



Reactivity of IgE to the allergen hyaluronidase from *Polybia paulista* (Hymenoptera, Vespidae) venom



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ABSTRACT

To date, there are no allergenic extracts or components available in Brazil to diagnosis and treatment of patients with venom allergy from social wasp (Vespidae Family; Polistinae Subfamily) despite of the great number of existing species. We evaluated the immunogenic potential of the Hyal recombinant protein (*Pp*-Hyal-rec) which was expressed in an insoluble form in comparison with the allergenic native protein (*Pp*-Hyal-nat) for recognition of immunoglobulin E (IgE) in the serum of allergic patients to venom of the endemic social wasp *Polybia paulista* from São Paulo State, Brazil. Hyal cDNA from the venom of the social wasp *P. paulista* (*Pp*-Hyal) (GI: 302201582) was cloned into the expression vector pET-28a in *Escherichia coli* DE3 (BL21) cells. Solubilization and purification of *Pp*-Hyal-rec from inclusion bodies were performed using Ni²⁺ affinity chromatography (Ni-NTA-Agarose) under denaturing conditions. Both the native (*Pp*-Hyal-nat) and the recombinant (*Pp*-Hyal-rec) purified allergens were used for Western blotting to assess the levels of *Pp*-Hyal-IgE specific in the serum of 10 patients exclusively reactive to the venom of the social wasp *P. paulista*. The immune sera specifically recognized the band corresponding to the *Pp*-Hyal-rec protein (40 kDa) at a higher intensity than the native allergen (39 kDa). The sera recognized other proteins in *P. paulista* crude venom extract to a lesser extent, likely corresponding to other venom allergens such as phospholipase (34 kDa), Antigen 5 (25 kDa), and proteases. The recognition pattern of the immune sera to the *Pp*-Hyal-rec allergen strongly suggests that this recombinant antigen could be used for developing a diagnostic allergy test as well as for specific immunotherapy (IT).

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1. Introduction

Brazil has more than 300 species of social wasp (Vespidae Family; Polistinae Subfamily) of which 104 are endemic (Carpenter and Marques, 2001). Brazilian species are typically neotropical, with very different venoms from those found in temperate species. The Polistinae subfamily is represented by three tribes: Polistini, Epiponini and Mischocyttarini. The Epiponini tribe is constituted by 23 genera and 405 species, including the *Polybia paulista* species, which belongs to the Hymenoptera order (Reisman et al., 1984; Carpenter and Grimaldi, 1997).

Because of the close coexistence of social Hymenoptera with humans, the incidence of stings has increased. When stings occur, victims who have been sensitized to the injected venom may experience severe allergic reactions to the venom at the sting site, potentially resulting in manifestations of systemic anaphylaxis such as anaphylactic shock. Systemic anaphylaxis is life threatening to the allergic patient, potentially causing arrhythmia, bronchospasm, respiratory and cardiac arrest, shock and death of the patient (Reisman and Livingston, 1989; Freeman, 2004).

An estimated 25% of the general population suffers from some type of allergy, including those triggered by incidents with insect venom (Przybilla and Ruëff, 2012). Estimates of the proportion of individuals who are sensitive to social Hymenoptera venoms fall between 9.3% and 28.5% (Antonicelli et al., 2002), indicating that these allergies are a significant public health concern. Systemic reactions to venom from this order of insects occur in approximately 0.3–7.5% of the overall population (Charpin et al., 1992; Grigoreas et al., 1997; Strupler et al., 1997; Novembre et al., 1998; Fernandez et al., 1999).

These manifestations are directly associated with IgE-mediated sensitization to the proteins present in venoms of social Hymenoptera species, such as bees, wasps and ants (Müller, 1993, 2010, 2011). Thus, knowledge about the structure and function of these insect venom components, especially in the fields of allergy and clinical immunology, is crucial (Ross et al., 2000; Dreschler et al., 2011).

Although the components of vespine venom are generally less well characterized than those from bee venom, the habitat and venom composition of the social wasp *P. paulista* have been well studied in the fields of biochemistry, proteomics and immunology due to the accidents arising from its stings (da Silva et al., 2004; Santos et al., 2007, 2010; Pinto et al., 2012). Its venom has a similar protein composition to most venoms of the social Hymenoptera, consisting of biogenic amines, basic peptides and proteins of high molecular masses, particularly enzymes, among them the hyaluronidase, one of the main proteins responsible for allergic reactions. The biologically active peptides, together with other venom proteins are the main responsible for the prolonged pain, edema, erythema and systemic reactions (Castro et al., 1994; Müller, 2002).

Diagnosis and treatment of such allergies is currently limited by the availability and quality of the allergenic extracts, which can be used as tools for diagnosis and for promotion of specific desensitization. Compared with these allergen extracts, the development of recombinant allergens for measuring specific IgE represents an important

advance in the diagnosis and treatment of IgE-mediated allergies (Karoui et al., 2013).

The use of recombinant DNA technology for gene sequencing and expression of the major venom constituents from various organisms has enabled the elucidation of the molecular mechanisms of immunological cross-reactions among venom allergenic components, as well as the development of diagnostic kits and allergen-specific vaccines. Although there have been great advances in various expression systems, including bacteria, yeast, insect cells, mammalian cells and even cell-free systems, the bacterial expression system is still the most used due to its easy handling, rapid growth and low cost of recombinant protein production (Song et al., 2012). In this study, Hyal cDNA from the venom of the social wasp *P. paulista* (Pp-Hyal) (GI: 302201582) was cloned, expressed and analyzed immunologically in comparison to the natural allergen. The results demonstrate that the allergen Pp-Hyal-rec has great potential for use in immunotherapy, as well as in diagnostic kits for allergies caused by the venom from this and other closely related wasps.

2. Material and methods

2.1. Allergic patients

The samples studied consisted of ten sera from patients injured by stings from the social wasp *P. paulista*, with exclusive reactivity to this venom. All patient samples were obtained from the Ambulatório de Anafilaxia do Hospital das Clínicas, Faculdade de Ciências Médicas da Universidade Estadual de Campinas-UNICAMP regardless of sex or age. This institution offers treatment to patients from both the capital and the bordering municipalities of São Paulo state and southern Minas Gerais, Brazil. The study was approved by the Ethics Committee-FCM-UNICAMP under n° 187/2006. Informed consent was obtained in written form to all participants of the study and participation was voluntary. The samples were taken by collecting 5 mL of additional blood during the administration of routine tests ordered by the attending physician.

2.2. Insects

P. paulista nests and individuals of other wasp species (*Polybia sericea*, *Polybia ignobilis*, *Polybia occidentalis*, *Polybia scutellaris*, *Polistes lanio lanio* and *Agelaia palipes palipes*) as well as bee (*Apis mellifera*) and ant (*Solenopsis invicta*), captured within and around the campus of Universidade Estadual Paulista “Júlio de Mesquita Filho” – UNESP, Rio Claro, SP, Brazil, were transported to the Laboratory of Molecular Biology of Arthropods (LBMA) – Department of Biology – Institute of Biosciences – IB. After being subjected to analgesia at –20 °C, the venom glands were dissected and used for RNA extraction.

2.3. Native allergen (Pp-Hyal-nat)

Native hyaluronidase from the venom of the social wasp *P. paulista* (Pp-Hyal-nat) was isolated and purified by cation exchange chromatography on AKTA-FPLC and analyzed by

mass spectrometry MALDI-ToF/ToF. All analyses were performed according to the protocol previously described (Justo Jacomini et al., 2013).

2.4. RNA extraction and cDNA synthesis

Total RNA preparations from *P. paulista* were obtained from 100 glands per reaction tube, according to the TRIzol® method (Life Technologies, USA), using fresh material (at collection time) or glands macerated in TRIzol® and stored for 7 days at -85°C . cDNA synthesis was performed by RT-PCR with the ImProm-II™ Reverse Transcription System kit (Promega, USA) according to the manufacturer's directions, using 1 µg of total RNA and oligo dT-primer adapter (5' GGC CAC GCG TCG ACT AC (T) 17 3', synthesized by Gibco-Life Technologies, USA).

2.5. Cloning of Hyal coding sequence

The *Pp*-Hyal coding sequence was obtained by PCR from venom of the *P. paulista* social wasp using specific primers based on the Hyal cDNA complete sequence (GI: 302201582). Restriction sites for *Nde*I and *Eco*RI were, respectively included in the forward (5' TATA CATATG TCCGAAAGAAAGACCGAAAAGAGTGTTCAC 3') and reverse (5'AGTG GAATTC CTAAAGTTCAGGGATGATCT 3') primers (underlined) to allow the cloning of the coding sequence into the pET28a expression system. The *Pp*-Hyal sequence was also cloned in frame with a 6xHis tag at the N-terminal of the recombinant protein to allow purification of the recombinant protein by affinity chromatography. PCR was performed using Platinum Taq DNA Polymerase® (Life Technol, EUA) following the protocol previously described (Justo Jacomini et al., 2013) and the *Escherichia coli* XL1 Blue [recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F proAB lacIqΔM15 Tn10 (Tetr)]] (Stratagene, USA) was used as the cloning strain. The nucleotide sequence of the cloned region was verified by sequencing.

2.6. Expression and purification of recombinant Pp-Hyal

For the optimization of recombinant protein production, several clones of *E. coli* BL21 (DE3) cells [F-ompThsdSB (r-B mB-) gal demΔ(srl-recA) 306:Tn10(DE3)] (Novagen, USA) transformed with the expression vector pET28a-*Pp*-Hyal were tested under different expression conditions. Briefly, each selected clone of BL21-DE3 pET-28a-*Pp*-Hyal was inoculated into 5 mL of Luria Broth (LB) medium supplemented with kanamycin (50 µg/mL) and allowed to grow for 16 h at 37°C . These pre-inoculums were used to inoculate 50 mL of fresh medium. After cultures reached OD_{600nm} 0.3–0.6, they were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, Invitrogen, EUA) at different final concentrations (0.1, 0.5 and 1 mM) and different induction temperatures (37°C , 30°C and 20°C). Samples of 1.5 mL were collected at several times post-induction (0, 12, 24, 30, 48 and 72 h) and tested to determine the best induction conditions to produce recombinant protein *Pp*-Hyal.

Cells were collected by centrifugation (2600 g, 10 min), resuspended in phosphate-buffered saline pH 8.0 and then lysed by sonication in an ice bath for 2 min (Chubby,

GA450) using three pulses of 20 s, with 30 s between each pulse. The soluble and insoluble fractions were separated by centrifugation (9200 g, 4°C , 30 min), analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 15%) and stained with Coomassie Brilliant Blue R-250 (CBB).

After optimization of expression conditions, one *E. coli* BL21-DE3 clone carrying pET-28a-*Pp*-Hyal was selected for the production and purification of *Pp*-Hyal-rec protein. Ten milliliters of pre-inoculum, prepared as described, were used to inoculate 500 mL of LB/kanamycin (50 µg/mL) and the culture was incubated at 37°C under agitation (200 rpm) until OD_{600nm} 0.6 was reached. To induce protein expression, IPTG was added at a final concentration of 1 mM and cells were incubated at 37°C for 48 h. Cells were collected by centrifugation (10,000 g, 10 min, 4°C) and lysed by sonication. Because recombinant protein was expressed in the insoluble fraction as inclusion bodies, its purification by affinity chromatography was performed under denaturing conditions, following the Ni-NTA-Agarose manufacturer's protocol (Qiagen, Germany). In brief, the cellular pellet was resuspended in 25 mL of lysis buffer (50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, 5 mM imidazole and 0.2 mg/mL lysozyme) and incubated at 20°C with gentle agitation for 25 min before the sonication procedure (5 × 30 s pulses, maintaining the samples in ice bath between the pulses). After centrifugation (15,000 g, 15 min, 4°C), the insoluble fraction was resuspended in 10 mL of inclusion body solubilization buffer (8 M urea, 0.5 M NaCl, 50 mM Tris-HCl pH 8.0, 5 mM β-mercaptoethanol and 5 mM imidazole) and incubated overnight at 8°C under gentle agitation. The sample was then centrifuged (20,000 g, 15 min, 4°C) and the soluble fraction incubated (1 h at room temperature) with Ni²⁺-resin (Ni-NTA-Agarose, Qiagen, Germany) that had been previously equilibrated (100 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea, pH 8.0). This suspension was then applied to the column (5 mL) and washed with 10× column volume of washing buffer (100 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea, pH 6.3). Recombinant protein was eluted using elution buffer (100 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea, pH 4.5). Several samples were collected during the purification process for subsequent analysis by 12% SDS-PAGE.

2.7. IgE analysis

Sera from 10 patients allergic to *P. paulista* venom were evaluated for specific IgE recognition using 80 µg of purified *Pp*-Hyal-nat and *Pp*-Hyal-rec proteins, as well as 100 µg of *Pp*-crude-venom extract that had been previously submitted to 12% (w/v) SDS-PAGE along with a pre-stained standard molecular weight protein (High-molecular Weight Range Rainbow Markers, Amersham Biosciences, GE Healthcare, USA). The proteins were transferred to a nitrocellulose membrane using a semi-dry system (Nova Blot Unit, Multiphor II, Pharmacia Biotech). Transfer efficiency was assessed by staining the gel with Coomassie Blue G-250. The sera were previously diluted (1:50) in blocking solution (TBS-20% Tween containing 1% non-fat milk) and incubated overnight at 4°C . Immunodetection was performed using monoclonal antibody Anti-human IgE

alkaline phosphatase conjugated (Sigma–Aldrich, USA) diluted 1:5000. Bands were visualized with alkaline phosphatase/BCIP®/NBT (Sigma–Aldrich, USA).

2.8. IgE cross-reactivity

To analyze the IgE immune cross-reactivity of the allergic patients sera, 5 sera among the 10 previously tested were randomly chosen. Venom extracts were prepared from other wasp species (*P. sericea*, *P. ignobilis*, *P. occidentalis*, *P. scutellaris*, *P. lanio lanio* and *A. palipes palipes*) as well as bee (*A. mellifera*) and ant (*S. invicta*) and quantified for total protein, as described previously (Justo Jacomini et al., 2013). From each one of these extracts, 80 µg of total protein was subjected to gel electrophoresis for immunoblotting analysis.

3. Results

3.1. Cloning of the *P. paulista* venom hyaluronidase (*Pp-Hyal*) cDNA (GI: 302201582)

The pair of primers designed containing restriction sites for the enzymes *Nde*I and *Eco*RI amplified a fragment of 1017 bp. Following amplification, purification, digestion with these enzymes and sequencing, the fragment showed 100% identity with the *Pp-Hyal* deposited in GenBank (GI: 302201582).

3.2. *Pp-Hyal-rec* expression in *E. coli*

The allergenic protein *Pp-Hyal-rec* was heterologously expressed with a 6xHis tail at the N-terminal for its purification by affinity chromatography with Ni²⁺-agarose (Ni-NTA-Agarose) resin. So, the resulting *Pp-Hyal-rec* protein was 344 amino acids residues long with a theoretical MW of 40.48 kDa, in contrast with the 338 residues and theoretical MW of 39.65 kDa of the *Pp-Hyal* native. Here, it is important to mention that, although the concentration of polyacrylamide gels was always maintained at 15% in this

study, most likely due to intrinsic differences in each gel preparation or running, or the presence of different salts and buffers in each experimental step, the marker molecular weight standard, as well as native and recombinant proteins show differences in their electrophoretic profiles and therefore, bands with apparent molecular masses very different from their theoretical values calculated.

Different expression conditions were tested, including induction with varying concentrations of IPTG as well as different times and temperatures of induction. Analysis of *Pp-Hyal-rec* protein in the soluble and insoluble fractions of bacterial lysate showed that expression occurred in inclusion bodies, as seen in Fig. 1A, B, independent of the conditions used for induction of expression.

The best results were obtained with clone 8 under conditions of 1 mM IPTG and growth at 37 °C for 48 h, which resulted in the highest quantity of protein expressed (Fig. 1B). In times less than 24 h, low levels of protein expression were observed.

3.3. Inclusion body solubilization and *Pp-Hyal-rec* purification

Several protocols were tested for solubilization of inclusion bodies to obtain a higher level of recombinant protein purification. Best results were achieved using cell lysis in denaturing buffer with 8 M urea. Stepwise elution of the solubilized fraction rich in *Pp-Hyal-rec* from the column with two decreasing pH values (6.3 and 4.5) yielded better results (Fig. 2A, B) than when elution was performed with an imidazole gradient (data not shown) because a greater percentage of contaminating protein bands was observed in the latter.

3.4. Specific IgE immunodetection

The IgE immunoreactivity of the *Pp-Hyal-rec* protein was assessed by Western blotting with sera from patients exclusively reactive to the venom of the social wasp *P. paulista*. Specific reactivity was tested against *P. paulista*

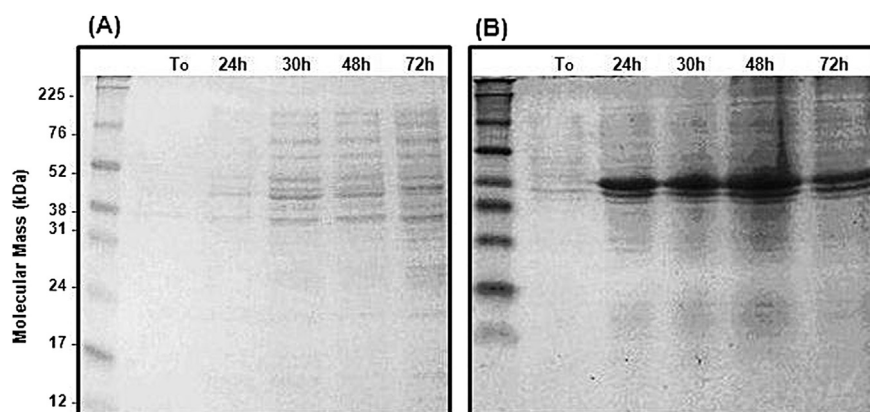


Fig. 1. SDS-PAGE (12%) showing the expression profile of *Pp-Hyal-rec* in *E. coli* BL21-DE3 cells as a function of the cultures' growing time at 37 °C and after induction with 1 mM IPTG. (A) represents the soluble fraction of the bacterial lysate, indicating the absence of *Pp-Hyal-rec* in all cultivation periods tested (24 h, 30 h, 48 h and 72 h). (B) represents the insoluble phase (inclusion bodies) showing that expression of *Pp-Hyal-rec* with 6xHis tag (344 amino acids residues; theoretical MW 40.48 kDa) reached the highest levels after 48 h of cultivation.

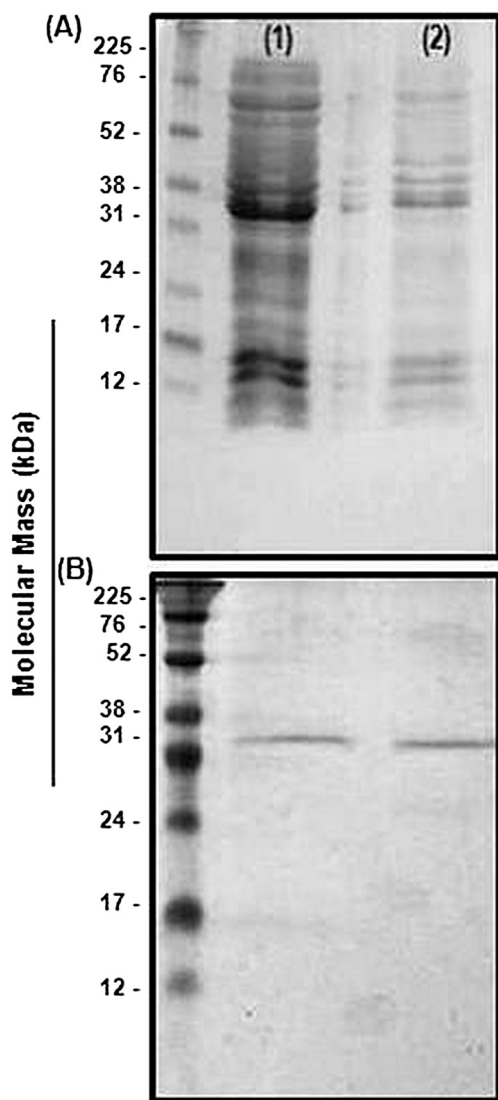


Fig. 2. Protein profile in 12% SDS-PAGE representing steps of solubilization and purification of the *Pp*-Hyal-rec attached to 6xHis, in an Agarose-Ni²⁺ (Ni-NTA-Agarose) affinity column previously equilibrated with buffer (50 mM Tris-HCl pH 8.0, 0.5 M NaCl, 5 mM β -mercaptoethanol, 5 mM imidazole and 8 M urea). (A) depicts in channel (1) proteins that did not bind to the resin and in (2) the fraction obtained after washing the column with washing buffer (100 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea, pH 6.3). (B) shows the purified recombinant protein that was eluted with 100 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea, pH 4.5. (M) Represents a standard molecular mass marker. Gels were stained with Coomassie Brilliant Blue R-250 (CBB).

crude venom extract as well as the purified native and recombinant forms of the *Pp*-Hyal allergenic protein. Serum from a non-sensitive individual was used as negative control. Fig. 3A–C shows that sera from the 10 patients tested recognized the crude venom extract as well as both native and recombinant *Pp*-Hyal proteins, with the band corresponding to recombinant protein *Pp*-Hyal recognized at a higher intensity than that of the native allergen. In *P. paulista* crude venom extract, sera also recognized additional

proteins besides Hyal with less intensity; these proteins are likely other allergenic components of the venom.

3.5. Analysis of IgE immune cross-reactivity

Immune cross-reactivity of 5 randomly chosen sera was tested against venom extracts from several related species. As shown in Fig. 4A–E, we can verify the existence of immune cross-reactivity due to IgE recognition of a protein with molecular weight between 39 and 43 kDa, likely corresponding to the allergenic protein Hyal, in venom extracts of *P. sericea*, *ignobilis* and *scutellaris*, as well as in *P. lanio lanio*. No IgE binding was detected with crude venom extracts of *A. mellifera* and *S. invicta*. The different profiles and intensities of reactions observed in the patients tested demonstrate the differences in IgE specificity and production levels. Additionally, we can observe in both Figs. 3 and 4A–E the recognition of other proteins in addition to the Hyal isoforms as described by Santos et al. (2010) and Pinto et al. (2012).

4. Discussion

Scientific studies of Hymenoptera stings have expanded into different areas of the biological sciences due to the diverse aspects of the reactions presented by victims after injury, especially those that trigger severe systemic manifestations that may lead to death (Bilò and Bonifazi, 2009). The social wasp *P. paulista*, endemic to southeastern Brazil, has a characteristically urban habitat and a very aggressive profile. Its stings result in many clinically significant incidents due to the complexity of its venom composition, which includes important allergens such as hyaluronidase (*Pp*-Hyal).

Dotimas and Hider (1987) reported that the allergens hyaluronidase and phospholipase are capable of provoking severe immunological reactions; these allergens are the major proteins responsible for the onset of allergic manifestations, triggering the production of specific IgE and causing hypersensitivity reactions in individuals stung by Hymenoptera. In turn, this class of antibody can bind to the high affinity Fc receptor (FcεRI) on the surface of mast cells and basophils, which consequently become sensitized, causing degranulation and the release of other factors (Kay, 2006; Larchè et al., 2006). In this study, the *Pp*-Hyal-rec, obtained by recombinant DNA techniques, was retained in bacterial inclusion bodies. Alternatives to minimize the formation of these aggregates, such as decreasing temperature and changing the concentration of the expression inducer, were tested, but did not successfully increase the expression of the soluble form of the *Pp*-Hyal-rec protein.

Proteins expressed in bacterial inclusion bodies are usually expressed at high levels, in some cases reaching 30% of the total protein from the host cell. These proteins thus exhibit lower levels of degradation, resistance to proteolytic attack by cellular proteases and greater homogeneity of the protein of interest, all of which help to reduce the number of purification steps for recovery of the pure protein (Walsh, 2004; Ventura and Villaverde, 2006). Furthermore, the purified proteins can be used with increased reliability for the production of specific

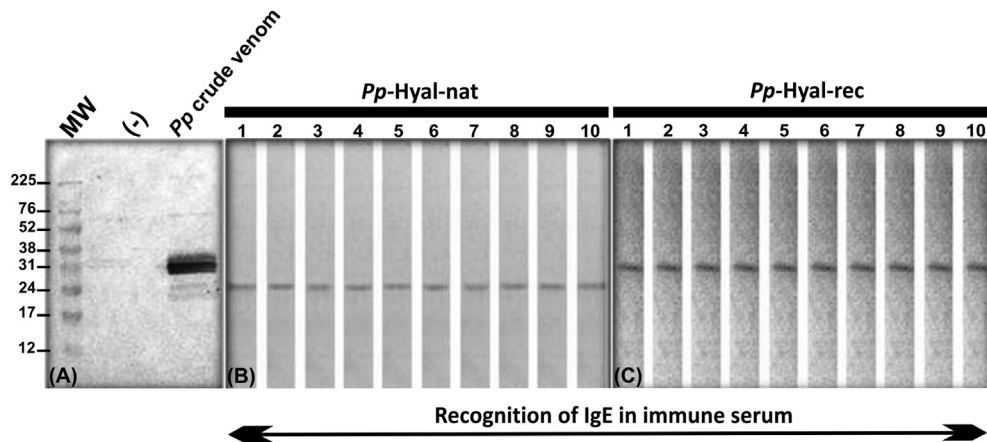


Fig. 3. Western blot from a 12% SDS-PAGE demonstrating the reactivity of IgE immune sera from 10 allergic patients to 80 μ g of purified native and recombinant forms of *Pp*-Hyal as well as to 100 μ g of total protein from *P. paulista* crude venom extract. In (A), the channel (–) represents a negative control containing sera from an insensitive patient, and the channel (*Pp*_crude venom) represents the IgE-mediated reactivity of serum of 1 of the 10 samples (randomly selected) against the allergenic protein *Pp*-Hyal (39 kDa, higher intensity band), and the lesser intensity of IgE recognition to other allergenic proteins present in the venom crude extract of this wasp (e.g., Ag5, PLA1 and proteases). (B) and (C) show allergen-specific IgE binding to the native protein (*Pp*-Hyal-nat, theoretical MW 39 kDa) and to the recombinant protein (*Pp*-Hyal-rec, theoretical MW 40 kDa), respectively, for 10 immune sera. Note that all sera recognized the recombinant allergen at a higher intensity than the native one. (MW) represents the standard molecular mass marker. Immunodetection was performed using Anti-human IgE alkaline phosphatase conjugated monoclonal antibody (Sigma–Aldrich, USA) diluted 1:5000, followed by visualization with the alkaline phosphatase/BCIP[®]/NBT colorimetric system (Sigma–Aldrich, USA).

polyclonal antibodies, as well as for the production of diagnostic tests and possibly allergen-specific immunotherapy.

The immunogenic potential of *Pp*-Hyal-rec expressed in *E. coli* was compared to the native allergen (*Pp*-Hyal-nat) by measuring the IgE reactivity of sera from ten patients

known to be allergic to *P. paulista* venom, as well as by determining the cross reactivity of these IgEs with venoms from different species of Hymenoptera (wasp, bee and ant).

Despite being in denatured form, after solubilization and purification the recombinant protein was able to bind to serum IgE of all allergic patients tested, and to a greater

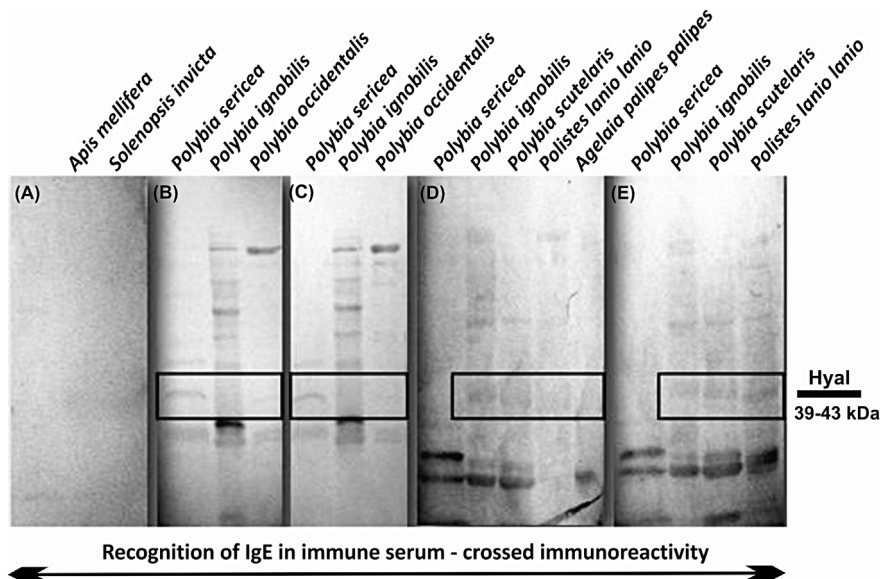


Fig. 4. Immunoblotting showing the IgE cross-reactivity profile of 5 samples of immune sera randomly chosen from the 10 studied against crude venom extracts from species of wasps, bees and ants. (A) shows the absence of IgE reactivity in one of the five sera against proteins of the extracts from bee venom (*Apis mellifera*) and ant (*Solenopsis invicta*). The sera shown in (B–E) (within the rectangles) displayed different intensity levels of recognition to a band between 39 and 43 kDa, most likely corresponding to the allergenic protein Hyal for the three species of *Polybia* (*sericea*, *ignobilis* and *scutellaris*) as well as *Polistes lanius lanius*. The recognition of other protein bands reveals the occurrence of a reaction of sera IgE with other allergenic proteins from these wasp venoms. Immunodetection was performed using monoclonal antibody Anti-human IgE alkaline phosphatase conjugated (Sigma–Aldrich, USA) diluted 1:5000, followed by visualization with the alkaline phosphatase/BCIP[®]/NBT colorimetric system (Sigma–Aldrich, USA).

extent than the binding observed with *Pp* Hyal-nat. These results strongly indicate that the linear epitopes, already described by structural modeling (Justo Jacomini et al., 2013) remained available in *Pp*-Hyal-rec and could be recognized by all sera IgEs from the allergic patients previously exposed to *P. paulista* venom. This also implies that the *Pp*-Hyal-rec protein is immunologically reactive even in its denatured form and thus has great potential to be used in specific immunotherapy.

A single band was detected in Western blot analysis for both *Pp*-Hyal-rec and *Pp*-Hyal-nat in 100% of patients tested, demonstrating the high degree of sensitivity and specificity of the IgE antibodies to the Hyal allergen and indicating that Hyal in *P. paulista* venom may be the primary protein responsible for triggering allergic symptoms caused after accidents with this wasp.

Regarding the IgE cross-reactivity of the sera studied here, with venoms of other species of Hymenoptera, we found that except for the reactions observed only with wasp venoms, IgE-mediated cross-reactivity with venoms from *A. mellifera* and *S. invicta* was not detected under the conditions tested. These results confirm those previously obtained with *Pp*-Hyal-nat (Justo Jacomini et al., 2013) but differ from those obtained by Aalberse et al. (1981), Hoffman et al. (1988), Hoffman (2006) and Egner et al. (1998) showing the occurrence of cross-reactivity with bee and wasp venoms from temperate climates in diagnostic tests applied to Hymenoptera allergic patients before the beginning of immunotherapy. In these studies, the authors indicate that, similar to our results, hyaluronidase is the main protein responsible for this response. According to King et al. (1996), patients allergic to wasp venoms exhibit responses to more than one venom due to cross-reactivity among homologous allergens present in the venoms of different species. This phenomenon is due to the presence of common epitopes reactive to specific IgE (Hoffman, 1985) because the antibodies are raised against highly conserved regions in these proteins (Barlow et al., 1986).

The specific IgEs from patient sera recognized *Pp*-Hyal in the same way and were highly specific for the *Pp*-Hyal allergen in both native and recombinant forms. Recognition occurred even though the recombinant protein obtained from bacteria was analyzed in its denatured state, and therefore non-glycosylated, which in principle could imply in a reduction or complete absence of any immunogenic activity. These results suggest that the IgE cross-reactivity to the Hyal allergen from the venom of another species of genus *Polybia* is likely due to similar amino acid residues present in epitopes of this allergenic component and not due to any residues of carbohydrate determinants (CCDs) because they are absent in the *Pp*-Hyal-rec expressed in bacteria.

Recombinant protein technology has allowed for the production of a large number of individual allergens and pools of allergens to produce new therapeutic products, as well as tools for diagnosis and for allergen-specific immunotherapy. Considering that robust reactivity of allergic patient sera against *Pp*-Hyal-rec was verified by our immunoblotting results, this recombinant allergen has potential for use in the development of more sensitive and

standardized specific immunodiagnostic tests to assist in the monitoring and immunotherapy (IT) of patients allergic to this social insect that is quite abundant in southeastern Brazil.

Ethical statement

The study was approved by the Ethics Committee of Faculty of Medical Sciences of Universidade Estadual de Campinas-UNICAMP FCM-UNICAMP under n° 187/2006.

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Conflict of interest

The authors have no conflicts of interest to declare.

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