

RESEARCH ARTICLE

Proteomic characterization of the multiple forms of the PLAs from the venom of the social wasp *Polybia paulista*

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The phospholipases A₁ (PLA₁s) from the venom of the social wasp *Polybia paulista* occur as a mixture of different molecular forms. To characterize the molecular origin of these structural differences, an experimental strategy was planned combining the isolation of the pool of PLAs from the wasp venom with proteomic approaches by using 2-D, MALDI-TOF-TOF MS and classical protocols of protein chemistry, which included N- and C-terminal sequencing. The existence of an intact form of PLA₁ and seven truncated forms was identified, apparently originating from controlled proteolysis of the intact protein; in addition to this, four of these truncated forms also presented carbohydrates attached to their molecules. Some of these forms are immunoreactive to specific-IgE, while others are not. These observations permit to raise the hypothesis that naturally occurring proteolysis of PLA₁, combined with protein glycosylation may create a series of different molecular forms of these proteins, with different levels of allergenicity. Two forms of PLA₂s, apparently related to each other, were also identified; however, it was not possible to determine the molecular origin of the differences between both forms, except that one of them was glycosylated. None of these forms were immunoreactive to human specific IgE.

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1 Introduction

The venoms from social Hymenoptera (wasps, bees and ants) are important instruments in the defense of individuals or of the colony [1]. Stinging events involving wasps, honeybees and ants are frequent worldwide; a single sting may cause a transient local inflammation characterized by pain, redness and swelling in humans [2]. About 20.7% of population in general develops a

hypersensitivity reaction of type 1 [3]. Massive envenomation can result both in immediate and delayed reactions in man [4], sometimes causing death [5]. Mass stinging events may be life-threatening via the toxic action of the venom when injected in large amounts [6]. Systemic reaction is reported to occur in 5% of population in general [7].

The Hymenopteran venoms consist of a complex mixture of low molecular mass toxins [1], a series of polycationic peptides [8–10] and proteins, which are recognized as important allergens [11, 12]. The major allergens of wasps and honeybee venoms have been identified as phospholipase A₁ (PLA₁) and phospholipase A₂ (PLA₂), antigen-5, hyaluronidase, major royal jelly proteins and acid phosphatases [13, 14, 11, 15, 16]. Phospholipases are quite common enzymes in animal venoms; PLA₂s are very

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Abbreviations: PLA₁, phospholipase A₁; PLA₂, phospholipase A₂

abundant in snake venoms, while PLA₁s are relatively common in Hymenopteran venoms [17].

However, PLAs are not only allergic factors, since they may also act as toxic principles of the wasps venoms. PLA₁/PLA₂ catalyze the hydrolysis of ester bonds at the C₁/C₂ positions of 1,2-diacyl-3-*sn*-glycerophospholipids into their corresponding lyso compounds with the release of free fatty acids. Thus, these enzymes are able to disrupt the phospholipid packings from several types of biological membranes, leading to pore formation and/or cell lysis [18]. PLA₁/PLA₂ from wasp venoms have been demonstrated to be lethal factors, causing cardiac dysfunction and death of the animal [19].

PLA₂s were biochemically characterized in honeybees and in some neotropical wasp venoms [20]. PLA₁s may be activated by the mastoparan peptides, inducing the release of TH2 cell-associated mediators and possibly other antigen-presenting cells [21, 22]; these proteins generally present variable IgE cross-reactivity, with multiple antigenic determinants [23]. PLA₁ activities have been detected in venoms, platelets, bovine brain and fungi [22]. The most members of lipase family present surface loops called lids and β9 (one of each loop), which cover the active site and are implicated in substrate recognition [22]. Generally, Hymenopteran PLA₁ and PLA₂ have reduced sequence identity among them and differ in their specificity concerning the catalysis of the hydrolysis of fatty acid residues at positions 1 and 2 of phospholipids, respectively [24].

The intact form of PLA₁ from *Polybia paulista* corresponds to a protein presenting 305 amino acid residues, which was classified as a lipase of the GX class, lipoprotein lipase superfamily, pancreatic lipases (ab20.3) homologous family and RP2 sub-group of phospholipase. When compared to the PLA₁s from other Vespidae venoms, the sequence was 53–82% identical to these enzymes [25]. The literature about the wasp venom PLA₁s is focused on purification, biochemical characterization and cDNA cloning; however, only limited information is available on the characterization of the different PLA₁ forms [22].

Recently, a pool of PLAs were reported in *P. paulista* venom, corresponding to a mixture of PLA₁ and PLA₂ [25], but the components of this mixture were not individually characterized in that publication. Thus, to make the proteomic characterization of the different forms of PLAs, the mentioned pool of enzymes was isolated as previously reported [25] and proteomically characterized through the combination of 2-D, MALDI-TOF/TOF MS and classical protocols of protein chemistry, inclusively for post-translational modifications.

2 Materials and methods

2.1 *P. paulista* venom

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 and microscissors. The venom reservoirs were then carefully washed and suspended in small volumes of a solution containing a cocktail of protease inhibitors (2 mM AEBSF, 0.3 μM Aprotinin, 130 μM Bestatin, 1 mM EDTA, 14 μM E-64 and 1 μM Leupeptin; Sigma-Aldrich), punctured, washed three times with the protease inhibitors solution to extract the venom and centrifuged at 10 000 × g for 10 min at 4°C. The supernatants were collected, lyophilized and maintained at –80°C until use.

2.2 Protein assay

Protein concentration was determined by the method of Bradford (BioRad), using bovine albumin (Sigma) as standard [26].

2.3 2-DE

Samples (700 μg protein) were applied by rehydration to 13-cm IPG strips, pH 3–10. IEF was carried out on a Multiphor II System (GE Healthcare) at 3500 V for 17.000 Vh. IPG strips were incubated in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS) containing 0.5% w/v DTT for 15 min, followed by equilibration buffer containing 4% w/v iodoacetamide for 15 min. The second dimension was run on self-cast SDS-PAGE gels (15% w/v polyacrylamide and 0.8% w/v bis (*N,N'*-methylene bisacrylamide)) at 15 mA /gel for 15 min and 30 mA/gel for 3 h, at 10°C in a Ruby Red system (GE Healthcare). Gels were stained overnight with Coomassie Brilliant Blue R-250 (CBB) as reported elsewhere [27] and stored at 21°C in preserving solution (7% v/v acetic acid).

The 2-D gels were stained with CBB, scanned and digitized (BioImage, GE Healthcare) in the transparency mode at 24-bit red–green–blue colors and 400 dpi resolutions for documentation. Images were analyzed using Image Master Platinum software v.7 (GE Healthcare).

2.4 In-gel digestion

The protocol for in-gel digestion was based on a previous publication [28]. Briefly, gel pieces were destained twice for 30 min at 25°C with 50 mM ammonium bicarbonate/50% ACN, dehydrated in ACN, dried and treated with trypsin (20 μg/mL, Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate, pH 7.9, at 37°C for 18 h. Digests were extracted from gel pieces with 60% v/v ACN/water and 0.1% v/v formic acid, combined, desalted and cleaned with PerfectPure C18 pipette tips (Eppendorf, Hamburg, Germany) according to the manufacturer's instructions and

vacuum dried. The concentrated digests were mixed with 0.6 L of matrix (10 mg/mL CHCA in methanol/ACN (1:1, v/v) mixed with an equal volume of 0.2% v/v aqueous TFA) and spotted onto a MALDI plate target.

2.5 MALDI-TOF/TOF mass spectrometry data

Mass spectrometric analysis was performed by MALDI TOF/TOF-MS/MS on a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, USA). MS data were acquired in the m/z range of 800–4000, with an accelerating voltage of 20 kV and delayed extraction, peak density of maximum 50 peaks per 200 Da, minimal S/N ratio of 10 and maximum peak at 60. MS/MS data were acquired in the mass range from 60 Da until each precursor mass, with a minimum S/N ratio of 10; a maximum number of peak set at 65 and peak density of maximum 50 peaks per 200 Da.

2.6 The isolation of the pool of phospholipases A from *P. paulista* venom

The pool of PLA present in the venom of *P. paulista* was purified as previously reported elsewhere [25]. Briefly, the freeze-dried venom (10 mg) was solubilized in 5 mM ammonium acetate pH 6.8 and submitted to a SEC in a Sephacryl S100 column (60 cm $\times$ 26 mm, GE Healthcare) coupled to an AKTA-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. The fractions presenting PLA activity were pooled, lyophilized, dialyzed against distilled water and analyzed for protein homogeneity.

2.7 PLA 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 [29]. The reaction medium contained 15 mmol phosphatidylcholine, 18 mmol Triton-X, 5 mmol calcium chloride, 80 nmol phenol red and 7.5 mmol 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 mmol phosphatidylcholine/h in 1 mL of the reaction medium at pH 7.9 and 37°C.

2.8 N-terminal amino acid sequencing

N-terminal sequence analysis of intact PLA₁ and its different isoforms were carried out with pulse liquid-phase microsequencer (Shimadzu, mod, PPSQ-21) operating standard programs. The samples used in this protocol were the spots of PLA₁ electrotransferred to PVDF membranes.

2.9 C-terminal amino acid sequencing

The C-terminal sequencing was performed based on the derivatization with acetylisothiocyanate to yield amino acid thiohydantoin (TH-Aas); this simple chemistry was automated using an ABI 473A N-terminal sequencer (all reagents, R1: trimethylsilylisothiocyanate; R3: alkaline thio-cyanate for cleavage) and solvents required for sequencing were accommodated on the sequencer, which was modified to deliver liquid R2 (acetyl chloride) to the reaction vessel. The conversion flask was used for preparing the TH-AAs for analysis by on-line HPLC, using a graphitized carbon (Hypercarb) column. The samples used in this protocol were the spots of PLA₁ electrotransferred to PVDF membranes. This setting permitted to determine at least four residues from the C-terminus.

2.10 Protein identification

GPS Explorer (Applied Biosystems) was used to submit the combined MS and MS/MS data to MASCOT protein search engine version 2.2 (<http://www.matrixscience.com>) using the National Center for Biotechnology Information (NCBI) protein database, restricted to the taxa Metazoa. The search parameters were as follows: no restrictions on protein molecular weight, one tryptic missed cleavage allowed; peptide mass tolerance in the searches was 0.8 Da for MS spectra and 0.5 Da for MS/MS spectra. Iodoacetamide derivative of cysteine and oxidation of methionine were specified in MASCOT as fixed and variable modifications, respectively. Scaffold (version 2.04.00, Proteome Software, Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. The identifications were accepted when their score exceeded the specific database search engine thresholds. MASCOT identifications required ion scores greater than both the associated identity scores and 20, 30, 40 and 40 for singly, doubly, triply and quadruply charged peptides. The FDR assessment was estimated using the original decoy FDR approach from Mascot; a separate decoy database was generated from the protein sequence database with the decoy.pl Perl script provided by Matrix Science. This script randomizes each entry, while it retains the average amino acid composition and length of the entries. For protein identification, the maximum protein and peptide FDR rates were set to 0.01 and the maximum peptide FPR to 0.1; FPR was calculated based on the Mascot

Score. Proteins were considered identified with at least two peptides assigned to the respective sequence. All peptides/proteins corresponding to toxins were also confirmed by manual examination of the spectra.

2.11 Immunoblotting

Sera from five *P. paulista* sensitized patients were obtained from the Division of Clinical Immunology and Allergy from the Clinics Hospital, University of São Paulo Medical School, São Paulo, Brazil, with the approval of the University Ethical Board. Sera were collected before starting the specific immunotherapy treatment of each patient and stored at -20°C until used. The protein profiles obtained as described above were electrotransferred to nitrocellulose membranes (Hybond-C Extra, GE Healthcare) for 1 h at room temperature. The binding of IgE antibodies to the membrane-immobilized allergens was analyzed by Western Blot using the pool of sera from five *P. paulista* allergic patients. The membranes were blocked with PBS containing 0.5% Tween-20 and incubated with sera from each patient, diluted 1:10 in blocking buffer overnight at 8°C . After washing with PBS–0.1% Tween-20, the membranes were incubated with a secondary antibody (mouse IgG anti-human IgE) conjugated to peroxidase (1:10 000, Zymed) for 1 h at room temperature. Chemiluminescence detection reagents (ECL Chemiluminescence Reagent Plus Western; GE Healthcare) were added to the membrane according to the manufacturer's instructions. The membrane was incubated with Hyperfilm film (GE Healthcare) in an X-ray cassette and the film was submitted to conventional photographic film.

2.12 Protein glycosylation detection

Gels were incubated in 50% v/v methanol for 30–45 min, washed twice in 3% v/v acetic acid for 5–10 min each and the glycans were oxidized by incubation in 1% v/v periodic acid, 3% v/v acetic acid for 20–30 min at room temperature. The gel was washed four times for 5–10 min each in 3% v/v acetic acid to remove residual periodate and incubated in Pro-Q Emerald 300 dye solution (GE Healthcare) for 30–120 min following the manufacturer's instructions. Afterwards, the gel was incubated in 3% v/v acetic acid twice for 5–10 min each. Glycoproteins were visualized by scanning the gel at 300 nm in a photo-documentation system through the VDS Image Master (GE Healthcare).

3 Results

A previous publication reported in detail the purification of the intact PLA₁ from *P. paulista* venom [25]; this publication also described the experimental protocol for the isolation of

the pool of PLAs as mentioned above. Thus, this protocol was reproduced in the present investigation; a gel permeation chromatography in Sephacryl S100 column was carried out with the crude venom of *P. paulista* collected and manipulated in presence of a cocktail of proteinase inhibitors. The chromatographic profile (Fig. 1A) showed eight peaks, which were assigned from A to H and the PLA activity was assayed through all the protein profile; the activity was associated to the peak C (fractions 45–60), which contained about 80% of the total PLA activity present in the crude venom.

The fractions corresponding to the peak C (Fig. 1A) were collected, pooled, lyophilized and submitted to 2-D SDS-PAGE; the electrophoretic profile reveals ten protein spots, which were assigned from I to X (Fig. 1B). The proteomic analysis of these protein spots corroborated the identification of all proteins as PLAs (Table 1), in the MW ranging between 10 320 and 29 846 Da, and pI from 4.528 to 9.616. All these identifications presented MASCOT score equal or higher than 81, with sequence coverage from 15 to 29%. The spots II and V were identified as PLA₂, while the spots I, III, IV, VI, VII, VIII, IX and X were identified as PLA₁ (Table 1).

These results reveal eight different forms of PLA₁ identified with same accession code (A2VBC4), suggesting that they may represent different forms of the same protein, originating due to either post-translational modifications or by gene splicing regulation. Thus, it was decided to obtain the partial sequences of the N- and C-terminal regions of each protein present in these spots. This way, the protein spots of the 2-DE were electrotransferred to PVDF membranes, excised and submitted to N- and C-terminal sequencing as described in Section 2; the results are shown in the Tables 2 and 3. When the N- and C-terminal sequences of all PLA₁ forms were compared to the sequence of the precursor PLA₁ from *P. paulista* venom (accession code: A2VBC4 in Protein Database of National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/>), the spots I, III, IV, VI, VII, VIII, IX and X, fitted to the positions 19–170, 19–167, 149–303, 32–175, 220–322, 68–166, 220–312 and 18–321. The result obtained for the spot X indicates that this protein corresponds to the intact form of the PLA₁, since its N- and C-terminal sequences coincided with both termini of the mature protein. Meanwhile, the other seven spots may correspond to truncated forms of this protein, or even to products of alternative gene splicing. The comparison between the experimental MW values obtained in the 2-D SDS-PAGE (Table 1) and the corresponding hypothetical molecular masses, calculated from the results shown in Table 2, reveals a good agreement of values.

The amino acid sequence of the PLA₂ from *P. paulista* venom is unknown; however, even under this condition, the N- and C-termini of the two different forms of this protein in the present study were determined (spots II and V in Table 3). Despite the sequences of the peptides used to identify both forms of PLA₂s being similar to those found in

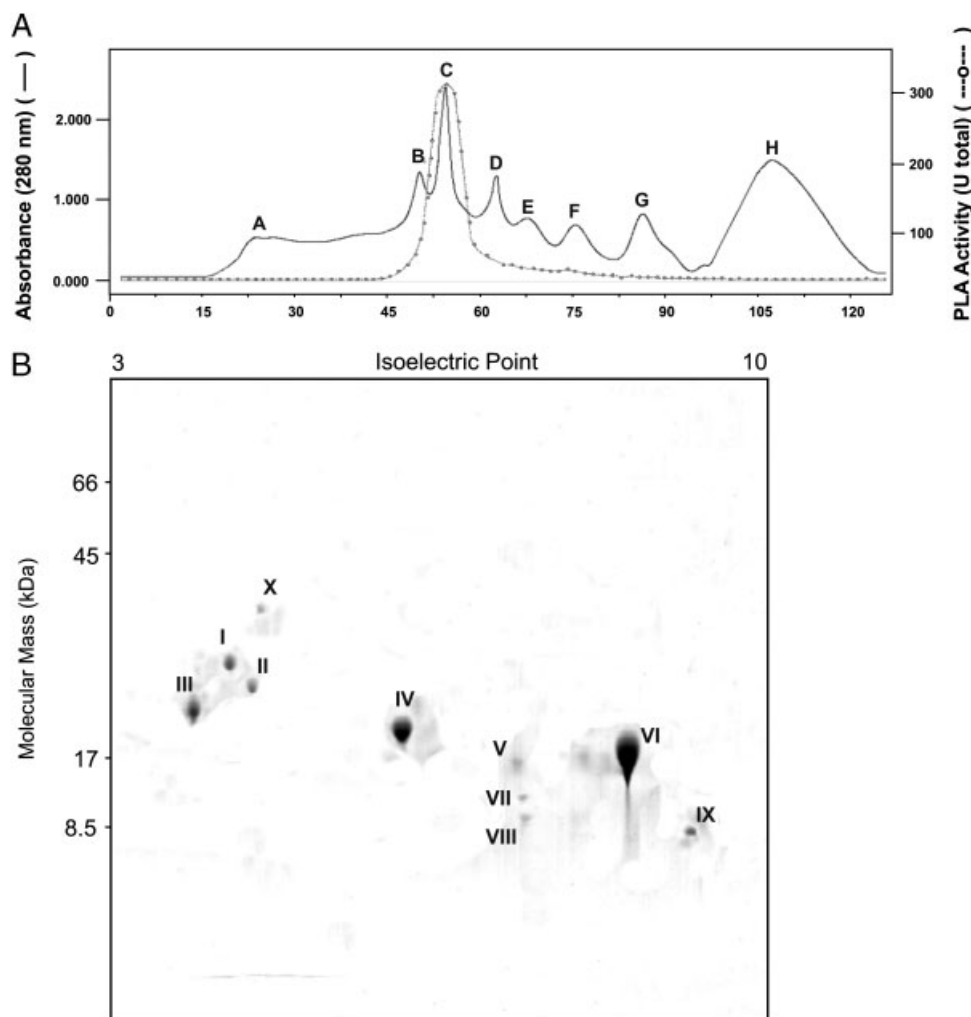


Figure 1. (A) Size-exclusion chromatography profile of the crude venom of the wasp *P. paulista* collected and manipulated in presence of a cocktail of proteinase inhibitors, carried out in a Sephacryl S-100 column (60 cm × 26 mm, 320 mL; GE Healthcare). Elution was performed with 5 mM ammonium acetate, 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; (B) 2-D SDS-PAGE profile of CBB-stained proteins from the peak "C."

honeybee venom PLA₂ (accession code AF438408; Table 1), the N-terminal sequences are very conserved in relation to that reported for the same enzyme from the venom of the solitary wasp *Orancistroceruss drewseni* [30], while the C-terminal sequences were conserved in relation to those found in the PLA₂ from the venom of the parasitic wasp *Chelonius sp.* [31].

To check the effect of the endogenous proteolysis of the venom in the integrity of the PLAs, the protocol of isolation of the pool of these enzymes was also carried out for the venom obtained and manipulated in absence of the cocktail of inhibitors of proteinases, in the same experimental conditions described above. The chromatographic profile (Fig. 2A) revealed 13 peaks, and the PLA activity was associated with fractions 45–50. However, the total PLA activity was about two-fold times lower than that obtained with the venom obtained in the presence of the cocktail of proteinase inhibitors. The fractions corresponding to the peak presenting PLA activity (Fig. 2A) were collected, pooled, lyophilized and submitted to 2-D SDS-PAGE; the electrophoretic profile reveals 26 protein spots (Fig. 2B), and

the position of the same ten spots observed in the presence of the cocktail of proteinase inhibitors were assigned in Fig. 2B for comparison with the results shown in Fig. 1B. Proteomic analysis corroborated the identification of all these proteins as new forms of PLA₁s (accession code: A2VBC4) and PLA₂s (accession code: AF438408), in the MW range from 6800 to 29 846 Da, and pI values from 3.905 to 9.830 (not shown results). All these identifications presented MASCOT score equal or higher than 60, with sequence coverage from 10 to 19% (not shown results). The proteomic analysis for the peaks A, B and from D to H (not presenting PLA activity) revealed the presence of hyaluronidases, antigen-5, acid phosphomonoesterases and serine proteinases among other proteins (not shown results), but no PLA was identified in these fractions.

The knowledge about PTMs such as glycosylation is an important structural information to understand the function, the cellular destination of some proteins and even the occurrence of the different forms of these proteins. Thus, the protein profile obtained in 2-D SDS-PAGE for the peak C from the gel permeation chromatography in Sephacryl

Table 1. Proteomic identification of the proteins present in the peak "C" from Fig. 1A

Spot	Protein	Acession Code	Protein score ^{a)}	Sequence coverage %	MW (Da)	pI	Peptides sequences
I	Phospholipase A ₁	A2VBC4	105	17	17 128	7810	DGIVLTEETLQNYDLFK, YVADFTK, DNFLVISVDWK, VSMSNIRLIGHSLGAHT, WCGHGNKSSGPNELGR
II	Phospholipase A ₂	AF438408	131	29	17 906	6975	IYPGTLWCGHGNK, IGDNEEER, FKHTDACC, R, DGIVLTEETLQNYDLFK, YVADFTK,
III	Phospholipase A ₁	A2VBC4	108	20	16 850	5357	DNFLVISVDWK, VSMSNIRLIGHSLGAHT, LLVEQYK, LCETDAEYVQIIHTSNILGVYSK
IV	Phospholipase A ₁	A2VBC4	84	16	17 030	8585	DTCVCVGLNAK, LIGHSLGAHTSGFAGK, WCGHGNKSSGPNELGR
V	Phospholipase A ₂	AF438408	110	25	22 016	5119	IYPGTLWCGHGNK, IGDNEEER, FKHTDACC, R, YFNLDITKCYK, DGIVLTEETLQNYDLFK
VI	Phospholipase A ₁	A2VBC4	114	26	10 320	9616	DNFLVISVDWK, VSMSNIRLIGHSLGAHT, LLVEQYK, LIGHSLGAHTSGFAGK, YVADFTK, SKIGTVDFYMNNGSHQPGCGR
VII	Phospholipase A ₁	A2VBC4	90	17	12 750	7791	YLTECIK, FFSPSCSHTK, DTCVCVGLNAK, DNFLVISVDWK, YVADFTK
VIII	Phospholipase A ₁	A2VBC4	89	18	10 928	7754	HVGKYVADFTK, LLVEQYKVSMSNIR, ALIEK, YLTECIK, FFSPSCSHTK
IX	Phospholipase A ₁	A2VBC4	89	18	11 308	4528	YFSTPKPISQCTK, IGTVDFYMNNGSHQPGCGR, GSFYVPVEATAPYCHNEGK
X	Phospholipase A ₁	A2VBC4	81	15	29 846	5102	GLIPECPFNEYDILFFVYTR, DGIVLTEETLQNYDLFK, DNFLVISVDWK

a) Mascot score: Protein scores greater than 76 are significant ($p < 0.05$).

Table 2. N- and C-terminal sequences of the protein spots corresponding to the different forms of PLA₁, their hypothetical molecular masses and their location in the amino acid sequence of the precursor protein

Spot	N-terminal sequence	C-terminal sequence	Hypothetical Molecular mass (Da)	Residues
I	LIPECP....	GKEVQ	17 182	19–170
III	LIPECP....	GFAGK	16 826	19–167
IV	NIRLIGHS....	PARGS	16 996	149–303
VI	FVYTRQ....	ELKLN	16 029	32–175
VII	SKIGTV....	EGIKL	11 415	220–322
VIII	IDHGFL....	SGFAG	10 741	68–166
IX	SKIGTV....	VGATA	10 260	220–312
X	LIPECP....	HNEGKIL	34 018	19–321

S100 was also submitted to the detection of glycosylation with Pro-Q Emerald 300 dye. The result shown in Fig. 3A reveals that the PLA_s from the spots II, IV, VII and IX were detected in the glycosylated form. The comparison of these results with those of Table 1 reveals that three PLA₁ forms (spots IV, VII and IX) and one PLA₂ form (spot II) are glycosylated.

Considering the well-known allergenicity of the wasp venoms proteins to humans, the protein profile obtained in 2-D for the peak C from the gel permeation chromatography in Sephacryl S100 was also submitted to an immunoblotting with the sera from *P. paulista*-allergic patients. The result shown in Fig. 3B indicates that the PLA_s from the spots III, VII, IX and X were reactive to the pool of human specific-IgE, revealing the high allergenic potential of this

protein. Apparently, no PLA₂ was immunoreactive to specific-IgE. The preparation obtained in the absence of the cocktail of proteinase inhibitors was submitted neither to the analysis of glycosylation nor to the immunoblotting assay.

4 Discussion

P. paulista represents a latent risk for the people who are allergic to the venom of social wasps, for which antigen-5, hyaluronidase and PLAs are recognized as the major allergens [32]. PLAs are relatively common in Hyme-

nopteran venoms, occurring mainly as -A₁ and -A₂ types [25]. PLAs are able to disrupt the phospholipid packings from several types of biological membranes, leading to pore formation, cell lysis and tissues damage [18].

PLA₂ was previously reported in social wasps venoms [13, 14] and in honeybee venom [33, 34], being described as highly hemolytic. PLA₁s are generally associated to allergic and inflammatory processes, but are not well characterized biochemically [15, 21].

When compared to each other, PLA₁s and PLA₂s present no sequence homology; apparently, these proteins have distinct functions. In the present investigation, eight natural different forms of PLA₁ (spots I, III, IV, VI, VI, VIII, IX and X; Fig. 1B and Table 1) and two forms of PLA₂ (spots II and V; Figure 1B and Table 1) were identified in *P. paulista* venom. Meanwhile, PLA₁s have been partially characterized in the venoms of some wasp species from northern hemisphere [21], in ants [32] and in the neotropical social wasp *P. paulista* [25].

Table 3. N- and C-terminal sequences of the protein spots corresponding to the different forms of PLA₂

Spot	N-terminal sequence	C-terminal sequence
II	MSSKFS....	TENV
V	MSSKF.....	DISVN

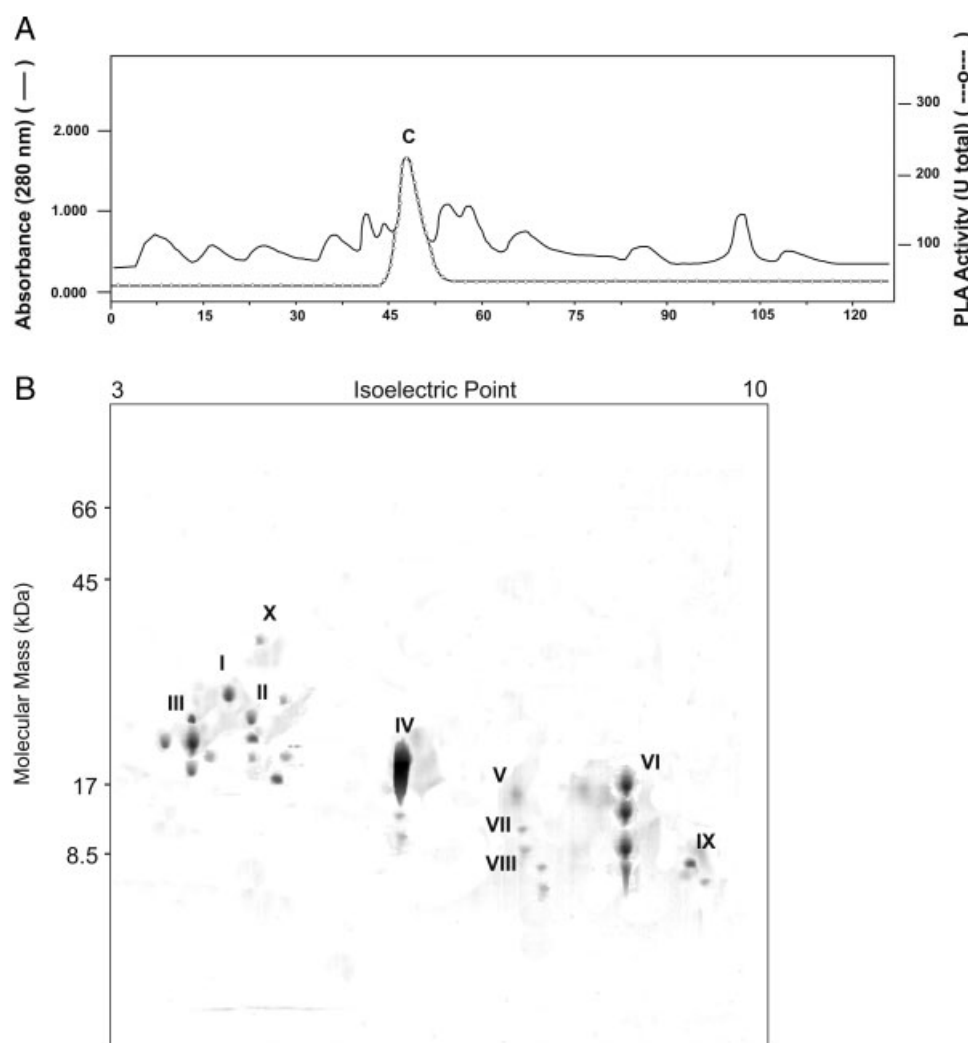


Figure 2. (A) Size-exclusion chromatography profile of the crude venom of the wasp *P. paulista* collected and manipulated in absence of a cocktail of proteinase inhibitors, carried out in a Sephacryl S-100 column (60 cm × 26 mm, 320 mL; GE Healthcare). Elution was performed with 5 mM ammonium acetate, 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; (B) 2-D SDS-PAGE profile of CBB-stained proteins from the peak “C”.

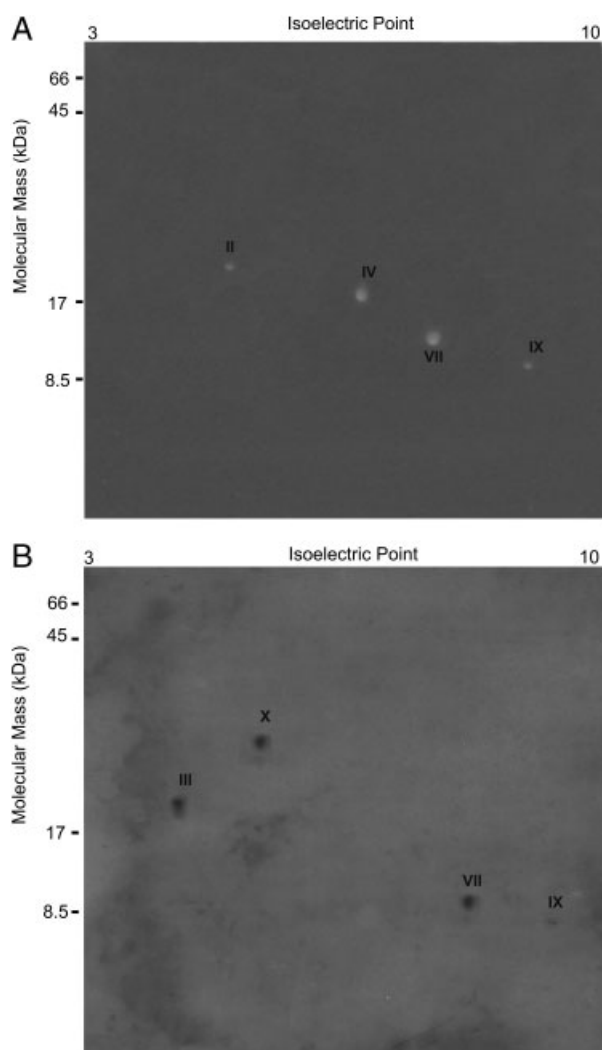


Figure 3. (A) Profile of glycosylated proteins from the peak “C,” after reaction with Pro-Q Emerald 300 dye; (B) profile of proteins immunoreactive to IgE from the sera of *P. paulista*-sensitive patients.

Considering the high number of different forms of PLA₁ in the venom of *P. paulista*, it was decided to characterize biochemically each one of these forms. Proteomic analysis confirmed the identification of eight forms of PLA₁ and two forms of PLA₂. All the PLA₁s were identified with the same accession code (A2VBC4), as well the PLA₂ (AF438408), suggesting that these enzymes may occur as a series of either different isoforms, or post-translationally modified forms, or even forms produced by alternative splicing. The forms, which were identified with a different accession code, probably present different molecular origins; however, for those proteins sharing a common accession code, it may be wondered if the different forms were created by some experimental artifact during sample manipulation, or if they were naturally created within the venom. The results shown in Fig. 2A and B clearly demonstrate that in the absence of

the cocktail of proteinases inhibitors, at least 16 new forms of PLAs were created, probably as products uncontrolled proteolysis occurred after venom collection and/or during its manipulation.

The most common types of PTMs are glycosylation, controlled proteolysis and phosphorylation [35]; the first two types were investigated in the present study. Considering that all the venom samples were obtained and manipulated in the presence of a cocktail of proteinases inhibitors, apparently it may be suggested that the different forms of PLA₁ reported in the present study in presence of the cocktail of proteinase inhibitors were naturally produced by the action of endoproteinases before venom collection and extraction. The analysis of the N- and C-terminal sequences of the eight natural forms of PLA₁s revealed that one of these forms corresponds to the mature and intact PLA₁ (spot X), while the other seven proteins seem to correspond to truncated forms of the PLA₁ (spots I, III, IV, VI, VII, VIII and IX), probably originated from a common protein sequence, i.e. the intact one. The results of the same analysis for both PLA₂ forms (spots II and V) cannot be compared to a reference sequence from social wasp venoms, since no PLA₂ of this origin was sequenced up to now. However, the two PLA₂ forms reported in the present investigation (Fig. 1B) presented N- and C-terminal sequences similar to the sequences found in the venoms from the solitary wasps *O. drewseni* and *Chelonus sp.*, respectively, while the internal sequences were conserved in relation to the enzyme from honeybee venom. These results cannot be explained by the natural occurrence of truncated forms; but it would be possible to speculate that the alternative gene splicing could explain the occurrence of these forms of PLA₂s. However, to proof this hypothesis, it would be necessary to sequence completely all the PLAs detected and to compare with the DNA sequences found in the genome of *P. paulista*, which currently is unknown.

Glycosylation was also investigated in the present study; three forms of PLA₁ (spots IV, VII and IX) and one form of PLA₂ (spot II) were identified in the glycosylated form. Other types of PTMs also could contribute for the observed structural differences between these proteins, but they were not investigated in the present study.

The heterogeneity of the PLAs is relatively well known in Hymenopteran venoms, but they were not properly investigated up to now. Peiren et al. [36] reported the occurrence of 24 different forms of PLA₂ in honeybee (*Apis mellifera carnica*) venom on gel electrophoresis; it has been proposed that either photo-oxidation of the histidine imidazole group or variations in the carbohydrate content of each individual form (or even both processes together) could contribute for this heterogeneity [37]. However, no specific investigation was previously developed to explain these observations in Hymenopteran venoms. In a recent publication, an extensive proteomic assignment of the crude venom from *P. paulista* [38] was reported, confirming the existence of the same eight forms of PLA₁s and two forms of PLA₂s

described above in the present manuscript. Thus, apparently, no other natural forms of PLAs seem to exist in the crude venom of *P. paulista*, except those reported in the presence of the cocktail of inhibitors, even in the fractions not presenting phospholipase activity.

The immunoblotting (Fig. 1D) revealed that four PLA₁s (spots III, VII, IX and X) were immunoreactive to human-specific IgE; therefore, these forms of PLA₁ correspond to allergenic proteins; meanwhile, no form of PLA₂ was immunoreactive to specific human-IgE. The existence of different molecular forms for some venom proteins would benefit the wasps, permitting the insects to adapt to the same original molecules to different cellular/molecular targets. Concerning those proteins reactive to specific-IgE, the existence of different molecular forms creates potentially different allergens, which may be differently recognized as antibodies by the different animal species stung by the wasps.

The allergenicity of some venom proteins is probably the most well-studied action of the stinging accidents caused by social wasps. The identification of allergens from each venom, as well as their molecular characterization, certainly will contribute to the identification of the whole panel of the allergens from wasp venoms. The identification of novel allergens and/or different forms of known ones, will be useful to build a more complete microarrays of proteins for allergy diagnosis, or even to help the development of suitable protocols for the expression of recombinant forms of these proteins to be used in the immunotherapy of patients sensitive to wasps venom.

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