Hyaluronidase from the venom of the social wasp *Polybia paulista* (Hymenoptera, Vespidae): Cloning, structural modeling, purification, and immunological analysis

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**ARTICLE INFO**

**Article info:**  
Received 27 September 2012  
Received in revised form 18 December 2012  
Accepted 21 December 2012  
Available online 7 January 2013

**Keywords:** Hyaluronidase  
*Polybia paulista* venom  
cDNA cloning  
Structural modeling  
Protein purification  
Pp-Hyal-specific antibody

**ABSTRACT**

In this study, we describe the cDNA cloning, sequencing, and 3-D structure of the allergen hyaluronidase from *Polybia paulista* venom (Pp-Hyal). Using a proteomic approach, the native form of Pp-Hyal was purified to homogeneity and used to produce a Pp-specific polyclonal antibody. The results revealed that Pp-Hyal can be classified as a glycosyl hydrolase and that the full-length Pp-Hyal cDNA (1315 bp; GI: 302201582) is similar (80–90%) to hyaluronidase from the venoms of endemic Northern wasp species. The isolated mature protein is comprised of 338 amino acids, with a theoretical pI of 8.77 and a molecular mass of 39,648.8 Da versus a pI of 8.13 and 43,277.0 Da indicated by MS. The Pp-Hyal 3D-structural model revealed a central core (α/β)\(_7\) barrel, two sulfide bonds (Cys 19 – 308 and Cys 185 – 197), and three putative glycosylation sites (Asn79, Asn187, and Asn325), two of which are also found in the rVes v 2 protein. Based on the model, residues Ser299, Asp107, and Glu109 interact with the substrate and potential epitopes (five conformational and seven linear) located at surface-exposed regions of the structure. Purified native Pp-Hyal showed high similarity (97%) with hyaluronidase from *Polistes annularis* venom (Q9U6V9). Immuno-blotting analysis confirmed the specificity of the Pp-Hyal-specific antibody as it recognized the Pp-Hyal protein in both the purified fraction and *P. paulista* crude venom. No reaction was observed with the venoms of *Apis mellifera*, *Solenopsis invicta*, *Agelaia pallipes pallipes*, and *Polistes lanio lanio*, with the exception of immune cross-reactivity with venoms of the genus *Polybia* (sericea and ignobilis). Our results demonstrate cross-reactivity only between wasp venoms from the genus *Polybia*. The absence of cross-reactivity between the venoms...
1. Introduction

Hyaluronidases (Hyal) are a widely distributed group of enzymes that hydrolyze hyaluronic acid (HA), one of the primary components of the extracellular matrix in all vertebrates (Kreil, 1995). Hyal are also present in almost all venom classes, acting as a “diffusion factor” by facilitating the penetration of the other harmful venom components and enhancing their action in various tissues into the bloodstream (Kemparaju and Girish, 2006; Senff-Ribeiro et al., 2008).

Hyal have been described as “allergenic factors” in scorpion, bee, and wasp venoms, and are able to induce severe and fatal anaphylactic IgE-mediated reactions in humans (Lu et al., 1995; Kolarich et al., 2005). Hyal have already been characterized as glycoproteins (Kemeny et al., 1984; Jin et al., 2008) and analysis by high performance liquid chromatography and mass spectrometry revealed that the α,1-fucose-containing N-glycan is the fundamental structural unit responsible for their allergenicity (Kubelka et al., 1995; Kolarich and Altmann, 2000; Kolarich et al., 2005).

Since allergenic Hyal are phylogenetically more conserved among the other Hymenoptera allergens (e.g. Ag5 and PLA1), a significant degree of homology is observed among the sequences and 3D structures of these proteins, whether they are from different vespids or honeybee Apis mellifera venom (Jin et al., 2010). In addition, a large percentage of patients allergic to Hymenoptera venom show reactivity to both bee and wasp venoms (known as cross-reactivity) in tests for the presence of IgE-specific antibodies (Hemmer, 2008). This makes selection of the most suitable venom for immunotherapy difficult. However, it is unclear whether this cross-reactivity is due to (a) sequence homology between these hyaluronidases; (b) sensitivity to the specific IgE antibodies; or (c) cross-reactive N-glycans (cross-reactive carbohydrate determinants [CCDs]), which have been investigated in allergens from different sources (Jin et al., 2010; Eberlein et al., 2012; Al-Ghouleh et al., 2012).

In terms of the mechanism of action on the substrate, Hyal enzymes are classified into three types (Meyer, 1971): (a) the group of the endo-β-N-acetyl-d-hexosaminidase that hydrolyze the high molecular weight substrate (HA) to tetrasaccharide as the main end product, being this group represented by the testicular enzyme; (b) the β-endoglucuronidase group represented by hyase from leeches and hookworm (Hotez et al., 1994); (c) and finally the group of lyases that act via β-elimination, yielding disaccharides as the main end products represented by the bacterial hyalases. According to Laurent (1989), Cramer et al. (1994) and Takagaki et al. (1994) the enzymes of the first group also catalyzes transglycosylation reactions, producing hexa-, di-, and octa-saccharides during hydrolysis of HA. Hyaluronate-4-glycanohydrolase (EC 3.2.1.35), or Hyal type 1, is an endo-β-N-acetyl-d-hexosaminidase is also found in Hymenoptera venoms and mammalian spermatozoa. Unlike the other two types of hyaluronidases, this group acts not only on HA, but also on chondroitin 4-sulfate and chondroitin 6-sulfate (CS) (Fiszer-Szafarz, 1984; Fiszer-Szafarz et al., 1990; Kreil, 1995; Cherr et al., 1996; Stern and Jedrzejas, 2006).

The social wasp Polystyla paulistus (Hymenoptera, Vespidae) is endemic to Southeastern Brazil, especially São Paulo State, and is responsible for many incidents due to their venomous stings. Due to consequent and serious allergic reactions that may develop and lead to anaphylactic shock (Palma, 2006), the social wasp is thus of great medical importance.

Studies of crude extracts of P. paulista venom by chromatography, SDS-PAGE, and specific assays showed significant levels of hyaluronidase, phospholipase, and proteolytic, hemolytic and myotoxic activities (Silva et al., 2004). Recently, proteomic analysis by Pinto et al. (2012) detected four different glycoprotein forms of Hyal in P. paulistus venom and subsequently sequenced and structurally modeled the most abundant form, Hyal III.

In order to examine the molecular characteristics and immunogenic potential of the Pp-Hyal venom allergen, the complete cDNA sequence of another form of this enzyme was obtained, cloned, sequenced and its 3D-protein structural model constructed by comparative modeling. Furthermore, the native form of this Pp-Hyal was purified through high performance chromatography and analyzed by mass spectrometry. The protein was then used to produce a Pp-specific polyclonal antibody, which was tested by Western blotting to confirm its specificity and immune cross-reactivity with venoms from other Hymenoptera species.

2. Material and methods

2.1. Insects and crude venom extracts

P. paulistus nests were collected in the city of Rio Claro, SP, Southeastern of Brazil. Insects were anesthetized at low temperature (−20 °C) and their venom reservoirs were extracted with tweezers. Crude venom extract was prepared from 1000 reservoirs, which were macerated at a 1:1 ratio (reservoir:solvent) with ultra pure water containing 1 mM PMSF (Sigma–Aldrich, USA). The suspension was centrifuged at 10,000 × g for 15 min at 4 °C and Pp-Hyal protein was purified from the freeze-dried supernatant. For immunological assays, venom extracts were prepared by the same method with 100 venom reservoirs from each of the following species of Hymenoptera: P. paulista, Polybia sericea, Polybia ignobilis, Agelaia pallipes pallipes, Polistes lanio lanio, A. mellifera, and Solenopsis invicta.

2.2. Protein determination

Quantification of total proteins in the extracts and fractions from chromatography was performed by the...
modified Bradford method using bovine serum albumin (BSA) as a standard (Sedmak and Grossberg, 1977).

2.3. Preparation of total RNA and cDNA synthesis

RNA was extracted from 100 venom reservoirs with TRIZol® reagent (Life Technol, USA) and maintained at −85 °C for 7 days to increase the integrity of the total RNA. cDNA synthesis was performed by RT-PCR of 1 μg of RNA using a kit from Promega® (USA) and an oligo dT primer. The complete cDNA sequence of Pp-Hyal was obtained with a degenerated forward primer designed based on the Hyal precursor sequence (Gl: 5815250) of Polistes annularis venom (5’TCC RAA AGA CCG AAA AGA GTG TTC ARC 3’) and a reverse primer (5’ CTA AAA GTT CAG GGA TGA TCT TCT 3’) designed based on the results of previous 3’ RACE experiments. Both primers were synthesized by Sigma–Aldrich (USA). PCR was performed using Platinum®Taq DNA Polymerase (Life Technol, USA) to a final volume of 25 μL containing 2 μg of cDNA, 1U of Taq DNA polymerase, 0.2 mM dNTPs, 2.0 mM MgCl2 and 0.2 μM of the above primers under the following conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 47 °C for 1 min, and extension at 72 °C for 3 min, and a final extension at 72 °C for 15 min in an MJ Research PTC-100 Programmable Thermocycler.

2.4. Cloning

Pp-Hyal gene-specific PCR products were cloned into the pCR®8/GW/TOPO® vector (kit pCR®8/GW/TOPO® Cloning Kit, Invitrogen, USA) following the manufacturer’s protocol. Escherichia coli One Shot™ Mach1™ T1T1R cells chemically competent, were reared in SOC Medium (Tryptone 2.0%, yeast extract 0.5%, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, and 20 mM glucose) and used for transformation reactions. Cloning plasmids were plated on Luria–Bertani agar (1.0% Tryptone, 0.5% yeast extract, 1.0% NaCl, pH 7.0) containing 100 μg/mL spectinomycin and incubated overnight at 37 °C. Plasmid preparations were obtained using the QIAprep® Spin miniprep kit (Qiagen, Germany) and analyzed by restriction digestion with Eco Rl enzyme (Fermentas UAB, Lithuania).

2.5. Sequencing

The Pp-Hyal gene-specific primers, as well as the forward (GW1: 5’ GTT GCA ACA AAT TGA TGA GCA ATG C 3’) and reverse (GW2: 5’ GTT GCA ACA AAT TGA TGA GCA ATT A 3’) primers from the pCR®8/GW/TOPO® vector (Invitrogen, USA), were used in sequencing reactions in an Applied Biosystems 3730 sequencer at the Center for Social Insects Studies (CEIS), Univ. Estadual Paulista “Júlio de Mesquita Filho” (UNESP), Rio Claro, SP, Brazil. The obtained sequences were examined using DNASTAR®Lasergene Sequence Analysis software.

2.6. Structural modeling

Modeling of Pp-Hyal 3D-structure was performed based mainly on the solved X-ray Hyal 3D-structure of this allergen in the venom of Vespula vulgaris (PDB ID: 2ATM) due to its greater sequence similarity with Pp-Hyal (75%) in relation to the same protein of A. mellifera (PDB ID: 1FCQ) (54%). However, since that only the latter 3D-structure was solved with substrate HA, it was used for the identification of the Pp-Hyal active site. The deduced primary sequence of Pp-Hyal (PMDB ID: PM0077230) obtained in this study was used as the input parameter for analysis. One hundred models were built by Modeller Program version 9.8 (Sanchez and Sal, 1997) taking into account spatial restrictions (resolution ≤ 2Å, factor-R satisfactory ≤ 20), and the model with the lowest energy was selected. Potentially immunogenic regions (epitopes) on this structural model were analyzed by the Modeler Program and checked by the EnsembleGly Server. The programs PyMol (Delano, 2002) and Procheck (Laskowski et al., 1993) were used for editing models.

2.7. Ion exchange liquid chromatography

Freeze-dried venom extract (10 mg of total protein) from P. paulista was solubilized in 50 mM sodium acetate buffer (pH 5.2) and separated by cation exchange chromatography in a Hiprep FF CM column (160 mm × 10 mm, 20 mL – GE Healthcare) coupled to an Akta-FPLC system. Elution was accomplished by a linear gradient of 0–1 M NaCl in the same buffer above and monitored by measuring the absorbance at 280 nm and the hyaluronidase activity.

2.8. Hyaluronidase activity

Hyaluronidase activity was determined by the turbidimetric method (Long-Rowe and Burnett, 1994) modified by Silva et al. (2004). Because venom Hyals are classified as type I enzymes that act on CS in addition to HA (Fiszer-Szafarz, 1984; Fiszer-Szafarz et al., 1990), enzyme activity was determined by hydrolysis of CS (Chondroitin Sulfate A Sodium Salt from bovine trachea or C4-S, Sigma, Aldrich, USA) at pH 5.2. One unit of specific activity was defined as the amount of enzyme necessary to hydrolyze 1 nmol of chondroitin (U = nmol of CS hydrolyzed/mg of venom protein) per hour.

2.9. SDS-PAGE

Fractions showing hyaluronidase activity were collected, pooled, and lyophilized. The protein concentration was determined and 80 μg of total protein were separated by 15% (w/v) SDS-PAGE in a Mini-Protean II (BioRad) at 100 V. The gel was stained with Coomassie Brilliant Blue R-250 (CBB) and scanned. For Western blotting experiments, 80 μg of total protein from venom extracts of different insects were separated by 15% SDS-PAGE. A pre-stained protein marker (High-Range Rainbow Molecular Weight Markers, Amersham Biosciences-GE Healthcare, USA) was run in parallel. Runs were carried out at 75 V in the stacking gel and 100–110 V on the resolving gel over a period of 2 h. Following separation, the proteins were transferred from the gels onto nitrocellulose membranes.
2.10. In-gel digestion

Gel pieces containing FPLC-purified \( Pp \)-Hyal were destained twice for 30 min at 25 °C with 25 mM ammonium bicarbonate/50% (v/v) acetonitrile, dehydrated in 50% acetonitrile, dried, and treated with 20 µg/ml trypsin (Promega, USA) in 25 mM ammonium bicarbonate (pH 7.9) at 37 °C for 16 h. Digests were extracted from gel pieces with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, (Promega, USA) in 25 mM ammonium bicarbonate (pH 7.9) at 37 °C for 16 h. Digests were extracted from gel pieces with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried. The concentrated digests with 50% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined and vacuum dried.

2.11. Mass spectrometry

Mass spectrometric analysis was performed by MALDI ToF/ToF-MS (Matrix-Assisted Laser Desorption Ionization Time of Flight/Time of Flight-Mass Spectrometry) on an Axima Performance MALDI Mass Spectrometer (Shimadzu Scientific Instruments). MS data were acquired in the m/z range from 700 to 3500, with an accelerating voltage of 20 kV and delayed extraction, a peak density maximum of 50 peaks per 200 Da, a minimal S/N ratio of 10 and a maximum peak at 60.

2.12. Protein identification

LaunchPad 2.8.4 (Shimadzu Biotech) was used to submit the MS data to MASCOT Protein Search Engine (version 2.2) using the National Center for Biotechnology Information (NCBI) Protein Database. The search parameters were as follows: no restrictions on protein molecular mass, one missed tryptic cleavage allowed, mass tolerance to peptide and protein structure. The four peptides generated by tryptic digestion, analyzed by MALDI ToF/ToF-MS and identified by the MASCOT Software Protein Search Engine are also shown.

2.13. Systemic sensitization and \( Pp \)-Hyal-specific antibodies preparation

The \( Pp \)-Hyal-specific antibody was prepared in the Experimental Immunology and Allergy Laboratory-LIAE, Medical Clinic Department, UNICAMP, Campinas, SP, Brazil. A total of 12 BALB/c female mice at approximately 30-days-of age and a weight of 25 g were used in the experiments. From the \( Pp \)-Hyal purified sample obtained by ion exchange liquid chromatography, 1 mg of total proteins were separated by 15% SDS-PAGE. As only one 39 kDa band was visualized in the gel, it was cut out, macerated, diluted in sterile physiological solution and applied to the backs of mice (approved by the Ethics Committee for Animal Utilization-CEUA-No. 031/2010) to produce the \( Pp \)-Hyal-specific antibody. Immunizations were done on day 7, 21, and 28, and on day 30, the animals were sacrificed and the antibody collected. Six mice were used as controls, receiving applications of polyacrylamide gel free of proteins that had been macerated and diluted as described above.

2.14. Western blotting

Following SDS-PAGE, venom proteins were transferred to a nitrocellulose membrane (0.45 µ) at 0.8 mA/cm² and 60 V for 2 h in a semi-dry system (New Blot Multiphor II unit, Biotech Pharmacy). Transfer efficiency was confirmed by staining the gel with Coomassie Blue G-250. Immunodetection was performed with the \( Pp \)-Hyal-specific antibody diluted 1:1000 and anti-mouse Ig, alkaline phosphatase conjugate (Sigma–Aldrich, USA) diluted 1:5000 (2 µl in 10 mL of blocking solution) as the primary and secondary antibodies, respectively. Bands were visualized with alkaline phosphatase/BCIP/NBT (Sigma–Aldrich, USA).

3. Results

3.1. Analysis of \( Pp \)-Hyal: cDNA sequence, deduced protein and structural modeling

The complete cDNA sequence of \( Pp \)-Hyal was determined after sequencing 11 positive clones. A 1315 bp consensus cDNA sequence (GI: 302201582) showed the highest similarity with Hyal from the venoms of the four endemic wasp species of the Northern hemisphere: 90% similarity with \( P. \) annularis, 81% with \( V. \) vulgaris, Vespula germanica, Vespa magnifica, and 80% with Dolichovespula maculata.

The primary sequence of the deduced \( Pp \)-Hyal mature protein (Fig. 1) contained 338 amino acid residues (1017 bp) and was rich in the amino acids Asn, Gln, and Lys, with a theoretical pI of 8.77 and a predicted molecular mass of 39,648.8 Da versus the 43,277.0 Da indicated by MS. Fig. 1 shows the location of the forward and reverse primers, the three potentially immunogenic N-glycosylated sites (Asn79, Asn187, and Asn325) and the two disulfide bridges (Cys19–Cys308 and Cys185–Cys197) responsible for stabilization of protein structure. The four peptides generated by tryptic digestion, analyzed by MALDI ToF/ToF-MS and identified by the MASCOT Software Protein Search Engine are also shown.

When the GenBank Hyal amino acid sequence for \( Pp \)-Hyal (ADLO9135) from this study was aligned with the same allergen of \( V. \) vulgaris (PDB 2ATM), \( P. \) annularis (HUGA_POLAN), and \( A. \) mellifera (PDB 1FCQ_A), high levels of similarity were revealed (75%, 90%, and 54%, respectively). In Fig. 2, shaded blue areas indicate several regions of similarity mainly among the three first molecules. In addition, the amino acids DFE (highlighted by a red rectangle), which are present in the active site, are also highly conserved.

The two proteins – Ves v 2 (PDB ID: 2ATM) and Api m 2 (PDB ID: 1FCQ) – used for building the model of the 3D-structure of the \( Pp \)-Hyal had their 3D-structures already determined by X-ray crystallography at a resolution of 2.0 Å (Skov et al., 2006) and 2.7 Å (Markovic-Housley et al., 2000), respectively. Despite the greater similarity among sequences have been found between the proteins of \( P. \) paulista and \( V. \) vulgaris, only the 3D-structure of the Api m 2 was solved with HA as its substrate, reason why the latter was used in this study to identify the \( Pp \)-Hyal active site and points of contact with the substrate.

Based on its model (Fig. 3A,B), \( Pp \)-Hyal displays a structure comprised of a central barrel (β/α)7 containing seven α-helix and seven beta-sheets, in agreement with the expected
structure for all hyaluronidases belonging to family 56 of glycoside hydrolases (Henrissat and Bairoch, 1996; Markovic-Housley et al., 2000; Skov et al., 2006). This model also reveals two important characteristics of the \( Pp \)-Hyal structure: the presence of two disulfide bonds between Cys19–308 and Cys185–197 (Fig. 3A) and putative glycosylation sites on residues Asn79, Asn187, and Asn325 (Fig. 3B).

The sites Asn79 (50NITI30) and Asn325 (50NITI30) are also found in Hyal of \( V. \) vulgaris venom (Skov et al., 2006), indicating that they exert a direct influence on the immunogenicity of the molecule. Glycosylation is the most common post-translational modification of many eukaryotic intracellular proteins, contributing to biological activity, immunogenicity, solubility, stability, and protease resistance. Carbohydrate residues may be enzymatically attached to proteins through the N-glycoside bond via the amide nitrogen of asparagine, or through the O-glycoside bond via the hydroxyl group of serines, threonines, hydroxylysines or hydroxyproline, or by a glycosylphosphatidylinositol anchor, which is subsequently removed (Steinberg et al., 2001).

Fig. 4 shows the topology of the \( Pp \)-Hyal molecule (Fig. 4A), making evident its active site position when compared to that of Hyal from \( A. \) mellifera and the predicted amino acid residues in the model that establish interaction with the substrate Ser299, Asp107 and Glu109 (Fig. 4B). In addition to their hydrophilic characteristics, these residues are located on opposite sides of the cavity, which is likely of great importance for substrate transport into the active site through electrostatic interactions with the carboxylic groups of HA.

Sequences of potentially immunogenic regions were also identified (Fig. 5) by the Conformational Epitope Prediction Serve (CEP) (Kulkarni-Kale et al., 2005). According these authors for every antigen–antibody complex the total of antibody-binding sites corresponds to the sum of the residues that interact with the antibody plus those that are buried under the antibody. Using an implementation of Voronoi polyhedron (McConkey et al., 2002) to the calculation of percentage accessibility of residues and with base on the spatial distance cut-off
among the involved atoms, Kulkarni-Kale et al. (2005) have stipulated a correction factor of $\frac{1}{25}$ for identification of antigenic residues less accessible by the antibody binding. So, in the Pp-Hyal 3D-structural model twelve antigenic sites were identified, located in regions of both the internal and external loops revealing five conformational (displayed in green) and seven linear (presented in yellow) predicted epitopes. Thus, we can infer that in this allergen the presence of linear epitopes directly influences immune responses while the five conformational epitopes affect the humoral response mediated by B cells.

3.2. Purification of native Pp-Hyal

The chromatographic profile of P. paulista crude venom (Fig. 6) produced eight peaks, designated A through H. Hyaluronidase activity was associated with peak F, with a total activity of 1.1 U/h. This corresponds to a recovery rate of 30%, taking into account that the total activity in crude venom was 3.6 U/h (100%). Thus, satisfactory recovery of specific hyaluronidase activity was obtained. After collecting, pooling, and lyophilizing the samples with major Pp-Hyal activity (fractions 71–74 from peak F), 1.4 mg of total protein were obtained and 80 mg of which was subjected to SDS-PAGE to evaluate its level of purity, what was confirmed by the presence of only one band in the gel (Fig. 7). Fig. 8 shows the MALDI-ToF-ToF-MS spectra achieved after in-gel digestion of the Pp-Hyal protein band (from Fig. 7) with trypsin. Nine major tryptic peptide peaks were observed corresponding to ions with $m/z$ 1060.51, $m/z$ 1226.57, $m/z$ 1342.63, $m/z$ 1354.67, $m/z$ 1372.72, $m/z$ 1381.62, $m/z$ 1913.84, $m/z$ 2052.06, and $m/z$ 2151.20. From these results, four peptides were identified by the Protein MASCOT.
Search Engine version 2.2, using the NCBI Protein Database, which revealed that they were similar to four regions of Hyal from *P. annularis* wasp venom (Q9U6V9), covering approximately 17% of this sequence (Score: 91, \( p < 0.05 \); see Supplementary Material). Through this analysis it was also possible to determine a molecular mass of 43,277 Da and a calculated pI value of 8.13 for *Pp*-Hyal, while the values for the protein obtained by molecular cloning were a molecular weight of 39,648.8 Da and a pI of 8.77. These differences may result from the specificities of each technique and the degree to which the digested peptides retained their post-translational modifications, such as phosphorylation, acetylation, and glycosylation, which result in changes to the pI and molecular mass (Seo and Lee, 2004).

### 3.3. Immunological analysis

Western blotting was carried out using the specific *Pp*-Hyal-antibody, as previously described. As shown in Fig. 9, the specificity of *Pp*-Hyal-specific antibody was confirmed by Western blotting because it recognized the *Pp*-Hyal protein in purified fraction (Fig. 9A) and crude venom (Fig. 9B, lane I), but no reaction was observed with venom of *A. pallipes pallipes*, *P. lanio lanio*, *A. mellifera* or *S. invicta* (Fig. 9B, lanes IV–VII), although a significant amount of immune cross-reactivity was observed with venoms from the genus *Polybia* (*sericea* and *ignobilis*) (Fig. 9B, lanes II and III). Recognition of other protein bands in the extracts of *P. paulista* crude venom by the *Pp*-Hyal-antibody would most likely be due to the presence of four isoforms of *Pp*-Hyal, as recently described by Santos et al. (2010) and Pinto et al. (2012), which likely share some common epitopes.

### 4. Discussion

Hyaluronidase of wasp venom is an allergen that has been extensively studied in several genders and species of
European and American wasps, but few studies have been conducted in Neotropical social wasps.

A high degree of immunological cross-reactivity among the allergens in the venom of Hymenoptera insects makes identification of the insect responsible for the stings difficult. Patients previously sensitized to the venom of a specific insect (e.g. from wasp) who are then stung for a second time by a different insect, can exhibit the presence of non-specific IgE antibodies. This can result in false-positive due to cross-reactivity with the allergens of different venoms whose epitopes have similar conformations, thus rendering differentiation by B-1 cells impossible. In addition, false-negative results can be observed in skin tests due to the low amount of IgE detected by tests with low sensitivity (e.g. RAST) (Hemmer, 2008).

In this study, the deduced primary sequence of Pp-Hyal protein from cDNA cloning presented a high degree of similarity to the same protein from P. annularis venom. This species is phylogenetically closer to P. paulista than the other species used here for comparison, even though both Polistes and Polybia belong to the same Polistinae subfamily.

Hyaluronidases are also found in the venoms of snakes, lizards, and other arthropods, and are highly conserved in these species (Csóka et al., 1999). Silva et al. (2004) performed a comparison between the levels of Hyal-specific activity (using CS as substrate) in crude venom extracts from the Hymenoptera wasp species Polistes similimus, P. ignobilis, P. paulista, and A. pallipes pallipes, and found that the latter two species showed high levels of Hyal activity. Nevertheless, the activity levels of enzymes present in Hymenoptera venoms are known to vary in response to physiological and environmental factors.

The most studied hyaluronidases are those from bee venom, which are found in greater abundance in comparison to other venom components. The molecular mass of hyaluronidase in bee venom is 41 kDa (Gmachl and Kreil, 1993). Studies performed with snake, bee, and scorpion venoms have demonstrated that they possess hyaluronidases with molecular masses ranging between 33 and 110 kDa (Cevallos et al., 1992). In spider venoms, hyaluronidases exhibit different values of molecular weight, for example of 33 kDa as well as an isoform of 63 kDa in L. recluse (Wright et al., 1973) and 44 kDa in L. deserta, L. gaúcho, L. intermediate, L. laeta, and L. recluse (Barbaro et al., 2005). Kolarich et al. (2005) detected a major polypeptide with a molecular weight of 43 kDa in V. vulgaris venom and...
identified it as a novel isoform of hialuronidase. All these differences can be ascribed to genetic variability as well as post-translational modifications.

Santos et al. (2010) identified four different molecular forms of Hyl in the venom of *P. paulista* by two-dimensional SDS-PAGE followed by mass spectrometry. Recently, using proteomic analysis, Pinto et al. (2012) characterized, sequenced, and constructed a 3D structural model of the most abundant isoform, Hyl III, which is 288 amino acid residues long with a molecular mass of 44,340 Da and a pI of 9.50. In contrast, the *Pp*-Hyal determined in this study by two methods is 338 amino acids long and displayed different values of theoretical pI and molecular mass. The *Pp*-Hyal purified protein was confirmed to be another isoform by determination of specific activity and MALDI ToF/ToF-MS analysis. When the amino acid sequence of *P. paulista* Hyl III was aligned with this *Pp*-Hyal protein deduced here by a molecular approach, a difference in 27 amino acid residues was verified (data not shown), resulting in a degree of similarity of 74.8%. Differences in other characteristics, such as pl value, the number of disulfide bonds and tertiary structure were also observed. Because the venom extracts in both studies were prepared from *P. paulista* wasps from the same region, and the Hyl enzymes were purified by cation exchange chromatography on FPLC under identical conditions in order to ensure that the Hyl activity profiles were reproducible, we can affirm that the two proteins correspond to different forms derived from genetic polymorphism. It remains unknown which of the three forms identified by Santos et al. (2010) this enzyme corresponds to. However, the existence of multiple forms of Hyl may be an important strategy to deceive or escape detection by the immune system, since attacks tend to involve a large number of insects.

Determination of the primary sequence of the allergenic *Pp*-Hyal protein was crucial to design its 3D-structural model. The main requirement necessary to construct a reliable protein structural model from comparative modeling is a highly detectable similarity between the query sequence and the model, as well as the correct alignment between them. In our study, modeling of the *Pp*-Hyal 3D-structure was possible because only some changes in sequences were observed among Hyls from *V. vulgaris*, *A. mellifera*, and *P. paulista* venom.

The 3D structure of recombinant Ves v 2 (carried out by crystallography with an electron-density map) showed that this protein is most stable when two disulfide bonds have formed between the cysteine residues Cys19–Cys308 and Cys185–Cys197, which are strictly coincident to those found in the *Pp*-Hyal 3D-structural model in our study. These findings reinforce the reliability of the data represented by this model.

Comparative analysis and superpositioning between the structures of Api m 2 co-crystallized with the substrate HA and that of *Pp*-Hyal revealed the presence of three amino acid residues that make contact with the polar hydroxyl nitrogen atoms of HA: Asp111, Glu113, and Ser299. In most glycosidases, two acidic residues play a central role in catalysis of the substrate, one of which acts as a proton donor while the other acts as a nucleophile (Markovic-Housley et al., 2000). In Api m 2, the only two residues that are highly conserved in the substrate binding site are Asp111 and Glu113, both of which appear to act as proton donors.

**Fig. 7.** Protein profile in SDS-PAGE (15%; w/v) of the purified fraction of native *Pp*-Hyal allergen (fractions F71 to 74 from peak F of cation exchange chromatography). About of 80 μg of total protein were applied on gel that was stained with Coomassie Brilliant Blue R-250 (CBB).

**Fig. 8.** MALDI-ToF/ToF-MS spectra of another form of *P. paulista* venom hyaluronidase after digestion with trypsin.
In the structure of *Pp*-Hyal characterized in this work, these two residues correspond to Asp107 and Glu109.

Skov et al. (2006) identified four potential glycosylation sites in the rVes v 2 structure: Asn79 (also found in Api m 2); Asn99; Asn127; and Asn325. In the *Pp*-Hyal model, three potential glycosylation sites were identified: Asn79; Asn187; and Asn325, two of which are also found in rVes v 2. Based on this data, we can speculate that because *Pp*-Hyal is less glycosylated than rVes v 2, it could present a lower degree of CCD-dependent cross-reaction, since one of the causes of double positivity is due to the recognition of IgE specific to carbohydrate determinants. According to Jin et al. (2010), nearly 90% of the cross-reactivity observed in Western blotting with sera from allergic patients is due to CCDs.

Markovic-Housley et al. (2000) and Skov et al. (2006) agreed in stating in their works that the knowledge of the structure of the Hyal epitopes is an essential step to the characterization of the Hyal antigenicity and such knowledge is very important to establish strategies for treating allergy mediated by this allergen as well as to the understanding of immunological cross reactions, in which these epitopes may be involved. The protein structural modeling together with the CEP Server (Kulkarni-Kale et al., 2005) are trustworthy bioinformatics tools which allow to achieve this knowledge with great accuracy. Using these procedures, in this study we identified in the *Pp*-Hyal 3D-structural model the location of five conformational and seven linear predicted epitopes, thus corroborating with the results observed by Western blotting and contributing for a better understanding of the immunogenic potential of this *Pp*-Hyal venom allergen.

The structural superposition of the three molecules (data not shown) revealed that the folding of rVes v 2 (Skov et al., 2006), Api m 2 (Markovic-Housley et al., 2000), and *Pp*-Hyal-3D structures were similar as well as the active site location, but as described by Skov et al. (2006), the Hyal proteins from bee and wasps have significant structural differences in its surfaces related to topology and also in charge distribution, what may explain the unlikely occurrence of cross-reactivity between them.

These data could be confirmed in our study since cross-reaction was only observed between wasp venoms of the same genus, *Polybia*, and no reaction with the venoms of *A. mellifera*, *S. invicta*, *P. pallipes pallipes*, or *P. lanio lanio*. Meanwhile, these results differ from some reports of wasps in temperate climates, in which cross-reactivity has been observed between the venoms of wasps and bees, as an example the recent study of Eberlein et al. (2012) that estimated that approximately 59% of patients allergic to Hymenoptera venom show positive results for both bee and wasp. This is mainly due to the IgE-specificity of hyaluronidase, being that this allergen is the most conserved venom component. The absence of cross-reactivity is important, as it allows identification of the insect responsible for sensitization of the victim (or at least the phylogenetically closest insect), which is crucial to develop immunotherapy for allergic patients.

The production and use of allergen-specific antibodies (native and/or recombinant), such as the *Pp*-Hyal-specific antibody produced here, has been an ongoing strategy to overcome difficulties in the diagnosis and treatment of allergies. In this context, experiments for the production of the major allergens from the *P. paulista* venom (Hyal, Ag5 and PLA1) in the recombinant forms and the obtaining of its specific antibodies are being conducted.

**Acknowledgments**

This work was supported by FAPESP (Proc. N° 2009/51539-1) through a Doctoral fellowship to Débora Laís Justo Jacomini. The authors also thank the support by PROAP-CAPES from the Post-Graduation Program of Biological Sciences (Cellular and Molecular Biology) at the Univ...