocal identification of this protein would come from its primary sequence; thus, the complete amino acid sequence of *P. paulista* PLA₁ was determined by means of N-terminal sequencing. This way, different overlapping internal fragments were obtained from the proteolytic cleavage of the purified protein with trypsin and chymotrypsin; the resulting fragments were collected by using HPLC purification with a C₈-reversed-phase chromatography. Twenty-four tryptic fragments and 25 chymotryptic fragments were collected and sequenced by automated Edman degradation chemistry. Fig. 4 shows the pattern of proteolytic fragments obtained and sequenced, whose the overlapping covered all the protein sequences. When the complete amino acid sequence shown in Fig. 4 was submitted to BLAST analysis an unequivocal identification of a vespid venom PLA₁ was obtained.

ESI-MS analysis of the purified enzyme revealed a molecular mass of 33,961.40 Da, which seems to fit very well with the theoretical mass (33,962.53 Da) for the sequence shown in Fig. 4.

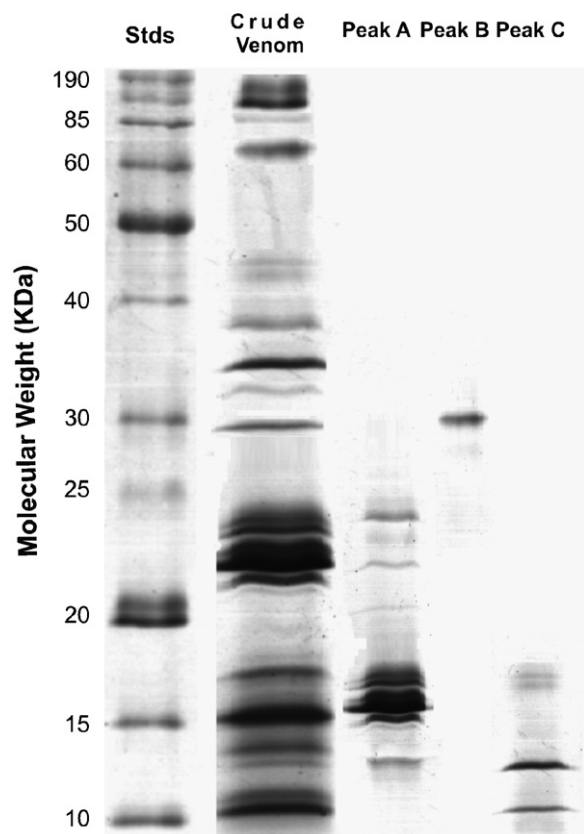


Fig. 3. CBB-stained SDS-PAGE showing the protein profiles of the standard MW markers (Stds), the crude venom of *P. paulista* and the peaks A, B and E from the cation exchange chromatography. Peak B corresponds to the purified PLA fraction.

The primary sequence of this PLA₁ presents 13 cysteine residues and the comparison of the molecular masses obtained by ESI-MS between untreated protein versus fully reduced and alkylated protein, indicated that 12 of these residues seem to be forming disulfide bridges among them, while a single free thiol group was detected (results not shown). Certainly, the formation of six disulfide bridges must contribute to the structural rigidity of *P. paulista* PLA₁, rendering it resistant to mild conditions for reduction and/or alkylation.

The alignment of the sequence of *P. paulista* PLA₁ (Poly p1) with 12 other known lipases, including some vespid phospholipases is shown in Fig. 5. The enzyme sequences shown in this figure are used as references for different sub-types of lipases: human PS-specific PLA₁, guinea pig phospholipase (PLRP2), human pancreatic lipase (PL), human endothelial cell-derived lipase (EDL), hu-

man lipoprotein lipase (LPL) and human hepatic lipase (HL); some vespid venoms PLA₁'s were included in this comparison and mentioned here with their respective allergens nomenclature: *Vespula germanica* (Ves g1), *Vespula maculifrons* (Ves m 1), *Vespula vulgaris* (Ves v 1), *Dolichovespula maculata* (Dol m1.01), *Polistes anularis* (Pol a 1) and *Polistes dominulus* (Pol d 1.01). *P. paulista* enzyme presents 63% identity with Ves v 1, 64% with Ves m1, 60% with *V. vulgaris* PLA₁, 53% with Dol m 1.01, 80% with Pol a1 and 82% with Pol d 1.

3.4. Investigating the presence of carbohydrates attached to the protein

The purified enzyme was submitted to the detection of carbohydrates attached to the protein molecule through the use of a fluorescent probe (Pro-Q Emerald 300 from Molecular Probes) which reacts with glycans of gel-separated proteins after oxidation to aldehydes. *P. paulista* PLA₁ did not present any carbohydrate staining (not shown results), suggesting that this enzyme is not a glycoprotein. In addition to this, the molecular mass of the enzyme obtained through ESI-MS analysis revealed a value of 33,961.40 Da, which fits very well with the theoretical mass (33,962.53 Da) for the sequence shown in Fig. 4, corroborating the hypothesis that *P. paulista*-PLA₁ is not a glycoprotein, since the carbohydrates attaching to the protein should have a higher value of molecular mass compared to the theoretical value.

3.5. Molecular modeling

Since PLA₁'s are important toxins in the venoms of the social wasps, it is necessary to make a complete structural characterization of them; thus, it was decided to perform a molecular modeling of *P. paulista* PLA₁. The homologous enzymes to the target PLA₁ were identified by using a PSI-BLAST (Schäffer et al., 2001) search in the Protein DataBase (<http://www.rcsb.org/pdb/>). *P. paulista* PLA₁ presents 32% identity and 46% similarity with the primary sequence of the selected template protein (guinea pig lipoprotein lipase; 1 GPL). The percentage of amino acids identity indicates that the crystallographic structure was a good model to be used as template for the target. The atomic coordinates of all water molecules were removed from the template (1 GPL) and this was solved to a resolution of 2.01 Å.

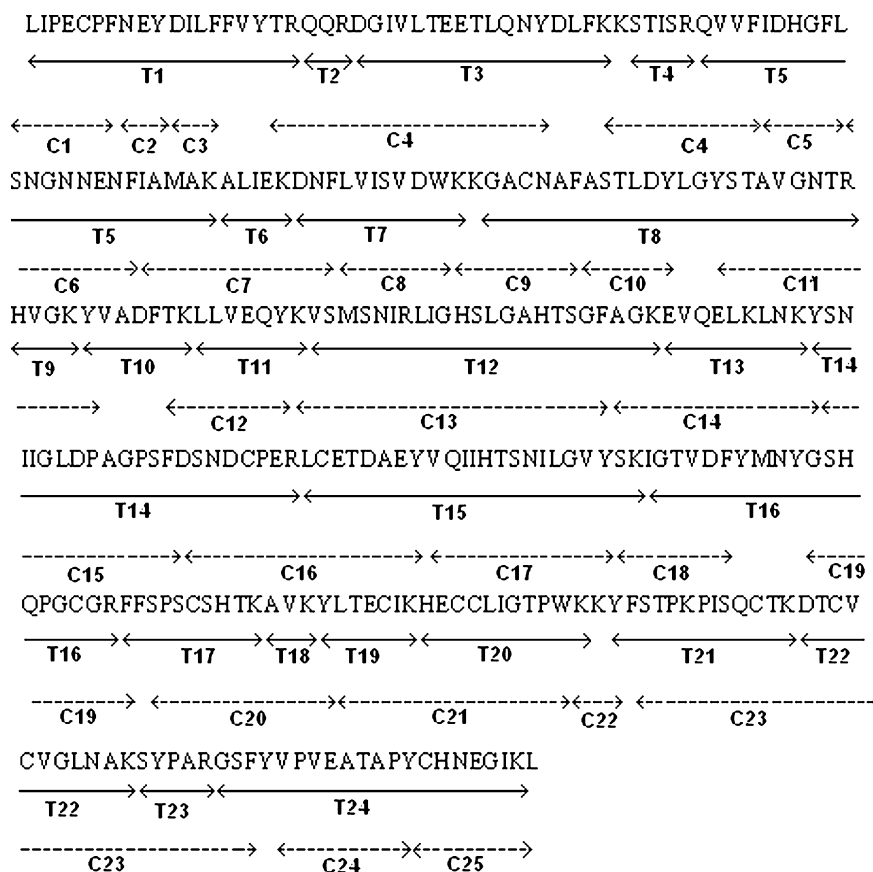


Fig. 4. Primary structure of the PLA₁ from the venom of the netropical wasp *P. paulista* obtained from the overlapping of the sequences of the peptides generated by trypsin (↔) and chymotrypsin (← -- →) digestions of the purified protein. The sequences were obtained by using automated Edman degradation chemistry.

The structural model for this allergen was analyzed by using the Procheck software (Schwitters et al., 2003) to estimate the secondary structure content: 23% α -helix, 28% β -sheet and 49% coil. The Ramachandran diagram of Φ - Ψ plots for the template and model indicates that over 90% of the residues are in the most favorable regions; the analysis of the structural quality of the homology model was performed by using PROCHECK (for Ramachandran diagram Φ - Ψ plots and G -factor), XPLOR (for RMSD from ideal geometry) and VERIFY-3D, which strongly indicated that the model was good enough for structural studies.

The ribbon diagram showing the structure of *P. paulista* PLA₁ (Fig. 6) reveals that this enzyme belongs to the α/β hydrolase fold common to many lipases/esterases; the core consists of a tightly packed β -sheet constituted of six-stranded parallel- and one anti-parallel β -strand, surrounded by four α -helices.

3.6. Hemolytic activity

The hemolytic activity of *P. paulista* PLA₁ was assayed and compared with other hemolytic toxins from honeybee, wasps and snakes venoms as shown in Table 2. *P. paulista* PLA₁ is not a potent hemolysin like the Polybitoxins (PbTX) from the same venom; it is important to emphasize that the PLA₁ caused direct lysis of membrane phospholipids per se, i.e., without combination with any other component.

3.7. Immunoreactivity to specific IgE

Since the venoms of social wasps are frequently associated to immunological reactions, the immunoreactivity to specific IgE was evaluated by submitting the purified protein to immunoblotting with the sera of *P. paulista*-sensitive patients. Fig. 7 shows a typical result of this biological

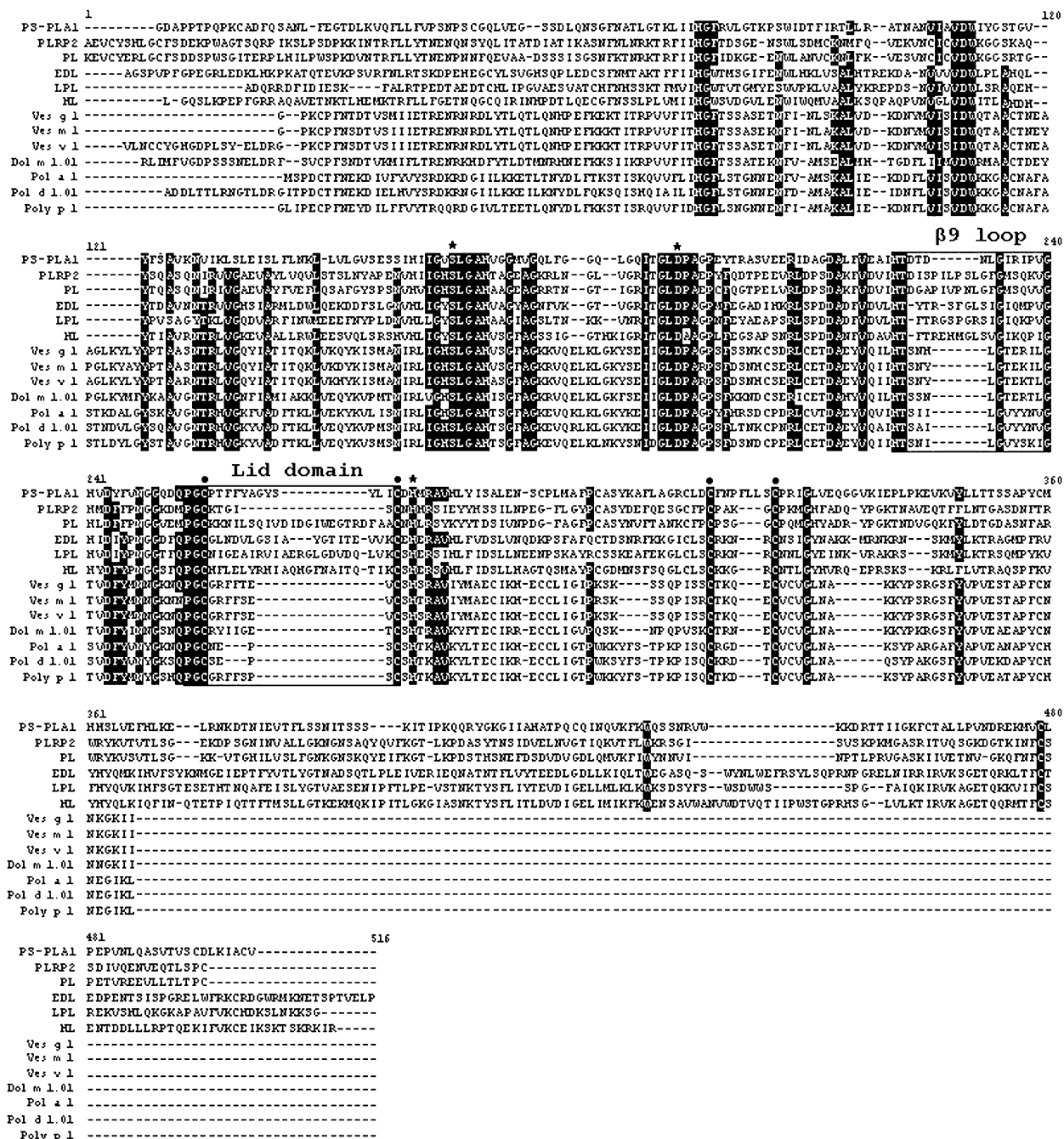


Fig. 5. Sequence alignment of *P. paulista* PLA₁ with other vespid venom PLA₁'s. Shadowed areas assign the most conserved regions/residues. The asterisks indicate the amino acid residues of the catalytic triad: Ser 165, Asp 194 and His 281. The lid domain and the β 9 loop are indicated in the boxes. The enzyme sequences used as references were: human phosphatidylserine (PS)-specific PLA₁, guinea pig phospholipase (PLRP2), human pancreatic lipase (PL), human endothelial cell-derived lipase (EDL), human lipoprotein lipase (LPL) and human hepatic lipase (HL). Some allergenic PLA₁'s from vespid venoms were also included in this comparison: *Vespa germanica* (Ves g1), *Vespa maculifrons* (Ves m 1), *Vespa vulgaris* (Ves v 1), *Dolichovespula maculata* (Dol m1.01), *Polistes anularis* (Pol a 1), *Polistes dominulus* (Pol d 1.01).

characterization, where the position corresponding to the purified PLA₁ is coincident with the IgE-immunoreactive protein in the immunoblots. A

control constituted of a negative human serum from a nonsensitive individual and was included in this immunoblotting in order to permit

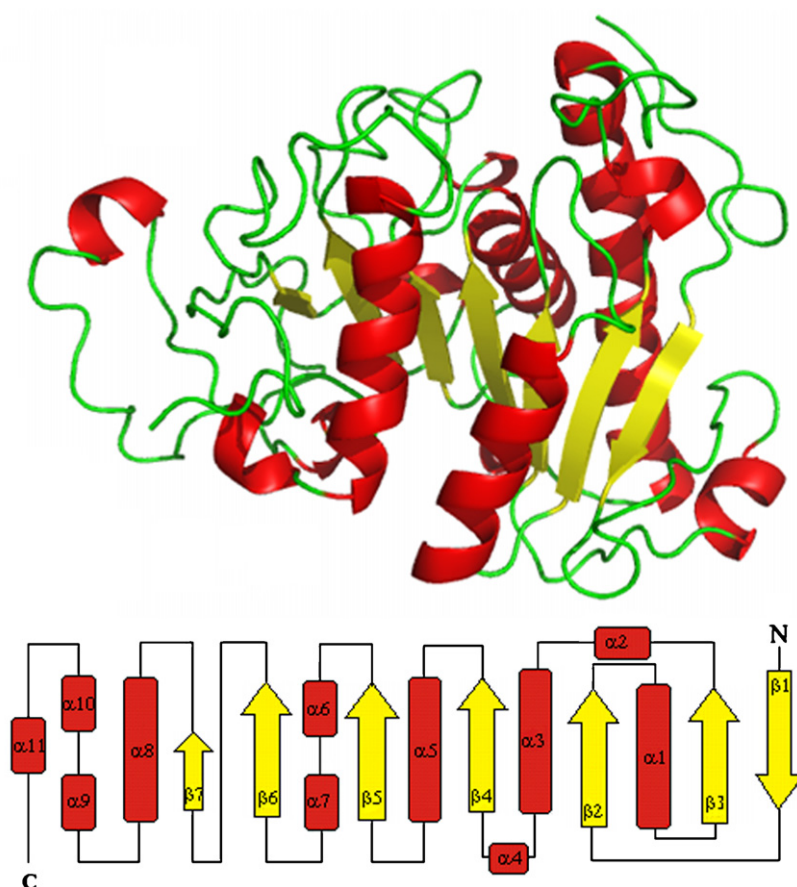


Fig. 6. Ribbon diagram generated by MODELLER (Sali and Blundell, 1983) of the PLA₁ from the venom of the social wasp *P. paulista*. The secondary structure elements are colored: helices in red and strands in yellow.

Table 2

Comparison of the direct hemolytic activity of *P. paulista* PLA₁ with hemolysins from honeybee, wasps and snake venoms

Toxin	Concentration (μg/mL)	Direct hemolysis (%) in WRBC
PLA ₁ from the wasp <i>Polybia paulista</i>	100	17 ± 4
PLA ₁ from <i>Vespa verutina</i> (VT-1) ^a	10	47 ± 17
PLA ₁ from <i>Vespa verutina</i> (VT-2a) ^a	1	86 ± 5
PLA ₁ from <i>Vespa verutina</i> (VT-2b) ^a	1	96 ± 2
PLA ₁ from <i>Vespa basalis</i> (lethal protein) ^b	100	96 ± 1
PbTX-I from the wasp <i>Polybia paulista</i> ^c	100	62 ± 4
PbTX-II from the wasp <i>Polybia paulista</i> ^c	100	57 ± 3
PbTX-III from the wasp <i>Polybia paulista</i> ^c	100	90 ± 6
PbTX-IV from the wasp <i>Polybia paulista</i> ^c	1	60 ± 3
Hornetin from <i>Vespa flavitarus</i> ^b	1	13 ± 2
PLA ₂ from <i>Apis mellifera</i> ^c	100	27 ± 3
Neutral PLA ₂ from <i>Naja nigricolis</i> ^b	100	35 ± 10
Basic PLA ₂ from <i>Naja nigricolis</i> ^b	100	92 ± 1
Cobra cardiotoxin from <i>Naja naja atra</i> ^b	100	16 ± 1

Data are shown as means ± SE.

^aHo and Ko (1988).

^bHo et al. (1999).

^cOliveira and Palma (1998).

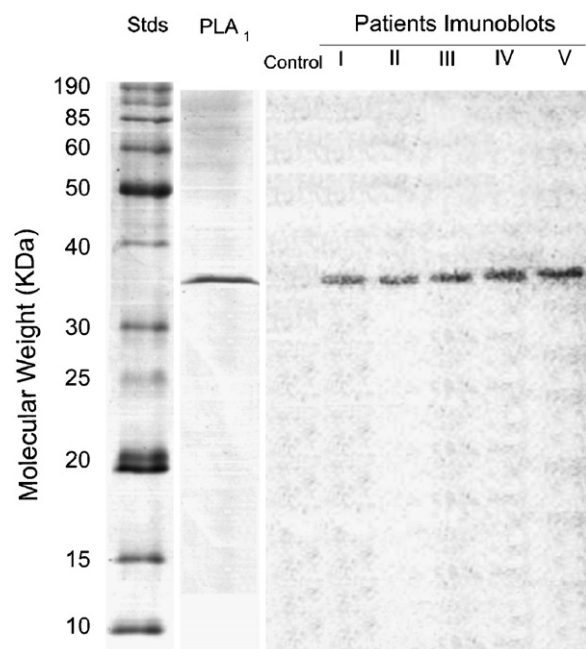


Fig. 7. IgE binding to *P. paulista* PLA₁. Purified PLA₁ was submitted to SDS-PAGE, transferred to PVDF membranes and incubated with the sera both from *P. paulista*-sensitive patients (lanes I–V) and nonallergic individuals (control). Specific IgE binding was detected by using chemiluminescence; the first two lanes correspond to the standard MW markers (Std) and to the purified enzyme (PLA₁).

comparison with the results of *P. paulista*-sensitive patients.

Fig. 8A shows the protein profile in 2-D SDS-PAGE of *P. paulista* crude venom, compared to the profile of IgE immunoreactivity obtained with the sera *P. paulista*-sensitive patients (Fig. 8B). About 237 different protein spots were detected after staining with CBB and 15 of these proteins were IgE-immunoreactive; *P. paulista* PLA₁ is one of these proteins (assigned with an arrow in Figs. 8A and B).

4. Discussion

P. paulista PLA₁ is an extracellular enzyme, containing 304 amino acid residues and presenting an experimental molecular mass of 33,961.40 Da. When the amino acid sequence of the active enzyme was evaluated in the Lipase Engineering Database (<http://www.led.uni-stuttgart.de>) (Fisher and Pleiss, 2003), it was classified as a lipase of the GX class, liprotein lipase superfamily, pancreatic lipases (ab20.3) homologous family and RP2 sub-group of phospholipase. All extracellular members of the

PLA₁ sub-family belong to the family of pancreatic lipase; this family is conserved in a wide range of organisms from insects to mammals (Aoki et al., 2007).

The members of the pancreatic lipase family generally exhibit the amino- (N-) and carboxy- (C-) terminal domains. The N-terminal domain is essential for catalytic activity, since it contains the amino acids residues involved with the active site; the function of the C-terminal domain is not well understood (Aoki et al., 2007). When compared to the other members of this family of proteins, the PLA₁'s from vespid venoms have only the N-terminal domain (Soldatova et al., 1993), which in turn contain the “catalytic triad”, composed of the residues of Ser, Asp and His, assigned with black stars in Fig. 5, at positions 165, 194 and 281, respectively.

The tertiary structure of the members of lipase family presents surface loops, called lids and $\beta 9$ (one of each type), which cover the active site and seem to be involved with substrate recognition and determination of substrate specificity (Aoki et al., 2002); the positions of these loops are assigned in Fig. 5. Generally, the lipases of this group present long lid and $\beta 9$ loops, whereas the vespid-venom PLA₁ have shorter lid and $\beta 9$ loops (Aoki et al., 2007). A comparison of the amino acid sequences and the substrate specificities of PLA₁'s and lipases in general, suggest that: (1) enzymes with short lid and short $\beta 9$ loop exhibit only PLA₁ activity, (2) enzymes with a short lid and a long $\beta 9$ loop exhibit both PLA₁ and TG lipase activities, and (3) enzymes with long lids and long $\beta 9$ loop exhibit only TG lipase activity (Aoki et al., 2007). Thus, a careful examination of Fig. 5 reveals that *P. paulista* presents short lid and short $\beta 9$ loop, suggesting that *P. paulista* PLA₁ seems to exhibit only PLA₁ activity.

Another structural feature observed in Fig. 5 is high the conservation of the positions of all cysteine residues in vespid venoms; *P. paulista* PLA₁ molecule contains six disulfide bridges, making it a relatively compact structure. The most important structural similarities are assigned as shadowed areas, emphasizing the highly conserved residues positions in this type of alignment.

Considering that this protein was immunoreactive to specific IgE from the sera of *P. paulista*-sensitive patients, it may be considered as an allergen, which according to the nomenclature rules of International Union of the Immunology Societies

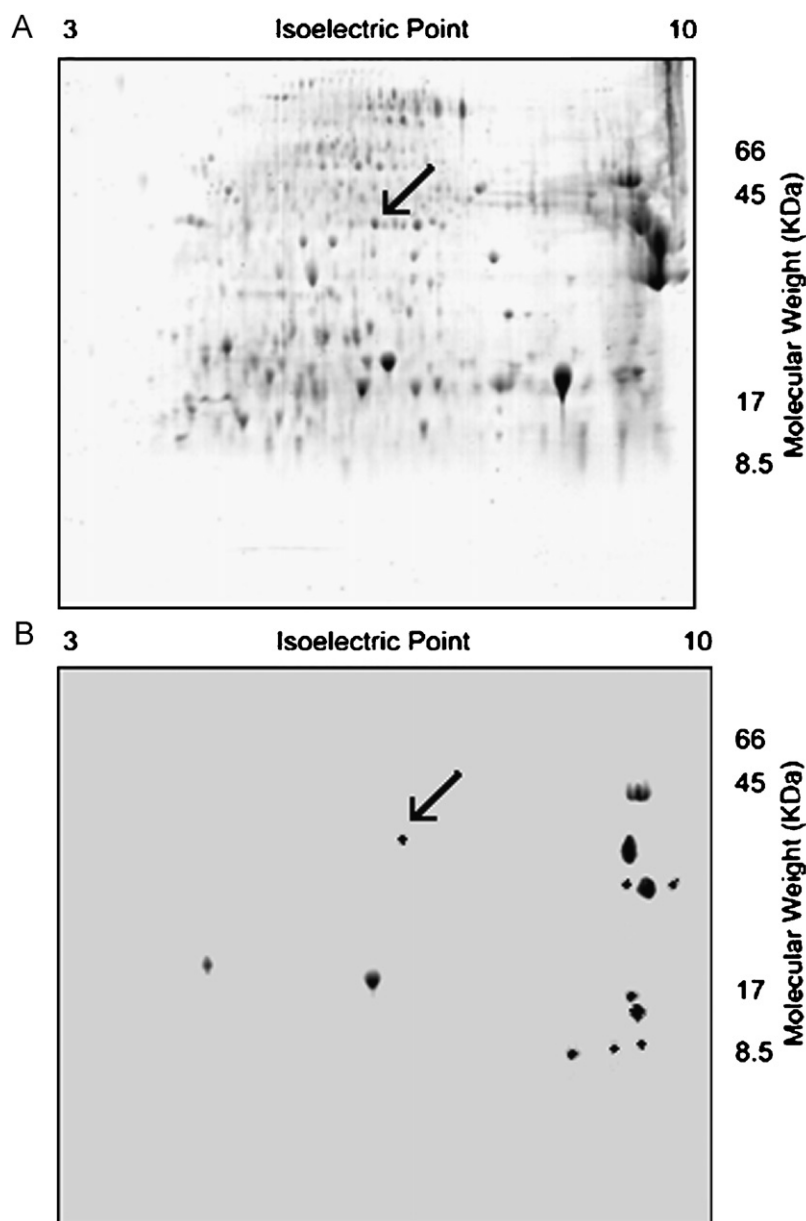


Fig. 8. (A) 2-D SDS-PAGE profile of CBB-stained proteins from the crude venom of the social wasp *P. paulista*; (B) profile of proteins immunoreactive to IgE from the sera of *P. paulista*-sensitive patients.

(<http://www.allergen.org/Allergen.aspx>) was named Poly p 1. However, these data are not enough to establish if this protein is a major allergen without investigating a large number of patients, this currently being done. The general structure of *P. paulista* PLA₁ with parallel β -strands (in combination with some α -helices linking two or three of these strands) represents a relatively common structural motif in nature; however, it seems to be

under-represented among the allergenic proteins (Aalberse, 2000).

P. paulista PLA₁ presents direct hemolytic action against washed red blood cells and despite being a hemolysin it is much less potent than the PLA₁'s from other vespid venoms such as the verutoxins from *V. verutina* and the lethal toxin from *V. basalis*. In addition to this, it is also less potent than the vespid PLA₂'s such as the PbTXs isolated from

the venom of same wasp specie, than Hornetin from *V. flavitarus* and than the PLA₂'s both from honeybee and some *Naja* snakes venoms. However, it is as potent as the Cobra Cardiotoxin from *Naja naja atra* (Table 2).

P. paulista is a very aggressive social wasp found in southeast Brazil, causing several stinging accidents throughout the year, especially in urban areas. The cases of multiple stingings are not uncommon and are generally followed by severe hemolysis and myocardial dysfunction; in this situation, the described symptoms may be caused by the accumulation of high levels of PLA₁ and PbTx, among other toxins. *P. paulista* crude venom presents 15 proteins immunoreactive to IgE, from which PLA₁ is one of these putative allergens, contributing to the occurrence of immunological/inflammatory processes generally associated with the social wasp stingings.

Acknowledgments

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