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Recombinant phospholipase A1 from *Polybia paulista* wasp venom for molecular diagnosis of allergy

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SETEMBRO 2017

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Tese de Doutorado apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista "Júlio de Mesquita Filho", como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Biologia Celular e Molecular).

Orientadora: Profa. Dra. Márcia Regina Brochetto Braga, PhD.

Co-orientador: Prof. Alexis Musacchio Lasa, PhD

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TÍTULO DA TESE: Recombinant phospholipase A1 from Polybia paulista wasp venom for molecular diagnosis of allergy

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Rio Claro, 22 de setembro de 2017

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RESUMO

Pérez-Riverol, A. **Fosfolipase A1 recombinante do veneno de *Polybia paulista* para o diagnóstico molecular de alergia.** [Tese]. Rio Claro: Instituto de Biociências da Universidade Estadual Paulista "Júlio de Mesquita Filho", 2017.

A fosfolipase A1 é um dos principais alérgenos identificados no veneno do *Polybia paulista* (Hymenoptera: Vespidae), uma vespa social de elevada importância clínica no sudeste do Brasil. A produção recombinante deste alérgeno contribuirá com o desenvolvimento do diagnóstico molecular de alergia. Neste trabalho é descrita a produção recombinante da fosfolipase A1 de *P. paulista* (rPoly p 1) no sistema celular *Escherichia coli*. Elevados níveis da rPoly p 1 na forma insolúvel foram obtidos após expressão na bactéria. A otimização das condições de solubilização permitiu incrementar os níveis de recuperação do alérgeno recombinante. A rPoly p 1 foi purificada (99%) até homogeneidade mediante cromatografia de afinidade em coluna de Ni^{2+}, mostrando valores de rendimento finais de 1.5 g/L de meio de cultura. A forma nativa do alérgeno (nPoly p 1) foi purificada mediante cromatografia de troca catiônica. A rPoly p 1 foi reconhecida pela IgE específica de soros de pacientes sensibilizados ao veneno de *P. paulista*. O uso da rPoly p 1 permite diferenciar a ocorrência de real dupla sensibilização ao veneno de vespa e formiga ou vespa e abelha da incidência de reatividade cruzada. Soros de pacientes com IgE específica ao veneno de abelha e formiga não reagiram com a rPoly p 1, enquanto que soros de camundongos sensibilizados com rPoly p 1 apresentaram reatividade cruzada exclusivamente com fosfolipases A1 (PLA1) de vespas Neotropicais ou de climas temperados. O alinhamento múltiplo do modelo 3-D da rPoly p 1 sugere que a base molecular desta reatividade é a presença de epitopos lineares e conformacionais compartilhados pelas PLA1s das espécies avaliadas. A presença de CCDs no veneno de varias espécies de vespas Neotropicais foi também analisada. Os resultados apresentados nesta Tese indicam que a rPoly p 1 pode ser utilizada no diagnostico molecular de alergia a veneno de *P. paulista*. O uso da rPoly p 1 ira a melhorar à identificação específica de veneno responsável pela sensibilização primaria e por tanto o desenho da imunoterapia.

Palavras chaves: *Polybia paulista*, fosfolipase A1 recombinante, sensibilicação, reatividade cruzada, IgE específica

ABSTRACT

Perez-Riverol, A. **Recombinant phospholipase A1 from *Polybia paulista* venom for molecular diagnosis of allergy**. [Thesis]. Rio Claro: Instituto de Biociências da Universidade Estadual Paulista "Júlio de Mesquita Filho", 2017.

Phospholipase A1 (PLA1) is one of the major allergens identified in the venom *Polybia paulista* (Hymenoptera: Vespidae), a clinically relevant social wasp from Brazil Southeast. The recombinant production of this allergen could result in the development of molecular diagnosis of allergy thus improving the outcomes of venom immunotherapy (IT). Here, we describe the heterologous production of the PLA1 from *P. paulista* venom in *Escherichia coli*. High levels of the insoluble recombinant allergen (rPoly p 1) were obtained after expression in the prokaryotic system. The downstream optimization of the solubilization process resulted in high levels of protein recovery. The rPoly p 1 was purified to homogeneity (99%) using an immobilized Ni^{2+} metal affinity chromatography while a single-step cation-exchange chromatography allowed the purification of native Poly p 1 (nPoly p 1) from the venom glands. Immunoblotting analyses showed the IgE-mediated recognition of the rPoly p 1 by sera from patients sensitized to *P. paulista* venom. The rPoly p 1 could allow the differentiation of true double sensitization to wasp/bee and wasp/ant venoms from cross-reactivity. The sera from patients with monosensitization to honey bee or fire ant venoms do not cross-reacted with the recombinant allergen. Meanwhile, the sera from rPoly p 1-sensitized mice cross-reacted with venoms of other clinically relevant wasps from Neotropical and temperate regions. The alignment of the 3-D model from rPoly p 1 with the PLA1s from some of these wasps suggested the presence of homologues epitopes as the molecular basis for the cross-reactivity. The presence of cross-reactive carbohydrates determinants (CCDs) in the venom of several Brazilian wasps, which is a major issue for understanding the incidence of cross-reactivity during diagnosis, was also analyzed. Overall, the results described in this work suggest that rPoly p1 is a feasible candidate for the rational design of molecular diagnosis of *P. paulista* venom allergy. The use of rPoly p 1 will allow the identification of the primary sensitizing insect thus leading to a significant improvement in the outcomes of the venom immunotherapy.

Keywords: *Polybia paulista*, recombinant phospholipase A1, sensitization, cross-reactivity, specific IgE

ABBREVIATIONS:

BAT: basophil activation test

CCDs: cross-reactive carbohydrate determinants

CRD: component resolved diagnosis

ESTs: expressed sequence tags

HBV: honey bee venom

YJV: yellow jacket venom

HPLC: high-performance liquid chromatography

HVA: Hymenoptera venom allergy

IPTG: isopropyl β-D-1-thiogalactopyranoside

LLRs: large local reactions

MIC: minimum inhibitory concentration

NanoLC-ESI-CID: Nano liquid chromatography electrospray ionization collision-induced dissociation

nPoly p 1: native phospholipase A1 from *Polybia paulista* venom

nPoly p 2: native hyaluronidase from *Polybia paulista* venom

nPoly p 5: native antigen-5 from *Polybia paulista* venom

PLA1: phospholipase A1

PLA2: phospholipase A2

PMNLs: polymorphonuclear leukocytes

PTMs: post- translational modifications (PTMs)

RMSD: root-mean square deviation

rPoly p 1: recombinant phospholipase A1 from *Polybia paulista* venom

sIgE: specific IgE

SIT: specific immunotherapy

SLIT: sublingual immunotherapy

vPLA1s, vespid phospholipases A1

FIGURES LIST

CHAPTER 1

Article 1: Wasp venomic: Unravelling the toxins arsenal of *Polybia paulista* venom and its potential pharmaceutical applications. **Journal of Proteomics** 2017, 161 88–103

CHAPTER 2

Article 3: Molecular cloning, expression and IgE-immunoreactivity of phospholipase A1, a major allergen from *Polybia paulista* (Hymenoptera: Vespidae) venom. **Toxicon 2016,** 124, 44–52.

Ves g 1) and the ant PLA1 from *Solenopsis invicta* venom (Sol i 1). In green are the amino acid residues of the catalytic triad (serine 137, asparagine 165 and histidine 226). Red triangles show the correspondent positions in the mature rPoly p 1. In grey are the identical residues.

9- **Figure 3.** SDS-PAGE (8-20%) analysis showing the expression profile of rPoly p 1 in *E. coli BL21* (DE3) cells at different times after induction (20 °C, 1 mM IPTG). T5-T10 represent hours after induction. (-) corresponds to a negative control (non-induced clone at T_{10}).

10- **Figure 4.** SDS-PAGE (12%) analysis of rPoly p 1 *E. coli BL21* (DE3) cell lysate. (S) soluble and (I) insoluble fraction after cell disruption and centrifugation. T8-T10 represent hours after induction. (-) corresponds to a non-induced rPoly p 1 clone at T_{10}, used as a negative control.

11- **Figure 5.** SDS-PAGE (12%) analysis of fractions obtained during rPoly p 1 purification process. Samples: S= Solubilized proteins (8M urea); NB = Protein not bound to the column; W1-W2= fractions corresponding to column-washing steps with 75 and 100 mM imidazole; E= fraction obtained after elution with 200 mM imidazole.

12- **Figure 6.** Cation-exchange chromatography profile of P. paulista crude venom extract (23 mg), obtained in a Hiprep FF CM column (16X10 mm, 20 mL; GE Healthcare) coupled to an AKTA-FPLC system. The column was washed with an isocratic buffer of 50 mM sodium acetate pH 5.2. The elution was performed under a linear gradient from 0 to 1 M NaCl in the same buffer, at a flow rate of 1 mL/min, collecting fractions of 5 mL. The protein was monitored by measuring the absorbance at 280 nm, represented by a continuous line. Phospholipase A1 enzymatic activity is represented with a shifting line-block.

13- **Figure 7.** SDS-PAGE (12%) analysis of crude venom extract, nPoly p 1 and rPoly p 1. Gel was staining with silver-nitrate.

14- **Figure 8.** (A, B, C). Antigenicity of Poly p 1 protein evaluated using a Western blot. Recognition of (A) Poly p 1 in crude venom, (B) native-purified (nPoly p 1) and (C) recombinant (rPoly p 1) by serum IgE in *P. paulista* venom-sensitized patients. Serum samples: P1-P10= individual sera of *P. paulista* venom-sensitized patients; P = pool of sera; N =serum from nonsensitized patient, as a negative control. The molecular weight marker (kDa) is indicated.

CHAPTER 3

Manuscript 4: Improved production of the recombinant phospholipase A1 from *P. paulista* wasp venom expressed in *E.coli* (Submitted to **Molecular Biotechnology**)

CHAPTER 4

Manuscript 5: Venoms from several clinically relevant Neotropical wasps are devoid of cross-reacted carbohydrate determinants (To be submitted on **Journal of Allergy and Clinical Immunology**)

Manuscript 6: Cross-reactivity analyses of the recombinant phospholipase A1 from *Polybia paulista* wasp venom (To be submitted on **Molecular Immunology**)

TABLES LIST

CHAPTER 1

Article 1: Wasp venomic: Unravelling the toxins arsenal of *Polybia paulista* venom and its potential pharmaceutical applications. **Journal of Proteomics** 2017, 161 88–103

CHAPTER 3

Manuscript 4: Improved production of the recombinant phospholipase A1 from *P. paulista* wasp venom expressed in *E.coli* (Submitted, **Molecular Biotechnology**)

CHAPTER 4.

Manuscript 6: Cross-reactivity analyses of the recombinant phospholipase A1 from *Polybia paulista* wasp venom.

INDEX

1. GENERAL INTRODUCTION

Polybia paulista (Hymenoptera: Vespidae) is a clinically relevant Neotropical wasp that causes a high number of sting accidents in Southeast Brazil (GUIMARÃES, 2009). The venom of this wasp comprises a complex mixture of bioactive toxins ranging from low molecular weight compounds to peptides and allergenic proteins (DOS SANTOS et al., 2010; DIAS et al., 2015). In sensitized individuals, *P. paulista* venom can cause a wide range of allergic reactions including life threatening anaphylaxis (DOS SANTOS et al., 2010). The early specific diagnosis of allergy is a mandatory prerequisite for starting the venom immunotherapy, the unique disease-causative treatment currently available (OLLERT; BLANK, 2015).

The diagnosis of *P. paulista* venom allergy is based on the history of allergic reactions, skin tests and *in vitro* IgE detection using crude venom extracts (JUSTO JACOMINI et al., 2014). The use of these allergenic materials for detection of specific IgE (sIgE) is often related to high levels of cross-reactivity caused by the presence of the cross-reactive carbohydrate determinants (CCDs) or/and common B-cell epitopes in homologues allergens from different insects (SPILLNER; BLANK; JAKOB, 2014). Furthermore, the use of crude venoms during immunotherapy could cause undesired side effects (BONIFAZI et al., 2005).

The production of recombinant allergens has remarkably improved the specific identification of the primary sensitizing insect (JAKOB; SPILLNER; MULLER, 2017). The expression of venom allergenic proteins in different cell systems including *E. coli* (JUSTO JACOMINI et al., 2014), *Pichia pastoris* (VINZÓN et al., 2010; BORODINA et al., 2011) and insect cells (SEISMANN et al., 2010a) allowed the production of large amounts of native-like allergens that can be used in component-resolved diagnosis (CRD) of allergy. Furthermore, the heterologous expression using *E. coli* and *Spodoptera frugiperda* Sf9 cells enables the production of CCD-depleted molecules, helping to decrease the cross-reactivity incidence (SEISMANN et al., 2010b). To date, dozens of venom allergens have been heterologously expressed and several are currently being tested in molecular diagnosis (SPILLNER; BLANK; JAKOB, 2014).

Despite the wide diversity of Hymenoptera identified in Brazil that includes 324 species of wasps (LOCHER et al., 2014), no recombinant forms of venom allergens from Brazilian clinically relevant insects are available for allergy diagnosis (JUSTO JACOMINI et al., 2014). The lack of allergenic components obtained as recombinant proteins hampers the development of molecular diagnosis, thus increasing the incidence of cross-reactivity and misidentification of the culprit insect. The inclusion of non-relevant venoms on immunotherapy caused by improper diagnosis often results in *de novo* sensitization of patients and increased risk of undesired side effects (SPILLNER; BLANK; JAKOB, 2014).

Venomic analyses of *P. paulista* led to the identification of three major allergens: phospholipase A1 (Poly p 1) (DOS SANTOS et al., 2007; SANTOS et al., 2010), hyaluronidase (Poly p 2) (PINTO et al., 2012; JUSTO JACOMINI et al., 2013) and antigen 5 (Poly p 5) (DOS SANTOS-PINTO et al., 2014). During envenomation, the native Poly p 2 (nPoly p 2) acts as a spreading factor that facilitates venom diffusion from the site of the sting (PINTO et al., 2012). Venom hyaluronidases cleave the hyaluronan, a polysaccharide of the extracellular matrix found in connective tissue facilitating the venom toxins diffusion (BORDON et al., 2015). Meanwhile, native Poly p 1 (nPoly p 1) activity disrupts the phospholipid packing of biological membranes and as other venom PLA1s could cause severe hemolysis leading to cardiac dysfunction and death in animals (SANTOS et al., 2007; HOU et al., 2016). In addition, nPoly p 5 is a highly ubiquitous protein of unknown physiological role during envenomation (DOS SANTOS-PINTO et al., 2014).

Previous to this work, the recombinant forms of Poly p 2 (rPoly p 2) and Poly p 5 (rPoly p 5) expressed in *E. coli* and *P. pastoris* cells, respectively, had been evaluated for IgE-mediated immunorecognition by sera of allergic patients (JUSTO JACOMINI et al., 2014; BAZON, 2017). The heterologous expression along with the structural and immunological characterization of the Poly p 1 will complete the set of the recombinant major allergens from *P. paulista* that could be included in panels of venom components for the development of molecular diagnosis of allergy.

The nPoly p 1 is a ~34 kDa, non-glycosylated, and therefore CCD-free enzyme, that belongs to the lipase GX class (SANTOS et al., 2007). The tridimensional model of the allergen showed an α/β fold common to many lipases: a core consisting of a

tightly packed β-sheet composed of a six-stranded parallel and one anti-parallel β-strand, surrounded by four α-helices. The primary sequence of the nPoly p 1 contains 13 cysteine residues with 12 potentially involved in disulfide-bonds formation (SANTOS et al., 2007). The presence of these disulfide-bonds in Poly p 1 structure could be critical for the proper folding and soluble production of the allergen in bacterial cells. It has been well documented that disulfide-bonds formation during protein expression in *E.coli* is compromised by the reducing conditions of bacterial cytoplasm (BERKMEN, 2012).

To date, the venom PLA1 from *Vespula vulgaris* (Ves v 1) which is one of the major cause of wasp allergy in Europe has been extensively used for distinction of true double sensitization to wasp and honey bee venoms (HBV) from cross-reactivity (SEISMANN et al., 2010a, 2010b; MÜLLER et al., 2012). Ves v 1 has been expressed in *E. coli* (KING et al., 1996), yeast (BORODINA et al., 2011) and insect cells (SEISMANN et al., 2010a). The expression in the eukaryotic systems resulted in the production of a native-like soluble allergen but with significantly low protein yields. Recombinant forms of the venom PLA1s from *Polistes spp.* have been also used for identification of the primary sensitizing insect (MONSALVE et al., 2012). Similar to these wasp PLA1s in the case of *Vespula* and *Polistes* spp., the recombinant production of Poly p 1 could be useful for the differential diagnosis of *P. paulista* venom allergy.

This work aimed to produce the Poly p 1 as a heterologous protein and to conduct several structural and allergomic analyses for the characterization of the recombinant allergen. Also, as a mandatory prerequisite for the introduction of rPoly p 1 in routine diagnosis, a comprenhensive analysis of the molecular basis for the cross-reactivity incidence during *P. paulista* allergy diagnosis and related to the use of this recombinant allergen, was performed. As noted, cross-reactivity could be caused by the CCDs and/or common B-cells epitopes in homologues allergens from different insects. Previous to this work, the presence of CCDs in the venom of *P. paulista* and several other clinically relevant Neotropical wasps remains unexplored, hampering the understanding of the molecular mechanism underlying the cross-reactivity incidence during diagnosis. Also, little was known about the PLA1s-based cross-reactivity in wasp venoms.

The results obtained in this thesis will be presented in the format of six scientific papers (three published articles and three manuscripts) divided in four chapters. In the first chapter, comprehensive revisions on the venomic analyses of *P. paulista* and trends on the diagnosis of Hymenoptera venom allergy are provided. The early results obtained after the recombinant expression of the rPoly p 1 in *E. coli* cells, along with the chromatographic procedures used for the purification of rPoly p 1 and nPoly p 1 are described in Chapter 2. This chapter also shows a preliminary analysis of the sIgE-mediated immunorecognition of rPoly p 1 by sera from patients previously diagnosed with sensitization to *P. paulista* venom. In Chapter 3, an improved strategy for the recombinant production of the allergen in *E.coli* along with the results obtained after the evaluation of the expression in the methylotrophic yeast *P. pastoris*, are described. Finally, in Chapter 4, the molecular basis for the incidence of cross-reactivity are analyzed and discussed in two different manuscripts. For the first time the absence of CCDs in venoms of Brazilian wasps and the incidence of PLA1-based cross-reactivity among clinically relevant insects regarless the geographical origin was showed.

The results obtained in this thesis suggest that rPoly p 1 is a valuable marker to discriminate the occurrence of clinically relevant wasp/bee and wasp/ant sensitizations from cross-reactivity, a major goal for the development of molecular diagnosis of allergy. The combined use of rPoly p 1 with rPoly p 2 and rPoly p 5, which are also under evaluation in our project will improve the diagnosis of *P. paulista* venom allergy, the outcome of the venom immunotherapy and overall, the quality of life of the allergic patients.

2. OBJECTIVES

2.1 General objective

The recombinant expression, purification and downstream molecular and immunological characterization of the phospholipase A1 from *P. paulista* wasp venom, envisioning its use in molecular diagnosis of allergy.

2.2 Specific objectives

Chapter 1:

1. To review the state of the art of the major results obtained in peptidomic, proteomic and allergomic analyses of *P. paulista* venom and the trends in molecular diagnosis of Hymenoptera venom allergy.

Chapter 2:

2. Evaluation of the production of rPoly p 1 in *E. coli* BL21 (DE3) cells using the bacterial vector for cytoplasmatic expression pET_28a.

3. Purification of the expressed rPoly p 1 using an immobilized Ni^{2+} metal affinity chromatography and the 6xHis tag provided by the pET_28a vector.

4. Purification of the native form of the Poly p 1 from *P. paulista* venom glands and the quantification of the specific phospholipase A1 activity.

5. Analysis of the IgE-mediated immunoreactivity of sera from patients sensitized to wasp venoms with purified rPoly 1 and nPoly p 1.

Chapter 3:

6. Evaluation of different culture conditions for soluble expression of rPoly p 1 in the cytoplasm of *E. coli* BL21 (DE3) cells.

7. Molecular cloning and heterologous expression of rPoly p 1 in the methylotrophic yeast *P. pastoris*.

8. Optimization of the solubilization process of rPoly p 1-inclusion bodies to increase the overall yield of the solubilized allergen.

9. Preliminary evaluation of the sensitivity related to the use of rPoly p 1 using a cohort of sera (n=40) from patients previously diagnosed with sensitization to wasp venoms.

Chapter 4:

10. Determination of the CCDs presence in venoms from several clinically relevant Brazilian insects.

11. Analysis of the IgE-mediated recognition of nPoly p 1 and *P. paulista* venom by sera from rPoly p 1-immunized mice to test the immunogenicity of the heterologous allergen.

12. Analyses of the cross-reactivity of sera from rPoly p 1-sensitized mice with venoms of clinically relevant Hymenoptera from Neotropical and temperate regions.

13. *In silico* structural analyses of insect venom PLA1s from several clinically relevant Hymenoptera to elucidate the molecular basis of the cross-reactivity related to the use of rPoly p 1.

14. Analyses of the cross-reactivity of sera from patients monosensitized to honey bee or fire ant venoms with rPoly p 1 and *P. paulista* crude venom extract.

3. CHAPTER 1

Wasp venomic: Unravelling the toxins arsenal of *Polybia paulista* venom and its potential pharmaceutical applications. **Perez-Riverol, A**; dos Santos-Pinto, J.R.A.; Musacchio-Lasa, A.; Palma, M.S. and Brochetto-Braga, M.R. (**Journal of Proteomics** 2017, 161, 88–103).

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Review

Wasp venomic: Unravelling the toxins arsenal of *Polybia paulista* venom and its potential pharmaceutical applications

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abstract

Polybia paulista (Hymenoptera: Vespidae) is a Neotropical social wasp from southeast Brazil. As most social Hymenoptera, venom from *P. paulista* comprises a complex mixture of bioactive toxins ranging from low molecular weight compounds to peptides and proteins. Several efforts have been made to elucidate the molecular composition of the *P. paulista* venom. Data derived from proteomic, peptidomic and allergomic analyses has enhanced our understanding of the whole envenoming process caused by the insect sting. The combined use of bioinformatics, *-omics-* and molecular biology tools have allowed the identification, characterization, *in vitro* synthesis and recombinant expression of several wasp venom toxins. Some of these *P. paulista* - derived bioactive compounds have been evaluated for the rational design of antivenoms and the improvement of allergy specific diagnosis and immunotherapy. Molecular characterization of crude venom extract has enabled the description and isolation of novel toxins with potential biotechnological applications. Here, we review the different approaches that have been used to unravel the venom composition of *P. paulista*. We also describe the main groups of *P. paulista* - venom toxins currently identified and analyze their potential in the development of component-resolved diagnosis of allergy, and in the rational design of antivenoms and novel bioactive drugs.

Contents

Abbreviations: PLA1, phospholipase A1; PLA2, phospholipase A2; Poly p 1, phospholipase A1 from *Polybia paulista* venom; Poly p 2, hyaluronidase from *Polybia paulista* venom; Poly p 5, antigen-5 from *Polybia paulista* venom; HPLC, high-performance liquid chromatography; HVA, Hymenoptera venom allergy; SIT, specific immunotherapy; PMNLs, polymorphonuclear leukocytes; CRD, component resolved diagnosis.

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1. Introduction

Sting accidents caused by social Hymenoptera represent one of the three major causes of anaphylaxis among human population worldwide [1]. Venoms from these insects are mixtures of unique natural weapons evolved to ensure the defense of the colony against predators and prey capture [2]. Venom toxin cocktails include low molecular weight compounds - terpenes, biogenic amines -, highly abundant peptides, and allergenic proteins - phospholipases A1, A2, hyaluronidases, acid phosphatase and antigen 5 - [3]. Low molecular weight compounds, and peptides are often involved in toxic reactions causing pain, inflammation, tachycardia/bradycardia and cardiac arrhythmia [4]. Meanwhile, allergenic proteins are related to local and/or systemic allergic reactions including life-threatening anaphylaxis related to the occurrence of HVA.

More than 20,000 species of bees (Apoidea) and ants (Formicidae) along with 15,000 species of wasps and yellow jackets (Vespoidea) have been identified as clinically relevant [5]. To date, systemic analyses based on the use of biomolecule databases, bioinformatic tools and -*omics*- approaches including genomic, transcriptomic, proteomic, peptidomic and glycomics have been successfully used to unravel the toxins arsenal from these venomous animals [6,7]. Venomic analyses of bioactive molecules from social Hymenoptera have enabled the rational design of novel antitoxins [8], and the evaluation of venom components for potential biotechnological applications. Furthermore, detection and molecular characterization of allergenic proteins from Hymenoptera venoms have enabled the production of recombinant allergens then allowing the development of component resolved diagnosis (CRD) and molecular-defined immunotherapy (IT) of HVA [9]. Unlike traditional methods based on the use of crude venom extracts that showed an extremely variable composition [10], these novel alternatives for allergy diagnosis and SIT use defined panels of standardized and highly purified allergens with characterized physiochemical and immunologic profiles. Consequently, the use of recombinant allergens as the allergenic materials significantly improves the reliability of the diagnosis and the safety profile of the immunotherapy [3,7].

Brazil hosts nearly 320 species of social wasps which represent 57% and 33% of the species currently described in Latin America (552) and worldwide (974), respectively [11]. The close coexistence between social wasps and the human populations (Fig. 1) causes a high number of medically important stinging accidents, due to the highly aggressive behavior of these insects [12]. The victims may experience local toxic and/or immunological life-threatening reactions, depending on the number of wasps involved in the accident, the amount of venom

Fig. 1. Distribution of *P. paulista* wasp in South America.

injected and the levels of sensitization to venom allergens. Unfortunately, the vast majority of these accidents and the prevalence of HVA have been neglected for decades in Brazil as a consequence of the lack of data collected by health institutions and survey analyses. The World Health Organization (WHO) considered the accidents of envenoming caused by venomous animals (snakes, spiders, scorpions, wasps, ants and bees) as a neglected public health issue in the developing countries [13]; this was latter recognized by the Brazilian Academy of Sciences [14].

Epidemiological studies have estimated that 56.6%–94.5% of human populations have been stung by Hymenoptera, at least once in lifetime [15]. The prevalence of systemic allergic reactions related to HVA is 0.3–8.9%, with anaphylaxis occurring in 0.3–42.8% of the cases [1]. The prevalence of the accidents caused by insects sting have been predicted to be higher in developing and low-income countries since a higher percentages of humans develop their economic activities near to populations of Hymenoptera insects [16]. All these data strongly suggest that Hymenoptera stings and Hymenoptera venom anaphylaxis are outstanding human health problems not only in Brazil, but also in whole Latin America. This is particularly true for social wasps considering the remarkable diversity of species currently identified in the country cohabitating with humans. Moreover, at least one epidemiologic study conducted with Brazilian patients ($n = 138$) showed that wasp venom was the main cause of HVA in the population analyzed [17].

The epidemiological data currently available suggest that 10 to 15 thousands sting accidents related to bees and/or wasps occur annually in Brazil. Most of these are caused by *Polybia paulista* (Hymenoptera: Vespidae) with 35–42 deaths registered every year [18,19]. *P. paulista* –popularly *known* as "paulistinha"– is a clinically relevant neotropical wasp that belongs to the Polistinae subfamily which is divided in four tribes (Ropalidiini, Polistini, Epiponini and Mischocyttarini) [20]. Several studies showed that the species from Epiponini tribe are the most commonly founded in Southeast Brazil [11]. Moreover, members of the *Polybia* group -including *P. paulista*- have been reported as the most abundant species founded in urban garden from this region (Fig. 1) [21]. As a consequence, *P. paulista*-related sting accidents and venom allergy are highly frequent, and represent major causes of human morbidity in Southeast Brazil.

Considering these facts, *P. paulista* have been extensively stud- ied, and became a model for proteomic, peptidomic, immunological and pharmacological studies in insect venom toxinology during the last two decades. Several efforts have been made to explore and pro- file the toxin arsenal of this endemic wasp as a prerequisite to devel- op antivenoms and to treat *P. paulista*-related toxic and allergic reactions.

Early attempts to elucidate the venom composition of this wasp were based on the use of low-resolution chromatographic methods, which were able to detect a limited number of toxins [22]. Nevertheless, recent studies [23,24] performed using more resolutive proteomic and peptidomic techniques have promoted a systemic comprehension of crude venom extract composition. The elucidation of *P. paulista*-toxins arsenal allows us to hypothesize about a general envenoming mechanism [23].

It has been informed that peptides comprise up to 70% of the weight of freeze-dried venom in social Hymenoptera [2]. The use of high-throughput techniques have boosted the identification of frequent as well as rare venom peptides generating a large quantity of data related to venom composition [25]. Thousands of venom peptides from snake [26,27], spider [28,29], scorpion [30], marine snails [31] and solitary/social Hymenoptera [32,33] have been detected and described after the implementation of these approaches. In the case of *P. paulista* venom, the use of peptidomic analyses resulted in the description and further molecular characterization of known and also novel wasp-venom peptides [24,34]. The peptides toxins characterized in *P. paulista* displayed diverse structural features and biological activities revealing the complexity of toxins arsenal produced by this social wasp.

Despite the great diversity of social Hymenoptera identified in Brazil, no component-defined allergenic materials are available for specific diagnosis of allergy [12,35]. Currently, HVA diagnosis is based on the use of crude venom extracts from native species or commercialized allergens from insects of Northern Hemisphere. The use of crude venoms increases the incidence of cross-reactivity due to the presence of cross-reactive carbohydrate determinants (CCDs) [36]. Meanwhile, diagnosis of Brazilian patients conducted with allergens from species of temperate regions lead to a decrease in sensibility and miss-identification of the culprit venom thus compromising the efficacy and safety profile of the specific immunotherapy (SIT). Inclusion of non-relevant allergens in immunotherapy leads to non-specific sensitization of patients and can cause life-threatening systemic side-effects [37]. The incidence of adverse side effects worldwide during immunotherapy has been estimated in 25.1% and 5.8% for honeybee and vespid venoms, respectively [38]. Overall, these facts set significantly challenges for the aim of decreasing the morbidity related sting accidents and HVA in Brazil.

Venomic analyses represent a powerful approach to face these challenges as they provide systemic information about the proteinaceous components comprised in social Hymenoptera venoms, including allergens. To date, more than 60 Hymenoptera venom allergens have been identified, cloned, heterologously expressed and are currently being tested [3] allowing the design of panels of recombinant allergens that can be used in allergy CRD and molecular-defined SIT. Proteomic analysis allowed the identification and purification of the three major allergens from *P. paulista* venom: phospholipase A1 (Poly p 1) [39], hyaluronidase (Poly p 2) [40,41] and antigen 5 (Poly p 5) [42]. These allergens are medium (Poly p 2) to highly abundant (Poly p 1 and Poly p 5) venom proteins [23,35,39,41,43]. The combined use of bioinformatics and proteomic tools as well as molecular biology techniques resulted in the cloning, sequencing and further heterologous expression in *E. coli* of both Poly p 1 [35] and Poly p 2 [12]. Also, nPoly p 5 is currently evaluated for the development of a peptide-based allergy diagnoses system and an engineered vaccine [44]. Both strategies could significantly improve the diagnosis and treatment of *P. paulista* venom allergy and then the patient's quality of life.

Finally, venomic-based uncovering of *P. paulista*-toxins arsenal has allowed the description of diverse pharmacological activities that could be evaluated for either development of antivenoms or novel biotechnological applications [45,46] (Fig. 2). Here, we review the main approaches used for systemic molecular profiling of *P. paulista* venom composition. Additionally, we describe the growing body of data related to the identification and functional characterization of bioactive molecules comprised in the venom of this wasp. We focus on the production of recombinant allergens toward improvement of the strategies currently used for specific diagnosis and treatment of allergy. Also, we discuss the potential use of *P. paulista*-venom toxins for the rational design of antivenoms and the development of novel biopharmaceuticals products.

2. Exploring *P. paulista* venom – *omics* approaches

Animal venoms comprise heterogeneous levels of low molecular weight compounds, peptides and proteins, ranging from high to almost undetectable concentrations. The heterogeneity of toxin levels represented a significant challenge for venom characterization. Low-abundance molecules remained undescribed for decades thus tackling our systemic comprehension of the envenoming process. Proteomic and peptidomic analyses based on the use of techniques such as LC–ESI-MS and nano-ESI-MS/MS have progressively reversed this situation enabling identification of thousands of novel venom proteins and peptides [24,47,48].

To date, *P. paulista* venom has been explored using mainly peptidomic, proteomic and allergomic approaches (Fig. 2). Early efforts to elucidate *P. paulista*-venom peptides composition were conducted using classical procedures directed to the identification and molecular

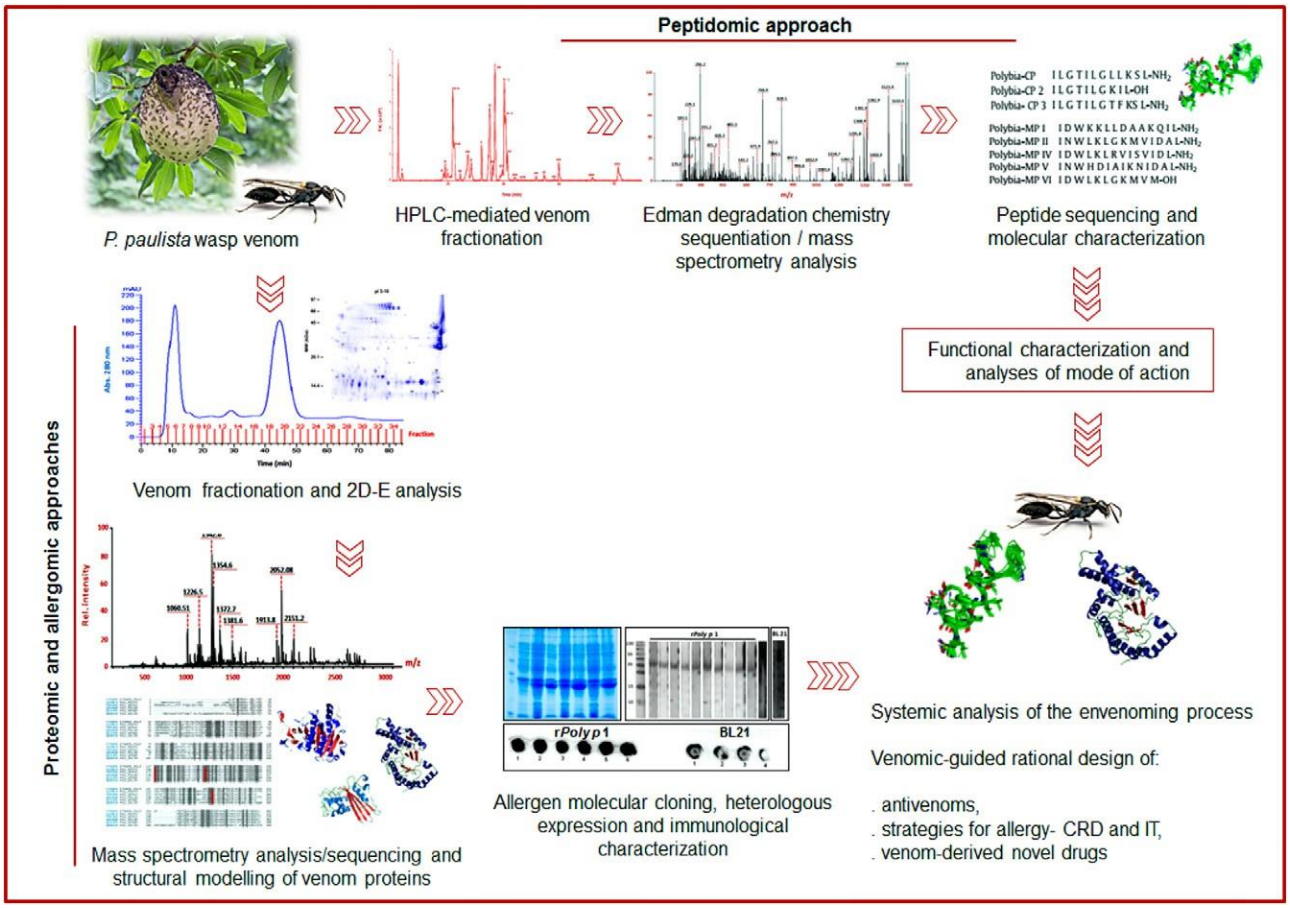

Fig. 2. Overview on the workflow used for the venomic analyses of *P. paulista*

characterization of a limited number of these molecules. The combined use of HPLC with mass spectrometry techniques (MALDI-Tof-PSD-MS and QTof-MS/MS) [49,50] resulted in the identification of two new peptides that are related to a subclass of mastoparan toxins. Both toxins showed a potent hemolytic activity, induced mast cells degranulation and were predicted to induce inflammation during envenoming process. Two low molecular weight peptides named Polybine-I and -II, that are also related to inflammation and chemotaxis of PLMNs were isolated using reverse phase-HPLC, sequenced using ESI-MS/MS and functionally characterized [51]. Another two peptides, Polybia-MPI and Polybia-CP with antibacterial and chemotactic activity, were described using a similar procedure [52].

Likewise to the identification of peptides, early attempts to describe proteinaceous components in *P. paulista* venom were conducted using traditional proteomic procedures. For instance, a gel filtration chromatography -Sephadex G 200- followed by ion exchange chromatography -DEAE Cellulose- enabled purification of Polybitoxins, a group of glycosylated phospholipases A2 [22]. Polybitoxins showed a high hemolytic activity in functional characterization bioassays. The native form of Poly p 1 (nPoly p 1) was initially isolated and characterized using a similar proteomic approach based on a double-step chromatographic purification protocol followed by Edmand degradation chemistry sequentiation [39]. The nPoly p 2 was purified using a single step ion-exchange chromatography, sequenced by mass spectrometry and, as nPoly p 1, structural modeled using several bioinformatics tools and protein structure databases [40,41]. Non proteinaceous or peptidic compounds such as Polybioside I, a low molecular weight neuroactive toxin was also described after analyses of *P. paulista* venom using a classical fractionation procedure [53].

The approaches described above ensured an accurate detection and molecular characterization of some *paulistinha* toxins. Nonetheless, its application reduced the possibility to obtain a systemic picture of *P. paulista* venom composition as a limited number of bioactive molecules have been identified and described in each study. To cope with this, some proteomics and peptidomic techniques that allowed detection of a large number of high, but also low-abundance, venom toxins has been performed recently. A proteome profile was obtained after submitted the *P. paulista* crude venom extract to bi-dimensional electrophoresis (2-DE) followed by identification of digested individual spots using matrix-assisted laser desorption ionization time-of-flight/time of-flight-mass spectrometry (MALDI TOF/TOF-MS/MS) [23].

The 2-DE revealed 237 ± 36 spots (five replicates) with MW ranging from 8 to 96 kDa and pH values from 3 to 10 (Fig. 3). Further mass spectrometry analysis resulted in identification of 84 venom proteins related to 12 different molecular functions. Proteins from the venom glands were classified into three different groups: typical venom proteins, muscle proteins and housekeeping proteins. The first group included 53 components, 23 similar to social wasp venom proteins previously described, most related to allergenicity. An immunoblotting analysis showed that 16 single proteins immunoreacted with sera from *P. paulista* venom-allergic patients (Fig. 3) revealing the potential of several venom toxins, and not only the three major allergens mentioned above, to be evaluated for improvement of CRD. Furthermore, this study showed for the first time the presence of antigen 5 and diverse metalloproteases as *P. paulista* venom components. As the authors mentioned [23], the discovery of novels allergenic proteins will greatly improve our understanding of the pathophysiological effects experienced by the victims after the wasp sting.

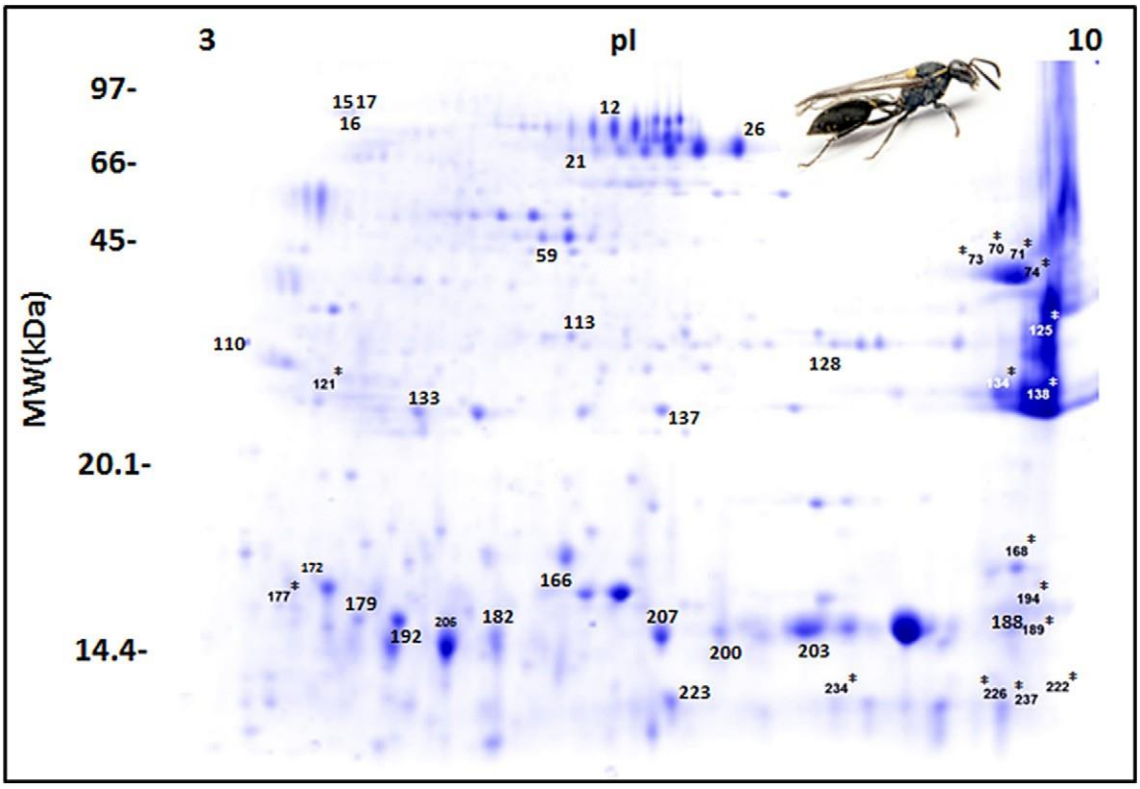

Fig. 3. Representative 2D-gel of *P. paulista* venom proteome stained with Coomassie Brilliant Blue G-250. Spots reactive to sera from allergic patients are marked with asterisk (*). (Image corresponds to a replicate of the experiment originally published by dos Santos et al. (2010)).

Venom proteome profiling of *P. paulista* has also allowed the description of a general mechanism for the envenoming process (Fig. 4) based on the different functions described for the identified proteins [23]. However, it is important to note that only 84/236 spots (~ 35%) were assigned with a putative protein function. The relative low number of identified spots is related to the limitations of the bottom-up gel based approach used in this study, as low-abundance proteins are difficult to identify [55,56]. Also, the large number of Hymenoptera proteins with unknown functions deposited in databases when the analysis was conducted hampered the identification of other venom toxins [57,58]. A reanalysis of the venom proteome of *P. paulista* using a gel-free shotgun proteomic approach combined with the use of updated protein databases could significantly enhance the identification of wasp venom toxins [48,64] with relevant biological functions. The use of this sensitive analytical alternative will allow the identification of the low-abundance "hidden proteins" in *P. paulista* venom thus helping to complete the model proposed for the envenoming process [48]. Proteinaceous compounds identified using shotgun proteomics could be also evaluated for the development of novel drugs with potential pharmaceutical applications.

For decades, the peptidome from *P. paulista* venom remained poorly explored. Before the application of high throughput screening approaches only few bioactives peptides were chemically characterized in the venom of this wasp [49,59,60]. Nonetheless, the use of advanced mass spectrometry techniques significantly changed that landscape and resulted in the description of dozens of novel *P. paulista* venom peptides [24]. An intra- and inter-colonial analysis of peptide diversity in *P. paulista* venom performed using LC-ESI-MS showed high levels of variability for both conditions. In the intra-colonial analysis (venom collected from one nest at three different seasons) a total of 146 peptides were detected. Interestingly, from that number only 44 (30%) peptides were common among the three samples. Similarly, from a total of 179 peptides detected in the inter-colonial analysis (venom collected from three different nests at the same time), only 36 (20%) were common across the three nests [34]. As the authors stated during discussion of these results, the high number of peptides in combination with the intra- and inter-colonial variations in the venom peptides´ composition might extends the spectrum of predators/preys against these toxins may be effective.

Interestingly, similar variations in other venomous animals have been suggested to correlate with differences in the pharmacological effects of the venom. For instance, intra-specific variability in *Conus vexillum* venom composition correlated with variations on venom potency and cytotoxic effects [61]. Furthermore, a systemic venomic analysis involving sixteen species of snakes from Costa Rica has showed a profound diversity on venom composition [27]. The analyses of the snake venom-proteome/peptidome profiles have helped to partially explain and even predict the potential variations on the pharmacological and pathophysiological effects suffered by the victims. The diversity in clinical symptoms caused by *P. paulista* venom is mainly related to allergenic proteins [23,62]. Despite the diversity of pharmacological activities suggested for *P. paulista* venom peptides [2], it is difficult to correlate intra/inter-colonial variations in *P. paulista* venom peptidome with differences in clinical manifestations as peptide activities is often related to limited number local toxic reactions [24]. However, a similar analysis for exploring the intra/inter variability on the *P. paulista*-venom proteome, including other medically relevant neotropical Hymenoptera could be extremely helpful for understand differences in clinical manifestations, particularly in the case HVA [60]. In fact, it has been informed that the frequency and intensity of the venom-caused allergic reactions may vary not only as a consequence of intra-individual variations along the victims, but also, along the qualitative and quantitative composition of the insect venoms [48,65].

A peptidome profile of *P. paulista* venom was recently obtained using a reversed-phase chromatography coupled to electrospray ioniza- tion-ion trap-time of flight mass spectrometry (LC-ESI-IT-TOF/MS) [24]. The profiling included peptides detection, sequencing, *in vitro* synthesis and biological evaluation. In summary, fourteen unambiguous peptide

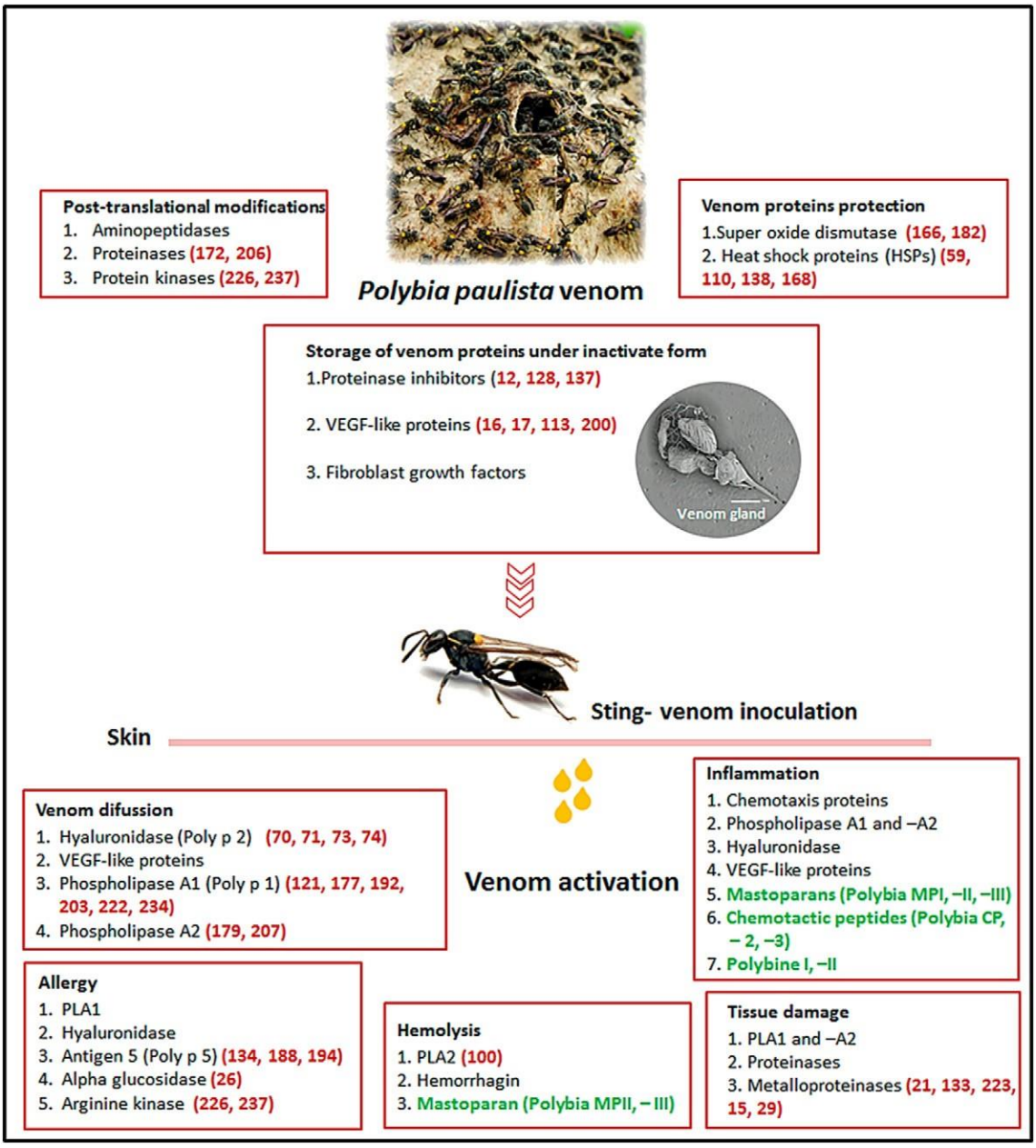

Fig. 4. Proteins (black) and peptides (bold green) identified in venomic analyses of *P. paulista* and their proposed biological functions during envenoming process (red bold numbers indicate corresponding spot of the protein in 2D-electrophoresis, see Fig. 3).

sequences were assigned. From that group, nine represented novel peptides including two wasp kinins, two chemotactic components, three mastoparans and two with unknown function. The peptides were further synthesized on solid-phase and submitted to different bioassays. Analysis of biological activity showed that *P. paulista* kinins (i.e. - Thr^{6}-Bradykinin, RA-Thr^{6}-Bradykinin, RA-Thr^{6}-BradykininDT) caused weak mast cell degranulation, oedema formation and pain suggesting that the toxins are used as a defense weapon causing discomfort in natural predators. Meanwhile, the novel peptides Polybia-CP 2 and Polybia-CP 3 promoted a medium to strong chemotaxis of polymorphonuclear leukocytes (PMNLs). Finally, as previously characterized *P. paulista* venom mastoparan, novels Polybia-MP IV, V and IV are related to inflammation during envenoming process.

Using a proteometabolomic approach, Esteves et al. (2017) [66] identified several volatiles compounds (as metabolites), such as tetradecanol, tridecanoic acid, tetradecanoic acid, tetradecanoic acid methyl ester, hexadecanoic acid methyl ester, octadecanol, octadecanoic acid, eicosanoic acid and eicosanoic acid methyl ester in the *Polybia paulista* venom. The authors suggested that these compounds are produced by Dufour's gland and secreted/mixed into the venom reservoir for use as alarm pheromones in a coordinated colony defense, including a massive attack against the predators. Dufour's gland and the venom gland are associated with the sting apparatus in female aculeate wasps and bees; despite this, no role have been attributed to the former in terms of venom activity [67].

The peptidome and proteome profiling of the *P. paulista* venom have contributed to hypothesize about a general mechanism for envenoming process caused by insect sting [23]. The mechanism proposed (Fig. 4) includes proteins and/or peptides involved in venom production, protection and storage in the venom gland and reservoirs along with those acting after the inoculation on the victim/prey. The actions of the toxins during the envenoming process have been divided into five basic

functions: venom diffusion, hemolysis, tissue damage, inflammation and allergy.

A more detailed analysis of the proposed mechanism suggests that, similar to other venom hyaluronidases [68–72], Poly p 2 could acts as a spreading factor that facilitates venom diffusion from the site of the inoculation. Hyaluronidases cleave the hyaluronan, a polysaccharide of the extracellular matrix found in connective tissue [40,69]. Degradation of hyaluronan potentiates the action of the venom by facilitating the toxins diffusion into the blood stream and the tissues of the victim [68,69,73,74]. In fact, hyaluronidases are widely distributed in animal venoms [70,75] highlighting the important role of these enzymes during the envenoming process. The Poly p 2, which showed a significant enzymatic activity *in vitro* [40] and structural homology with other Hymenoptera venom hyaluronidases described as spreading factors [41,72], has been proposed to increase the absorption rates and diffusion of the *P. paulista* venom into tissues after the insect sting [40]. The development of antivenoms or inhibitors that target this hyaluronidase could limit the diffusion of the venom thus preventing or delaying the occurrence of large tissue damages [70,71], potentially allowing a more efficient clinical management of the victim.

Despite that *P. paulista* has showed a potent hemolytic activity, intravascular hemolysis is rarely induced by insect stings. This pathophysiological manifestation has been informed mainly in veterinary accidents caused by snakes [76]. However, hemolysis has been occasionally described in massive attacks involving honeybees or wasps [23,77]. Hemolytic activity of *P. paulista* venom is related to peptides mastoparan II, III [60] and PLA2s [22]. *In vitro* assays have showed that both groups of toxins are direct hemolytic factors that disrupt the integrity of erythrocyte plasma membrane in the absence of any other agent [22,60]. As suggested for other venom hemolytic toxins such as mellitin [78], cardiotoxins [79] and venom snake PLA2s [76], the identification of these *P. paulista* venom toxins could be helpful for the analysis and further understand of pathophysiology effects caused by the wasp sting. Interestingly, at least one study [80] has showed that the activity of a PLA2 from the venom of a rattlesnake (*Crotalus durissus terrificus*) is facilitated by the activity of the snake hyaluronidase. A similar mechanism for enhancement of PLA2 activity could be proposed in the case of *P. paulista* venom.

Identification of snake venom-like metalloproteases (spots 21, 133 in Fig. 3) in *P. paulista* could represent a key finding to explain some symptoms experienced by the victims during envenomation caused by the wasp [23]. The activity of some snake-venom metalloproteases cause myonecrosis, skin damage, edema, and inflammatory reactions [81] in the victims which are clinical manifestations that have been informed in wasps' massive attacks [82]. Moreover, snake venom-like zinc-metalloproteinase-disintegrins also identified in *P. paulista* (spot 15 in Fig. 3) are known to cause hemorrhage, platelet aggregation, and some types of coagulopathies [83], which are clinical effects that have also been observed in victims of accidents involving multiple insect stings [23]. Structural and functional analysis must be performed to elucidate to role of *P. paulista* venom metalloproteases in the envenoming process [23]. As suggested for hyaluronidase, the production of metalloprotease antivenoms and the rational design of inhibitors could help to prevent the effects of these toxins after a *P. paulista* massive attack.

Besides these metalloproteases, *P. paulista* venom phospholipases A1 and A2 has been proposed to cause tissue damage. These enzymes disrupt phospholipidic packing of cellular membranes and are known to exert a wide diversity of toxic and pharmacological reactions. Venom PLA2s activity could produce edematogenic, hypotensive, cytotoxic and anticoagulant effects in the victims [84]. Meanwhile, some wasp PLA1s have showed a potent hemolytic activity [85] although their major consequence during envenoming is the induction of allergic reactions [35]. The role of these *P. paulista* venom toxins along with those involved in inflammation (mastoparans Polybia MPI, II, III, Polybia CP, 2, 3) and allergic reactions (Poly p 1, 2 and 5) mentioned in the proposed mechanism of envenoming will be addressed later in this review.

Overall the profiling of *P. paulista* venom composition using traditional and/or faster and more sensitive analytical techniques has rendered the identification of dozens of novel proteins and peptides [23,24]. Similar to other venomous animals [27,86], the wasp venomic analyses have expanded our knowledge about the arsenal of toxins produced by this social Hymenoptera. The venom composition suggests that the insect sting triggers a complex pathological scenario and that during the envenomation a wide range of molecular or/and cellular mechanisms are targeted by the wasp toxins. Further studies on these mechanisms will pave the way for the development of novel antivenoms and the design of specific immunotherapy strategies for induces venom tolerance in allergic patients [87]. Moreover, reanalysis of *P. paulista* venom using more sensitive analytical techniques and Hymenoptera protein databases currently available could increase the number of proteins identified thus helping to enlarge the panel of described venom composition and increase our understanding of the envenomation.

A transcriptome analysis of the *P. paulista* venom glands could also be used for exploring the toxin arsenal of the wasp. Transcriptome profiling provides large amounts of biological information and represent a feasible alternative to mine novel compounds in animal venoms [88,89]. As in the case of solitary [90] and other social Hymenoptera species [91,92], *P. paulista* venom-transcriptome profiling could significantly increases the number of toxins identified then allowing its structural and functional characterization. Consequently, the data derived for transcriptome analysis could help in the full decoding of the *P. paulista* venom composition and the pathophysiological caused by the insect sting.

3. *Polybia paulista* - venom peptide arsenal

As mentioned, the use of traditional venom fractioning approaches and second-generation sequencing techniques allowed the identifica- tion of several peptides comprised in *paulistinha* venom. Evaluation of biological activities of these toxins is a milestone for the understanding of it functions during envenoming and the further design of antivenoms. In the next two sections we describe the main groups of peptides (Fig. 5) currently identified in *P. paulista* venom and its biological activity. These findings are also summarized in Table 1.

3.1. Mastoparans (Polybia-MP)

Mastoparans are membrane-active polycationic molecules of 12–14 amino acids residues that represent the most abundant class of peptides in venom from both solitary and social hunting wasps [93]. These toxins caused histamine release from mast cells, serotonin from platelets along with catecholamines and adenylic acids from adrenal chromaffin cells. Mechanism of action involved either insertion in cell membrane, pore formation lately causing cell surface disruption and lysis; or direct interaction with G proteins on the cytoplasmatic face, thereby perturbing transmembrane signaling [94].

Two mastoparans, described as linear tetra-decapeptides of 1612.07 and 1658.60 Da. were the first group of peptides identified in *P. paulista* venom [49]. The analysis of the peptides primary sequence revealed that both toxins are similar to previously described mastoparans. In functional characterization bioassays both peptide showed a potent chemotactic activity as 90% of rat PMNL cells were chemo-attracted in the presence of each peptide. The toxins also showed a strong hemolytic activity causing *in vitro* disruption of 100% of rat red blood cells and also induced degranulation of mast cells.

Polybia-MPI is a typical mastoparan tetra-decapeptide of 1654.09 Da that caused lysis of mast cells, and shows chemotaxis to PMNLs. Unlike the two mastoparans previously described, Polybia-MPI does not have hemolytic activity in rat erythrocytes. A multiple alignment analysis

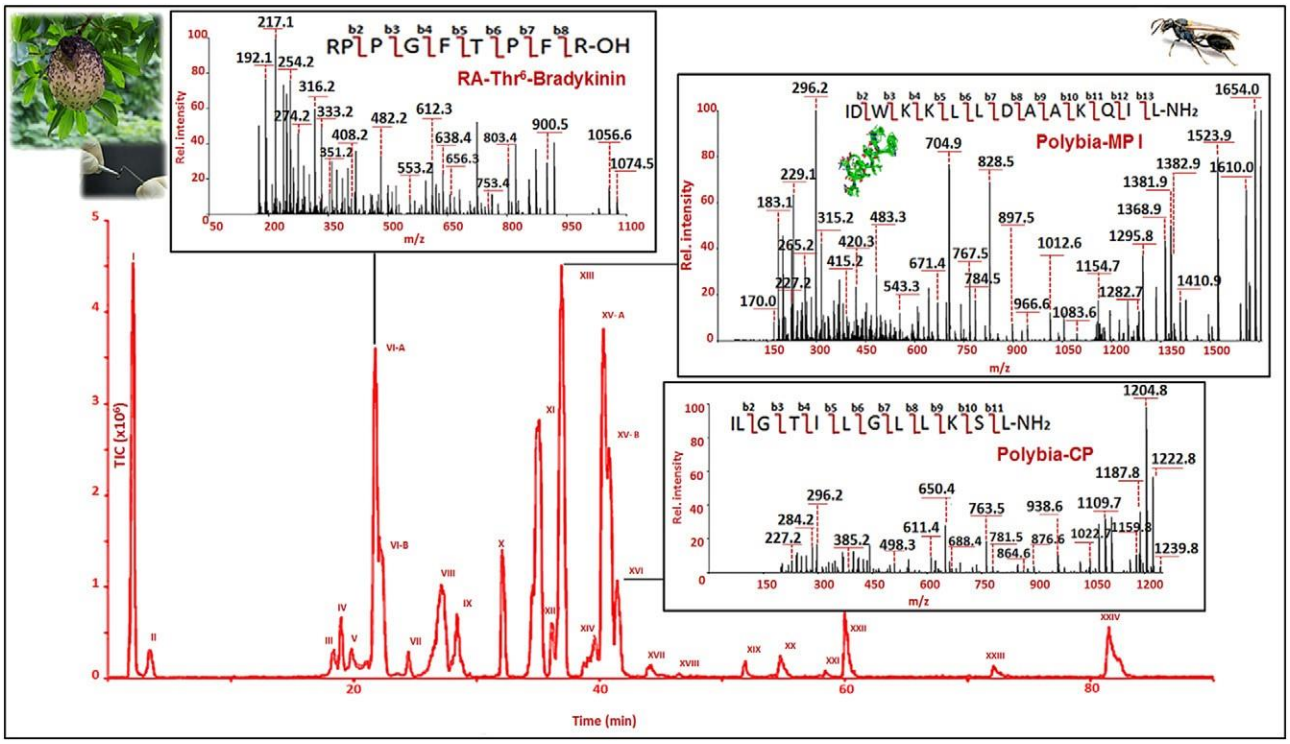

Fig. 5. Chromatographic separation (solid red line) of the peptides fraction from *P. paulista* venom, by RP-HPLC with a packed X-Bridge BEH 130C-18 column, eluted under a gradient from 5 to 60% (v/v) acetonitrile, containing 0.1% (v/v) trifluoroacetic acid. The tandem mass spectra of some of the major peptide components of this venom (RA-Thr6-bradykinin, Polybia-MP I, and Polybia-CP) are also shown. (Modified with permission from Dias et al., 2015).

showed different levels of identity with primary sequence of mastoparans from venom of other social wasps, with the higher homology (71%) related to the mastoparans identified in *Protonectarina sylveirae* venom [95]. Polybia-MPI also displayed a potent antimicrobial activity against Gram-positive and Gram-negative bacteria. In the case of *B. subtilis* (Gram/+) and *P. aeruginosa* (Gram/−) the minimum

Table 1
Summary of principal toxins from *P. paulista* venom identified using traditional or second generation proteomic approaches that are currently being evaluated for development of antivenoms, CRD and IT of allergy and novel drugs.

P. paulista venom toxins			
Type of molecule	Sequence	Biological activity/applications	References
Peptides			
Chemotactic			
Polybia-CP	CPILGTILGLLKSL-NH_2	Chemotaxis of PMNLs, antimicrobial and antifungal/development of antimicrobial, antifungal and antitumor drugs	de Souza et al., 2005
Polybia-CP 2	ILGTILGKIL-OH		Dias et al., 2015
Polybia-CP 3	ILGTILGTFKSL-NH_2		
Protonectin (1-6)	ILGTIL-OH		Mendes et al., 2004
Mastoparans			
Polybia-MP I	IDWKKLLDAAKQI L-NH_2	Mast cell degranulation, antibacterial and antifungal/development of antimicrobial, antifungal and antitumor drugs	de Souza et al., 2005 de
Polybia-MP II	INWLKLGKMVIDA L-NH_2		Souza et al., 2009
Polybia-MP III	INWLKLGKMVMDVL - NH_2		
Polybia-MP IV	IDWLKLRVISVID L-NH_2		
Polybia-MP V	INWHDIAIKNIDA L-NH_2		Dias et al., 2015
Polybia-MP VI	IDWLKLGKMVM-OH		
Proteins	Accesion number/entry		
name			
Phospholipase A1 (Poly p 1)	A2VBC4/PA1_POLPI	Allergen-hemolytic/CRD and molecular-based IT	Santos et al., 2007
Hyaluronidase (Poly p 2)	P86687/HUGA_POLPI E0AD89/E0AD89_POLPI	Allergen-Venom diffusion/CRD and molecular-based IT Justo-Jacomini et al., 2013	Pinto et al., 2012
Antigen 5 (Poly p 5)	P86686/VA5_POLPI	Allergen-Unknown/CRD and molecular-based IT	dos Santos-Pinto et al., 2014

inhibitory concentration (MIC) values were lower than those obtained for Tetracycline (positive control).

The *in silico* prediction of the Polybia-MPI secondary structure revealed the presence of about 71.43% of α-helix conformation and 28.57% of coil structure. The high content of α-helix in Polybia-MPI could be mediating the peptide interaction with cell membrane then promoting antimicrobial activity. Further analysis for characterization of the peptide interaction with bacterial membranes and cell wall could aid in the development of antibiotics based in Polybia-MPI, as predicted for other antimicrobial peptides (AMPs) [96].

Similar to Polybia-MPI, Polybia-MP-II and -MP-III were identified using a traditional peptidomic approach [60]. The chromatographic profile obtained after HPLC-mediated fractionation of *P. paulista* venom showed 13 peaks, with these peptides contained in the last fraction (F13). Further separation of this fraction followed by Edmand degradation chemistry and MS/MS analysis enabled the sequencing of both toxins. Circular dichroism analysis of Polybia-MP-II for secondary structure determination showed an α-helical and β-sheet content of 30 and 20%, respectively. Meanwhile, Polybia-MP-III has an α-helical and β-sheet content of 24 and 34%. Considering physiological concentrations of both venom peptides and results derived from functional characterization assays, Polybia-MP-II and -III were proposed to cause mast cell lysis disruption but no significant degranulation during envenoming process. Similarly, the mastoparans showed hemolytic (ED_{50} = 5 × 10^{-5} M) but not chemotactic activity at physiological conditions. In antimicrobial activity evaluation the two peptides inhibited growth of Gram (+) and Gram (−) bacteria. As expected, given the α-helical content in peptides secondary structure, Polybia-MP-II showed the lower IC_{50} values.

Diverse bioassays for functional characterization of Polybia-MP II showed that it causes sarcolemma disruption, cytoskeleton degradation, mitochondrial damage and has mytotoxic activity [97,98]. Further biological activity evaluation showed that the mastoparan causes myonecrosis, inflammation and apoptosis in muscle cells [99]. The mechanism of action includes mitochondrial damage related to potent mitochondrial permeability transition, activation of caspase 3, 9 and induction of TNF-α, IFN-γ, CD68 and CD163.

The levels and diversity of biological activities described for mastoparan peptides in Hymenoptera venoms show that they are key players during the envenoming process. Mastoparans induce secretion of histamine from mast cells, oedema formation that causes pain, toxin reactions and a remarkable physiological discomfort in predators. The detection and molecular characterization of this group of peptides partially explain several symptoms experienced by victim after *P. paulista* sting. Structural and functional data derived from proteomic analysis and bioassays described here may help to design antivenoms to counteract the toxic effects caused by these toxins. Furthermore, *P. paulista*-mastoparans, particularly Polybia-MP-I, could be used for the development of novel antimicrobial drugs.

3.2. *Chemotactic peptides*

Wasp venom chemotactic peptides are involved in massive recruitment of PMNLs followed by the release of large amounts of oxygen peroxide near to the site of sting [2]. The resulting hyperoxidative environment induces local inflammatory responses, cell death and pain. Polybine-I and -II were the first *P. paulista* venom peptides described with active PMNLs chemoattractant activity [51]. The toxins have an acetylated blocked N-terminus, showed by the inability to interact with the phenylisothiocyanate reagent in Edmand degradation chemistry. In bioassays, the native-acetylated form of the peptides showed higher PMNLs chemoattractant activity than the synthetized, non-acetylated variants.

Polybia-CP is a chemopeptide of 1239.73 Da that, unlike Polybine-I and -II, has a non-blocked N-terminus. Also, unlike chemopeptides described in wasp venom from species of temperate regions (13), Polybia-CP has 12 amino acid residues and lacked a characteristic FLP tripeptide at the amino terminal side [59]. The duo-decapeptide induced no degranulation in mast cells and presented no hemolytic activity at physiological concentrations. By contrast, Polybia-CP showed high levels of chemotaxis of PMNLs cells (1.7×10^4 cells/mL) at concentration of 10^{-5} M. Polybia-CP inhibited Gram positive bacterial growth with MIC values = 15 μg/mL for both *B. subtilis* and *S. aereus*. As discussed later in this review, several animal venoms has recently showed remarkably antibacterial activity and as a consequence venoms are gained attention as a suitable source of novel antibiotics [100].

As noted, in a systemic peptidomic analysis of *P. paulista* venom another two chemotactic peptides (Polybia-CP 2 and Polybia-CP 3) were described [24]. Both molecules are typical duo-decapeptides often identified in venom of wasps from neotropical regions [63]. They belong to the group of chemopeptides that contains a single K residue between positions 8 and 10 in the primary sequence. In the same study, a second group of chemotactic peptides (Protonectin 1–6), previously described in the social wasps *Agelaia pallipes pallipes* [59,101], was also detected and sequenced. These protonectin-like toxins are small chemotactic peptides - 4 to 8 amino acids residues - obtained as a consequence of serine protease-mediated proteolysis of mastoparans. Protonectins are involved in regulation of leukocytes chemoattraction during envenoming process [24].

The mechanism of action of chemotactic peptides from *P. paulista* remains unknown. However, it has been postulated that chemoattraction of leukocytes induced by these toxins is mediated by the interaction with G-protein coupled receptors (GPCRs) in the cell membrane [59]. G-protein-coupled receptors are a family of ubiquitous proteins with 7 transmembrane domains involved in a large range of pathological process [102]. The direct interaction between GPCRs and the peptides activates a cascade of molecular events resulting in cell migration to the sting site. Thus, Polybia-CP, -CP 2 and -CP 3 could be acting in envenoming process by eliciting massive leucocytes-cell migration and promoting a potent pro-inflammatory response.

4. *Polybia paulista* venom allergens

HVA is the most common cause of life-threatening outcomes after a Hymenoptera sting [5]. It is a classical IgE-mediated allergic disease triggered by the binding of allergen-specific IgE to their own receptors (FcεRI) at the surface of mast cells and basophils [43]. The subsequent re-exposure to venom allergens induces degranulation of mast cells and basophils and the release of inflammation mediators such as histamine, leukotrienes, and several cytokines. Mediators release causes type-1 hypersensitivity allergic reactions with symptoms ranging from generalized urticaria, angioedema, blood pressure fall to bronco-spasms, cardiac and respiratory arrest, and anaphylactic shock [87,103]. To date, the molecular basis of the variations in the susceptibility to Hymenoptera venoms and the symptoms experienced by the victims after the insect sting remain unclear. In atopic patients a genetic predisposition that increases production and/or persistence of allergen-specific IgE and consequently the incidence of HVA has been proposed [15]. Typically, in non-responsive individuals the IgE levels transiently rise after a sting and then return to basal values during the next 1–3 months [15,104]. For this group of victims a mechanism of peripheral tolerance development mediated by the regulatory T (Tr1) cells [87] has been described. While in non-responsive individuals, Tr1 cells is the most common cell subtypes, allergen-specific IL-4 secreting Th2 cells predominates in allergic patients. Immunotherapy with the culprit venom comprising the allergens responsible for sensitization has proven to induce tolerance in 75–98% treated patients [104].

A reliable diagnosis of HVA based on the detection of IgE-mediated reaction against the culprit venom is a critical prerequisite for initiation of venom immunotherapy [43]. Misidentification of venom responsible for sensitization leads to treatment with irrelevant venoms thus causing

de novo non-specific sensitization and increase the risk for occurrence of undesired side effects during SIT. Thus, continuous efforts have been made to improve the available systems used for specific diagnosis of HVA. Diagnoses using crude venoms or native allergens are hindered by the potential incidence of IgE-mediated cross-reactivity mainly related to the presence of the CCDs in the structure of some allergenic proteins [36]. As mentioned below, the use recombinant allergens produced after identification and characterization of their native counterparts in venomic analyses have helped to cope with these pitfalls, thus enabling the specific identification of culprit venom [3]. The use of recombinant allergens lacking CCDs in CRD has resulted in a significant decrease on the incidence of cross-reactivity, with is responsible for 70–80% of misidentification of double sensitization informed during HVA diagnosis [105,106]. By preventing misdiagnoses these CCDs-depleted recombinant allergens avoid the inclusion of irrelevant venoms in SIT.

Venom immunotherapy is the only disease-modifying treatment for HVA and induces long-term tolerance in allergic patients [37]. As noted, SIT with insect venoms is highly effective in inducing tolerance in allergic patients [104]. However, the use of crude venom in immunotherapy have also been related to the occurrence of undesired side effects caused by non-allergenic, toxic compounds comprised in the extract [37]. The use of recombinant allergens could also improve the safety profile of SIT by preventing the inclusion of these non-allergenic components present in whole venom. Furthermore, venomic analyses have allowed identification of hypoallergenic variants of venom allergens [107]. Recombinant production of hypoallergenic proteins lacking IgE-epitopes but maintaining structural determinant for T-cell recognition and induction of allergen-blocking antibodies have been suggested as a novel alternative for improvement of SIT [108].

As noted, *P. paulista* is involved in a large number of medically important stinging incidents with potential life-threatening outcomes in allergic patients [18,19,35]. However, as for the others Hymenoptera species in Latin America, *P. paulista*-related sting accidents and venom allergy are neglected human health problems. The lack of reliable surveillance studies is partially due to the lack of commercial systems of diagnosis designed using allergens from endemic species [12,109]. To date, clinical interventions related to HVA in Brazil are mainly based on the use of crude venom extracts or commercialized allergens obtained from species of temperate regions. As discussed above, the use of these allergenic materials on Brazilian patients often resulted in miss-identification of culprit venom which lead to non-specific sensitization [7,35], increased risk of side effects and limited induction of tolerance during SIT [43]. Thus, it is mandatory to continue exploring the venom of Brazilian and in general Neotropical wasps using intra and inter-specific analyses. These studies may help to identified novel allergenic proteins that could be heterologously expressed and evaluated for improvement of specific diagnosis and SIT of HVA caused by endemic native species. Furthermore, the analyses of intra and inter-species variations in wasp venoms and the correlation with differences in clinical symptoms could be used to establish a reliable prediction of the most probable effects on the victim triggered by a particular insect venom [27,65].

Considering all these facts, the major allergens from *P. paulista* venom identified in venomic analysis have been extensively character- ized, cloned and expressed. In this section we describe previous and re- cent analysis performed with the allergens envisioning their uses in the development of component resolved diagnosis and SIT of allergy.

4.1. Phospholipase A2 (PLA2)

As mentioned, early attempts to explore the proteinaceous arsenal of *P. paulista* venom resulted in identification of a group of highly glycosylated PLA2s-named polybitoxin (PbTX)-I, II, III and IV. Unlike PLA2 from *A. mellifera* (Api m 1) and from other social wasps [110,111], the polybitoxins induce no significant allergic reactions in the victim [23]. The total amount of the four PbTX in the crude venom of *P. paulista* represents only 1.1%, a low value compared with those observed in the venoms from *A. mellifera* (10–12%) and *Vespa basalis* (6%) [22]. Nonetheless, this group of toxins showed levels of hemolytic activity higher than those of Api m 1 or phospholipase A2 from venom of *Naja nigricolis* and *Naja naja atra* snakes. Given these, the role of PbTXs during *P. paulista* envenoming process is to cause hemolysis, a common feature of venom PLA2s [112,113], rather than to induce allergenic reactions.

4.2. Phospholipase A1 (Poly p 1)

Phospholipase A1 has been identified as a major allergen of several wasp venoms from neotropical [39,114,115] and template regions [116–118]. The native PLA1 from *P. paulista* venom (Fig. 6A) is a ~ 34 kDa, non-glycosylated protein with 51–83% of homology with vespid PLA1s from Northern Hemisphere. The enzyme catalyze the specific hydrolysis of ester bonds of 1,2-diacyl-3-*sn* glycerophospholipids, at the position *sn*-1 which disrupts phospholipid packing in cell membrane leading to pore formation and cellular lysis [39]. The nPoly p 1 showed levels of hemolytic activity similar to those reported for cobra cardotoxin *Naja naja atra* and reacted with sera of patient's allergy to *P. paulista* venom. Further analysis of nPoly p 1 showed that the allergen appears as a mixture of multiple molecular forms, some truncated, and with different levels of allergenicity [119].

Purification and sequencing of nPoly p 1 enabled the subsequent cloning and expression of the allergen. The combined use of bioinformatic, proteomic and molecular biology tools resulted in characterization of nPoly p 1 and further heterologous production in *E. coli* cells [35]. Furthermore, structural data derived from molecular characterization of nPoly p 1 contributed to optimize the strategy for production of the recombinant allergen. The expression of Poly p 1 in *E. coli* resulted in the production and purification of an immunological active

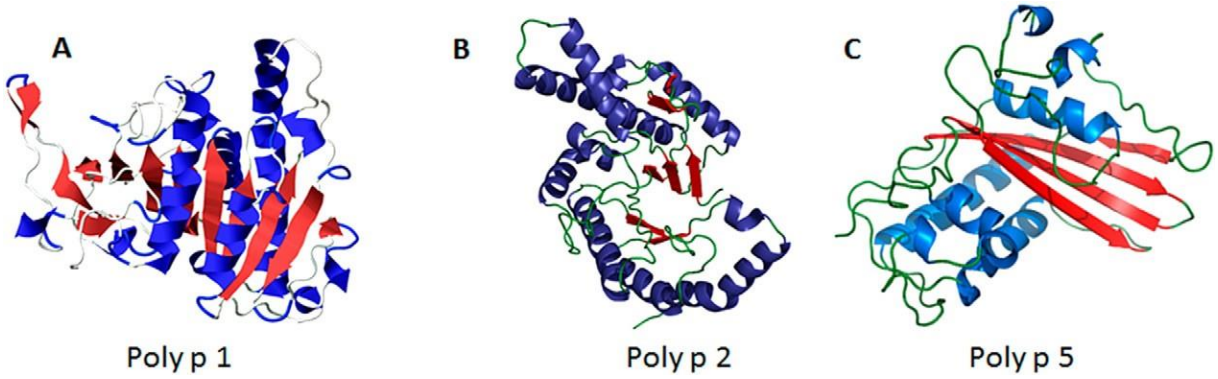

Fig. 6. Three dimensional molecular model of native phospholipase A1 (A), hyaluronidase (B) and antigen-5 (C), the major allergens from *P. paulista* wasp venom.

recombinant allergen (rPoly p 1) that was immunodetected by sera of allergic patients at a similar extend than *P. paulista* crude venom extract and nPoly p 1 [35]. Furthermore, sera from rPoly p 1-sensitized mice specifically recognized nPoly p 1 and showed no reaction with *A. mellifera* crude venom or commercial form of the major HBV allergens Api m 1, Api m 2 and melittin (Api m 4) [120]. Currently, rPoly p 1 has been evaluated to elucidate the incidence of cross-reactivity with venoms of wasp from neotropical and temperate climate zones. Overall, these findings suggest that rPoly p 1 is a good candidate for the development of *P. paulista* CRD and molecular-defined IT.

4.3. Hyaluronidase (Poly p 2)

Proteomics analysis of *P. paulista* venom also enabled the isolation, sequencing and further molecular cloning of the native Poly p 2 (nPoly p 2), the first hyaluronidase from neotropical wasp ever characterized [40]. Like other hyaluronidases from venomous animals, nPoly p 2 hydrolyze hyaluronic acid, a high molecular weight glycosaminoglycan which is one of the major components of the extracellular matrix of vertebrates [41]. Thus, the enzyme acts as a diffusion factor by facilitating the penetration of the others venom components in tissues and blood stream of the victim and finally enhancing the harmful effects of the toxins [93].

The nPoly p 2 variant described by Pinto et al., (2012) [40] is a glycosylated protein of 288 amino acid residues long with molecular mass of 44,340 Da. In contrast, nPoly p 2 purified, sequenced and modeled by Justo-Jacomini et al., (2013) [41] (Fig. 6B), is a 10 residues longer protein that showed 74.8% of identity with the nPoly p 2 previously described. As authors mentioned, the differences informed in these studies are rather due to the presence of several nPoly p 2 isoforms in *P. paulista* venom, previously described by dos Santos et al., (2011) [119], than to the variability of sites from wasps' collection or to modification in the experimental methods used.

As in the case of nPoly p 1, venomic analysis contributed to obtain a recombinant form of Poly p 2 (rPoly p 2) in *E. coli* [12]. rPoly p 2 was expressed in inclusion bodies, solubilized and purified to homogeneity using Ni^{2+} affinity chromatography (Ni-NTA-Agarose) under denaturing conditions. IgE-mediated immunodetection of the produced rPoly p 2 was confirmed by western blotting using sera from allergic patients. Interestingly, the immune sera from *P. paulista*-allergic patients recognized rPoly p 2 at higher intensity than nPoly p 2 showing that Poly p 2 lineal-IgE epitopes elicited a potent immune response in sensitized victims. This finding is particularly relevant in the case of Poly p 2, as the recombinant form expressed in *E. coli* lacked the CCDs thus decreasing the potential negative impact of cross-reactive occurrence during diagnosis based on the crude venom extract or native form of the allergen.

4.4. Antigen 5 (Poly p 5)

Antigen 5 is a ubiquitous protein of ~23 kDa that belongs to the CAP superfamily of secreted proteins which are involved in diverse biological process (reproduction, cancer, immune defense, allergic reactions) [121]. In social Hymenoptera, antigen 5 was initially reported as a venom component of ants and wasps from Northern Hemisphere [122]. Although it is one of the most abundant proteinaceous component of wasp venoms [3,123], no physiological role has been described for the protein [43]. Unlike the two others major allergens, the first clue about the presence of antigen 5 in *P. paulista* was obtained from the systemic analysis performed by dos Santos et al., (2010) [23] after 2D-E separation of the venom proteome followed by mass spectrometry analysis. In this study, six isoforms of nPoly p 5 were identified and the most abundant, which corresponds to the intact form of this protein, was recognized by a pool of sera from *P. paulista*-allergic patients. The authors suggested that the six isoforms of the allergens are originated from a controlled proteolytic processing of the intact protein or by gene splicing. The production of different isoforms could represent a strategy to avoid the immune response of the victim against a specific variant of the allergen thus enabling immune system evasion during envenoming process.

Given these results, further efforts were made to sequence and characterize Poly p 5. Using a gel based mass spectrometry strategy combined with traditional techniques from protein sequencing and post-translational modifications (PTMs) analysis, a complete sequence and structural model of the allergen were obtained. A series of PTMs modifications such as hydroxylation, phosphorylation, and glycosylation were observed on the Poly p 5 sequence. Alignment of nPoly p 5 primary sequence revealed a 59.3–93.7% identity with antigen 5 of other wasp venoms. Meanwhile, the 3D model showed the presence of three α-helices, one 3_{10} helix, and four β-sheets (Fig. 6C) [42]. The high levels of identity with antigen 5 of others social Hymenoptera partially explain the broad cross-reactivity reported for these allergens during allergy diagnosis [118,124].

The allergenicity of antigen 5 from Hymenoptera venoms and the lack of knowledge about enzymatic or other physiological activities have encouraged the efforts for heterologous production of this aller- gen. In addition to be helpful in allergy diagnosis and immunotherapy, the recombinant forms of the antigen 5 could be used in different assays for structural and functional characterization. To date, antigen 5 from venom of social Hymenoptera has been expressed in bacteria [125, 126], yeast [107] and insect cells [118,127]. Some of the heterologous forms of the allergen have proved to significantly improve CRD of Hymenoptera venom allergy [105,128].

Based on these findings and the data obtained from proteomic characterization of nPoly p 5, two studies are currently conducted to express the allergen in prokaryotic and yeast cells. Interestingly, a novel alternative for the development of molecular-defined IT has been suggested recently after the description of several B-cell linear epitopes of nPoly p 5 [44]. By combining SPOT-synthesis technique and structural modeling of Poly p 5, the authors identified nine linear B-cell epitopes immunoreactive to human IgG, one of which, peptide 7 (WAKTKE) also reacts with human IgE. It was suggested that peptide 7 is settled at a loop on the protein surface representing the major B-cell binding epitope of the *P. paulista* venom antigen 5. The use of synthetic peptides based on the described epitopes could provide a safe and effective strategy to induce tolerance in the allergic patients without the occurrence of adverse effects [129].

The combined use of rPoly p 1, 2 and 5 in panels of recombinant allergens could significantly improve the specific diagnosis of *P. paulista* venom allergy in sensitized patients. However, further analyses are required for immunological characterization of the produced heterologous forms. Moreover, other allergenic proteins that must be under-represented in the crude venom extract but are still recognized by specific IgE in allergic patients [130], should be characterized, heterologously expressed and tested. It has been well stablished that the use of a reduced number of recombinant allergens in CRD is associated with a decrease in sensitivity and a consequently increase of false negative results [131]. As mentioned, in a systemic profiling of the wasp venom, Santos et al., (2010) [23] found 16 single spots that reacted with sera of *P. paulista* venom-allergic patients which could be evaluated and included in CRD and venom immunotherapy.

5. Potential biotechnological applications

Animal venoms are unique cocktails of bioactive toxins that target a wide variety of physiological processes. Most of these toxins specifically interact with vital enzymes, ion channels and cellular receptors [54]. Due to these, animal venoms have been explored as a source of potential therapeutic agents. *P. paulista* venom comprised a rich mixture of peptides and proteins that could be evaluated not only for the rational design of antivenoms, but also as a source for the development of novel drugs.

To date, mainly *P. paulista* venom peptides have been evaluate as candidates for the development of novel drugs. Meanwhile, major allergenic proteins have been extensively characterized, produced as recombinant protein and are currently under evaluation envisioning their use in commercial system for CRD of *P. paulista* venom allergy. As mentioned, the bottom-up gel based proteomic approach used for the systemic analysis of the wasp venom resulted in the identification of a limited number of proteins (84/236 spots) [23] which potentially hampered the detection of novel toxins. In addition to expand our understanding of the envenoming process the use of a gel-free shotgun proteomic approach could potentially allow the discovery of proteins with novel pharmaceutical applications.

5.1. Antimicrobial and antifungal activity

Antimicrobial resistance (AMR) has progressively turned into a seri- ous threat for modern medicine as recognized in a global report recently published by the World Health Organization [132]. Current situation has prompted the search for new antimicrobial compounds with alternative mechanisms of action that could be used solely or combined with traditional drugs to tackle bacterial, fungal, parasites and virus drugs resistance. Antimicrobial activity is a common feature of animal venoms and is related to a wide range of peptidic toxins such as mastoparans [32], cardiotoxins [133], cecropins [134], mellitin [135] and some enzymes [136]. Unlike conventional antibiotics, which interact with specific bacterial targets, some venom AMPs act by direct disruption of cell surface [136–138]. This microbicidal mechanism usually prevents the appearance of AMR-mediated by gene mutations and is less likely to induce resistance [139]. Due to this, animal venoms could be explored as potential sources of novel and non-easily susceptible to resistance antibiotics [140].

Several antimicrobial compounds with unique structure and pharmacological properties have been described in Hymenoptera venoms [86,141]. As mentioned, some *P. paulista* venom-peptides including the mastoparans Polybia-MPI, -II, -III and chemotactic peptide Polybia-CP [52,60] has showed different levels of activity against Gram positive and/or negative bacteria. The analysis of the molecular mechanism of action is a mandatory prerequisite for the development of novel antibiotics using these compounds.

Unlike other venoms peptides, Polybia-MPI is a non-hemolytic toxin that showed significant and highly selective antimicrobial activity. The differential interaction of this venom toxin with bacterial and red blood cells was suggested to be related with the peptide structure and cell membrane composition [59]. Further analysis toward elucidation of Polybia-MP-I mechanism of action showed that the selectivity for bacterial cells is associated with the ratio of peptide and membrane lipids (P/L) concentrations needed to reach a point of starting a cooperative leakage process [142]. The permeabilization triggering (P/L)-ratio for Polybia-MP-I varies between different cell types as a function of the membrane lipid bilayer composition and partially explain the peptide specificity. *In vitro* analyses showed that Polybia-MPI preferentially interacts with anionic over zwitterionic vesicles. This finding is consistent with the selective antimicrobial activity of the peptide since outer leaflet of bacterial membranes are known to present a high negative charge density [143] whereas mammalian membranes are mainly formed by zwitterionic lipids. Overall, these findings pointed to Polybia-MP-I as an interesting candidate for development of potentially highly selective and effective antibiotics.

The antimicrobial mechanism of action of Polybia-CP has been described recently using a set of both Gram (+) and Gram (−) bacteria [144]. The *P. paulista*-chemotactic peptide showed a significant antimicrobial and bactericidal activity against several pathogenic strains. Polybia-CP effects over *E. coli* cells could be visualized by electron microscopy after 30 min of exposure. The action was characterized by the appearance of disrupted membrane surface likely related to pore formation. The venom toxin causes permeabilization of bacterial outer and inner membrane as showed by the incorporation of a hydrophobic fluorescent probe and a DNA-intercalating fluorescent dye, respectively. The authors also looked for peptide-bacterial DNA interaction in an attempt to determine alternative molecular targets. Polybia-CP showed no interaction with DNA reinforcing the suggestion that bacterial membrane is the unique target for the chemotactic peptide. Further analyses are required to evaluate the selectivity of Polybia-CP. However, the fact that its mode of action involves only physical interaction with cellular membranes and no enzymatic activities reduces the possibilities of drug-resistance emerging, a characteristic that is highly desired in novel antimicrobial compounds.

Polybia-MP-I [145] and Polybia-CP [146] also inhibits fungal cells growth. Both venom peptides have shown a significantly antifungal and fungicidal activity against different species of Candida. In addition to cause membrane disruption, Polybia-MP-I interacted with components of the fungal cell wall thus suggesting a concomitant mechanism of action for antifungal activity. Interestingly, further functional analysis showed that Polybia-MPI impaired biofilm formation which a typical defense strategy of pathogenic microorganisms against host immune system and drugs. The antifungal activity of Polybia-CP is also mediated by a membrane-active mode of action. Interestingly, bioassays also showed that the chemotactic peptide induce a remarkable increase of cellular reactive oxygen species (ROS) which damage proteins, lipids and DNA of fungal cells. Thus, both *P. paulista* venom peptides appear as good candidates for developments of antifungal drugs or as lead structure for the rational design of more active analogues.

5.2. Antitumor activity

To date, cancer remains the second most common cause of death among human population and it is expected to surpass heart disease as the leading cause during the next years [147]. Chemotherapy has been broadly used as an effective approach to prolong life and/or relieve symptoms in patients. However, as in the case of pathogenic microorganisms, the tumor cells-multidrug resistance is a major problem causing failure of anti-cancer therapeutic pharmacological interventions [148]. Moreover, current drugs have harmful actions over non-target cells, causing significant undesired-side effects. Thus, the search for novel, tumor-cell selective compounds with low probability of resistance emerging has turned into a priority for anti-cancer researches. Antimicrobial peptides have shown to exert cytotoxic activity against diverse cancer cell lines [141]. So far, the antitumor effects of *P. paulista*-venom toxins have been tested for the mastoparan Polybia-MPI. This peptide showed a highly selective cytotoxic and antiproliferative activity against prostate and bladder cancer cells [45]. As in the case of bacteria, Polybia-MPI antitumor activity appears to be related to the presence of α-helix conformation in the secondary structure of the venom peptide that mediates pore formation in membrane of tumor cells. Interestingly, proliferation of the non-tumorigenic murine fibroblast cell line NIH3T3 was almost unaffected by Polybia-MPI.

In a subsequent analysis conducted using sensitive and multi-drug resistant leukemic cell lines, Polybia-MPI showed a highly selective antiproliferative effect [138]. Cytotoxicity levels of Polybia-MPI were similar in both types of leukemic cells while no damage was detected in NIH3T3 cells. Further analysis of the mechanism of action showed that the peptide activity is mediated by the highest content of phosphatidylserine (PS) in the cell membrane of leukemic cells. In normal physiological conditions PS is located in the membrane's inner leaflet while tumorigenic cells experiment a significant increase of PS in the outer leaflet. As Polybia-MPI preferentially interacts with PS, the authors proposed that the differential distribution of PS in normal and tumorigenic cells promotes selective anti-cancer activity of the *P. paulista* venom mastoparan. Recently, Leite et al., (2015) [46] extended these results and showed that PS increase the Polybia-MPI binding to cell surface by a factor of 7–8. Moreover, this work found that not only PS but also phosphatidylethanolamine (PE) plays a critical role in the anti-

tumorigenic action of the peptide. Higher levels of PE content in cell membranes correlated to an increase in pore size and permeabilization. Since an increase in PE content is also observed in cancer cells, authors suggested that PS/PE synergistically enhances the activity of Polybia-MPI in cancerous cells.

As mentioned in this review, proteomic analysis of animal venoms combined with characterization of individual components could lead to rational design of novel biotechnological products. Using the structural and functional findings described here, Zhang et al., (2010) [149] further synthesized and characterized the *in vivo* antitumor activity of a Polybia-MPI analogue (MPI-1). In addition of a significant increase in resistance to enzymatic degradation, the modified peptide exhibited an enhanced lytic effect along with a remarkably lower mortality rates in mice when compared with Polybia-MPI. In summary, the results pointed to Polybia-MPI-1 as an improved variant of Polybia-MPI to be considered in the rational development of peptide-based anti-cancer drugs.

6. Concluding remarks

Venoms from Hymenoptera species have evolved as a defensive/predatory chemical weapon to be used against colony predators and to capture prey. Therefore, they represent unique cocktails of bioactive molecules with a wide range of biological functions that work synergistically during the envenoming process. Venom profiling and molecular characterization based on the combined use of different *-omic-* approaches is a mandatory prerequisite for the development of novel antivenoms and for the rational design of strategies to cope with HVA. For almost two decades, the venom of the neotropical social wasp *P. paulista* has been explored in order to elucidate the toxins-arsenal displayed in the victims after the insect sting. Like in the case of other venomous animals, the strategies used for biochemical and functional characterization of *P. paulista* venom have moved from traditional proteomic techniques, focusing in identification of a limited number of venom compounds, to systemic venomic approaches. *P. paulista* venom profiling resulted in the identification and molecular characterization of dozens of toxins, thus enhancing our understanding of the envenoming process. Overall, these findings enabled the development of allergy-CRD based in recombinant allergens and the search of novel *P. paulista*-venom derived biopharmaceuticals. Furthermore, venomic analyses of *P. paulista* represent a model for toxinology studies of other Hymenoptera taxon that could result in the identification of novel pharmacologically relevant molecules and the improvement of systems used for diagnosis of HVA caused by other clinically relevant insects.

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Review

Facing Hymenoptera Venom Allergy: From Natural to Recombinant Allergens

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Abstract: Along with food and drug allergic reactions, a Hymenoptera insect Sting (Apoidea, Vespidae, Formicidae) is one of the most common causes of anaphylaxis worldwide. Diagnoses of Hymenoptera venom allergy (HVA) and specific immunotherapy (SIT) have been based on the use of crude venom extracts. However, the incidence of cross-reactivity and low levels of sensibility during diagnosis, as well as the occurrence of nonspecific sensitization and undesired side effects during SIT, encourage the search for novel allergenic materials. Recombinant allergens are an interesting approach to improve allergy diagnosis and SIT because they circumvent major problems associated with the use of crude venom. Production of recombinant allergens depends on the profound molecular characterization of the natural counterpart by combining some "omics" approaches with high-throughput screening techniques and the selection of an appropriate system for heterologous expression. To date, several clinically relevant allergens and novel venom toxins have been identified, cloned and characterized, enabling a better understanding of the whole allergenic and envenoming processes. Here, we review recent findings on identification, molecular characterization and recombinant expression of Hymenoptera venom allergens and on the

evaluation of these heterologous proteins as valuable tools for tackling remaining pitfalls on HVA diagnosis and immunotherapy.

Keywords: Hymenoptera venom; allergy; recombinant allergens; "omics" approaches; diagnosis; immunotherapy

1. Hymenoptera Venom Allergy: General Epidemiological Considerations

Approximately 20,000 species of Hymenoptera from Apoidea (bees) and Formicidae (ants) together with 15,000 species of Vespoidea (wasps, yellow jackets) have been identified as clinically relevant [1]. Stinging events caused by these species account for 1.5%–34.1% of all types of anaphylaxis reported per year [2]. Hymenoptera venom can cause local and systemic allergic reactions including anaphylaxis. Several factors, such as the insect type, concomitant cardiovascular and respiratory disease, age, mastocytosis, high levels of serum tryptase, and previous use of angiotensin-converting enzyme (ACE) inhibitors, determine the severity of the clinical symptoms [2]. Systemic reactions occur mainly in allergic patients, compromising the proper functioning of the respiratory and circulatory systems. Clinical symptoms include generalized urticaria, angioedema, blood pressure fall, bronco-spasms, cardiac and respiratory arrest, and anaphylactic shock [3]. The estimated prevalence of systemic reactions associated with Hymenoptera venom allergy (HVA) is 0.3%–8.9%, while anaphylaxis occurs in 0.3%–42.8% of the cases [4]. Life-threatening cases occur in 0.1%−0.5% of inhabitants in Europe and the United States [5] and are mainly due to systemic hypersensitive reactions mediated by the specific allergen IgE, which binds to the Fc receptors on the surface of several cell types, including mastocytes and basophiles. The recognition of the allergen-IgE immuno complex by Fc receptors induces degranulation and release of inflammation mediators such as histamine, leukotrienes, and several cytokines [6].

To date, collected data about the incidence of Hymenoptera stings in humans have not been consistently documented. However, an estimated 56% to 94% of adults worldwide have been stung at least once in their lifetime [7]. Insect venom anaphylaxis incidence is highly influenced by geographic and economic backgrounds. An epidemiologic study developed on a pediatric population of Ireland [8] showed that 37.5% of children suffered at least one sting in their lifetime, while a proportion of 56.3% was observed in a similar survey conducted in Israel [9]. As mentioned by the authors, variations in geographic situation, weather factors, and flora/fauna composition between those countries may have caused the differences in the reported results.

Several studies showed that accident events are less frequent in the pediatric population than in adults [10]. Similarly, rates of insect sting hypersensitivity have been reported as lower in children (0.4%–8%) than in adults (3%) [11]. On the other hand, greater percentages of sting events are reported for the male compared to the female population [12], with beekeepers showing the highest incidence rate. Unfortunately, little is known about the incidence of Hymenoptera venom allergy in low-income countries, where most cases are not well documented, leading to an underestimation of their impact on

human health and quality of life. Most of the survey studies currently reported were conducted in developing countries where prevalence of HVA is assumed to be low, given the small percentage of people who live or develop their economic activities near populations of Hymenoptera insects.

2. Hymenoptera Venom Composition: "Omics" Approaches

Molecular characterization of Hymenoptera venom represents a milestone in the design and development of novel, safe and more efficient diagnosis and therapy strategies. Hymenoptera venom is a complex mixture of low molecular weight compounds such as biogenic amines, lipids and carbohydrates, cationic peptides, and high molecular weight proteins that represent the so-called allergens [13]. Usually, low molecular weight components are responsible for local toxic reactions, while systemic reactions are due to allergenic proteins. To date, hundreds of Hymenoptera venom allergens have been identified and characterized. Most of them are enzymes such as hyaluronidase, phospholipase A1 and A2, antigen 5, serine proteases, and acid phosphatases [5,14].

The application of high-throughput techniques along with several omics approaches are revealing novel, clinically relevant allergens and venom toxins that could be useful in order to understand the whole "venome" [14] and, subsequently, the allergenic and envenoming processes. Heterologous expression of these molecules, especially in the case of venom allergens, will help in improving component-resolved diagnosis (CRD) of venom allergy and specific immunotherapy (SIT). Here, we focus our analysis on Hymenoptera venom allergenic proteins recently discovered and characterized using different omics approaches and in some cases, high throughput screening techniques that, in conjunction, greatly contribute to their production as recombinant molecules.

1.1. Exploring Hymenoptera Venom Proteome

Most of the Hymenoptera venom allergens currently cloned and recombinantly expressed have been characterized using proteomic approaches, including the usual SDS-PAGE, 2D protein electrophoresis, mass spectrometry, and chromatographic techniques. To date, honeybee venom (HBV) and yellow jacket venom (YJV) remain the most explored among any other from the Hymenoptera species, mainly due to their high clinical and epidemiological impacts. Several pioneer works using chromatographic and immunochemical techniques led to the discovery of four major allergens from HBV: phospholipase A2 (*Api m* 1), hyaluronidase (*Api m* 2), acid phosphatase (*Api m* 3) and melittin (*Api m* 4) [15–17]. Meanwhile, early proteomic, biochemical, and immunological works with wasp venom enabled the identification of the three major allergens in *Vespula vulgaris* venom: phospholipase A1 (*Ves v* 1), hyaluronidase (*Ves v* 2), and antigen 5 (*Ves v* 5) [18]. Commercial recombinant forms of *Api m* 1, *Ves v* 5 and *Ves v* 1 are now available and have been successfully used for differentiation of true double sensitization from cross-reactivity cases during diagnosis [19,20]. Nevertheless, despite being widely used in allergy diagnosis, reports of low sensitivity levels of IgE detection when *Api m* 1 is used as the unique marker for HBV allergy [21] have prompted the search for novel *A. mellifera* venom allergens that could help in overcoming this inconvenience.

Peiren *et al.* identified three novel proteins by mapping the *A. mellifera* venom proteome using 2D gel electrophoresis and mass spectrometry (MALDI TOF/TOF-MS and Q-TRAP LC-MS/MS) techniques [22]. Full gene codifying for the novel protein 2, later named as icarapin (*Api m* 10), was subsequently amplified by 5′-Rapid amplification of cDNA ends (5′-RACE), cloned and expressed using a bacterial commercial system [23]. As *Api m* 10 is a low-abundance protein in HBV, a recombinant (r) form of the allergen was evaluated for the sIgE reactivity against a panel of allergy patients' serum [24]. r*Api m* 10 reacted with 80% of patients' sera, showing that it represents a novel allergen that is unrepresented in honeybee crude venom extracts but that could be used to improve specific allergy diagnosis by increasing the sensitivity rates. In fact, it was recently shown that r*Api m* 10 expressed in insect cells reacted with 61.8% of the sera from patients allergic to HBV [25]. Peiren *et al.* also identified an *A. mellifera* venom component similar to the major royal jelly protein 8 (MRJP 8) [22]. Further efforts to clone and express this MRJP 8 (*Api m* 11.0101) and a similar protein MRJP 9 (*Api m* 11.0201) in insect cells led to the identification of both molecules as minor allergens of *A. mellifera* venom [26].

The glycosylated *Api m* 11.0101 protein expressed on High Five *Tricolupsia ni* insect cells reacted with 53.2% of the allergic patients' sera, while the recombinant allergen expressed in *Spodoptera frugiperda* Sf9 cells, lacking α 1,3-core fucose (cross-reactive carbohydrate determinants (CCD-free), reacted with 14.9%. Moreover, MRJP9 expressed in High Five cells reacted with 61.7% of the patients' sera, while CCD-devoid recombinant allergen recognized 34% of them. Higher values of allergen sIgE reactivity with MRJP9 produced in High Five cells reflected the fact that this isoallergen has only three *N*-glycosylations, which is half of that of MRJP 8. Given these results, authors proposed that *Api m* 11.0101 and *Api m* 11.0201 are minor HBV isoallergens with IgE-sensitizing potential that could be contributing to improve the efficacy of CRD of HBV allergy by helping to identify the subsets of patients sensitized to these proteins. Furthermore, this result may also contribute to tackling the royal jelly-related allergic reactions (asthma, dermatitis and anaphylaxis) in individuals using cosmetics, food supplements, and commercial medical products derived from royal jelly (RJ) [27,28].

Allergens B and C, now *Api m* 3 and *Api m* 5, respectively, were initially identified in the high molecular fraction of *A. mellifera* venom [29] by combining chromatographic, proteomic and immunologic assays. Early attempts to clone *Api m* 3 failed to obtain a full-length gene sequence [30]. By combining an *in silico* analysis of *A. mellifera* venom peptides and available genome sequences, Grunwald *et al.* successfully designed PCR primers for the full gene amplification of *Api m* 3 [31] and its subsequent expression using insect cells. In that study, r*Api m* 3 showed reactivity with 37% of the sera from the HBV-sensitized patients analyzed. The sequencing of the purified natural *Api m* 5 using tandem mass spectrometry followed by an *in silico* comparative analysis with the *A. mellifera* genome also allowed its cloning and proper expression in insect cells [32]. Interestingly, by using a similar approach, authors were able to identify an acid phosphatase from *Vespula vulgaris* venom (*Ves v* 3) having a high percentage of identity with antigen 5 from HBV. *Api m* 3 and *Api m* 5 were also evaluated on CRD of HBV allergy [25] and reacted with 50% and 58.1% of the sera of bee venom allergic patients, respectively.

In a recent work, Blank *et al.* reported the identification of two novel vitellogenins in honeybee (*Api m* 12) and *Vespula vulgaris* (*Ves v* 6) venoms, also using tandem mass spectrometry-based protein sequencing [33]. Recombinant forms of both allergens expressed in insect cells are glycosylated but CCD-free, as no reaction was detected with anti-HRP (horseradish peroxidase) rabbit serum, specific for α 1,3-core fucosylation of N-glycans. This feature prevents the occurrence of cross-reactivity,

transforming *Api m* 12 and *Ves v* 6 as interesting alternatives for improving the diagnosis of HBV and YJV allergy.

For decades, classical proteomic approaches using 2D electrophoresis and mass spectrometry have uncovered around 30 defined compounds in *A. mellifera* venom, including allergenic proteins, low molecular mass peptides, and biogenic amides. In contrast, Van Vaerenbergh *et al.* recently identified in a single study 83 new molecules in HBV, including 17 putative toxins, by using a combinatorial peptide ligand library approach followed by shotgun LC-FT-ICR MS analysis [34]. In a similar study, also using a combinatorial peptide ligand and nano-LC/MALDITOF/TOF-MS system, four novel proteins in HBV were described [35]. These works reinforced the relevance of applied high-throughput techniques and novel proteomics methods in order to elucidate the whole proteome composition of Hymenoptera venoms as well as identify novel low-abundance allergenic molecules that could be used in panels of defined allergens for CRD.

Similar to studies with HBV, several proteomic approaches have also been used to determine the composition of venom from different wasp species. A classical bottom-up procedure using 2D protein electrophoresis and tandem mass spectrometry was used to elucidate *Polybia paulista* venom proteome in an attempt to understand the envenoming process [36]. In this work, authors describe the presence of more than 84 proteins in the venom of this Brazilian endemic wasp, most of them for the first time. In this regard, the proteomic characterization of a hyaluronidase from *P. paulista* venom (*Poly p* 2) [37] has allowed its cloning, sequencing, 3D-structural modeling, and recombinant expression using an *E. coli* system [38,39]. The authors demonstrated that the recombinant form reacts with sera from patients allergic to *P. paulista* venom in the same way as the natural allergen [39]. A similar approach is being used to obtain the recombinant forms of the two other major allergens—phospholipase A1 (*Poly p* 1) and antigen 5 (*Poly p* 5)—from the venom of this species as proteomic analysis and characterization of the referred proteins are already available [40–42]. In fact, data derived from proteomic characterization of *Poly p* 5 were used to identify linear B-cell epitopes in the structure of this allergen [43]. Using 66 peptides covering the full-length protein, authors were able to identify nine linear B-cell epitopes reacting with human IgG, one of which (peptide 7) also reacts with human IgE. Further analysis combining structural modeling of *Poly p* 5 and the peptide sequences revealed the presence of a critical fragment of peptide 7 (WAKTKE). Authors suggested that this sequence is settled at a loop on the protein surface representing the major B-cell binding epitope. Considering these findings, they proposed that it could be used as a candidate for peptide immunotherapy or production of modified recombinant Ag 5 (r*Poly p* 5), having reduced allergenicity and an improved safety and tolerance induction profile, a major goal for SIT success [43].

1.2. Genomic and Transcriptomic Approaches

Genomic approaches have also been used to clone and express genes from Hymenoptera venom. In this regard, the sequencing of the *A. mellifera* genome [44] represented a milestone for the identification of novel Hymenoptera genes, including those encoding venom allergens. Annotated gene sequences obtained from *A. mellifera* have been used in comparative analysis of the transcriptome-derived data from other Hymenoptera species. For instance, analysis of expressed sequence tags (ESTs) derived from the *Bombus ignitus* venom gland has recently allowed full-length gene amplification and sequencing of venom phospholipase 2 from this species [45].

As noted, relatively little information is available regarding gene and protein sequences from venom allergens of endemic species from the Southern Hemisphere. Consequently, few studies have been published on recombinant expression of venom allergens from Hymenoptera species of this geographic region. The integration of transcriptomic, peptidomic, and proteomic approaches will provide large amounts of data that may be used to reduce the lack of commercial recombinant allergens from typical species of this region and thus improve specific diagnosis of allergy. Interestingly, a recent study of allergic rhinitis in subtropical regions that combined transcriptomic, proteomic, and allergenomic approaches to grass pollen enabled the detection of more than 17,000 transcripts, several corresponding to putative allergenic proteins [46]. In addition, at least four novel allergens, namely *Sor h* 1, *Sor h* 13, *Sor h* 2, and *Sor h* 23, were identified in this study.

Transcriptomics has emerged as a common strategy for exploring venom composition and understanding the envenoming process. The use of this approach provides a large amount of biological data, helping to unravel the protein and peptide composition of venoms. A transcriptome analysis of the venom gland from *Tetramorium bicarinatum*, a widely distributed ant, led to the identification and sequencing of 364 high-quality ESTs, 18% corresponding to venom compounds, 62% of which match previously annotated allergens sequences [47]. Several identified ESTs match putative pilosulin-like peptides, the major allergens described in *Myrmecia pilosula* and also the main cause of HVA in Australia [48]. Another important finding was the detection of transcripts with *Sol i* 3-like peptide sequence, a major allergen from the *Solenopsis invicta* ant, which is a *Ves v* 5 3-D homolog. A similar transcriptomic analysis was conducted using venom glands of *Dinoponera quadriceps*, a giant ant from the Neotropical region [49]. A cDNA library of the venom gland was constructed and analyzed using the Sanger sequencing method and deep whole-transcriptome shotgun sequencing. From 420 independent clones derived from the cDNA library, 15% of contigs and singlets match previously identified venom toxins, while 5% represent hypothetical proteins. Deep RNA sequencing reveals 3807 contigs that were divided in 23 functional groups according to the Gene Ontology and Cluster of Orthologous Groups (COG) database. For both procedures, sequences matching annotated toxins and venom components were namely dinoponeratoxins, venom allergens, phospholipase-like toxin peptides and lethal-like proteins. Within venom allergens, sequences matching those encoding *Solenopsis invicta* phospholipase A1 (*Sol i* 1) and antigen 5 (*Sol i* 3) were found at high rates. Interestingly, 65% of the transcripts that were obtained using deep RNA sequencing procedure did not match any known sequence, showing the potential of this approach to identify novel venom compounds.

Transcriptomic coupled with proteomic analyses of venom glands have also been carried out to elucidate the molecular basis of envenoming processes after an accident with poisonous animal species including snake [50–54], jellyfish [55], spider [56,57] and scorpion [58]. These studies have resulted in the identification of several new venom toxins, allowing a greater understanding of the envenoming process.

Although venom composition of clinically relevant Hymenoptera species of the Northern Hemisphere has been elucidated, novel molecules are still being described mainly by using novel high-throughput screening assays combined with several omics approaches. This fact shows the potential of applying these strategies in the identification and characterization of novel venom

toxins and allergens that could be used in the pharmaceutical and biotechnology industries, particularly for improvement of diagnosis and treatment of venom allergy.

3. Hymenoptera Recombinant Allergens and Diagnostic Tests

Specific diagnosis of HVA represents a prerequisite step for the success and safety profile of immunotherapy procedures. For decades, the use of crude venom extracts predominated in the design of diagnostic strategies. However, high levels of cross-reactivity occur during diagnosis using crude extracts as allergenic material [59]. Homologous proteins in the venom of two different groups or species of insects may share specific IgE (sIgE) epitopes and/or can have CCDs. The latter accounted for up to 60%–70% of the patients' sera that cross-reacted with venom of different Hymenoptera species [60,61]. Cross-reactive occurrence prevents the proper identification of culprit venom leading to inclusion of non-relevant allergens on specific therapy schemes, compromising the safety profile of therapy intervention as nonspecific sensitization. Undesired adverse side effects can occur during SIT.

Diagnosis is based on patient history concerning allergic reactions, positive skin test, and *in vitro* tests that include allergen-specific IgE detection and basophil activation test (BAT) [62–64]. Double positive is a common issue during Hymenoptera venom allergy diagnosis using crude venom extracts, since up to 59% of the patients show reaction to both HBV and YJV [59]. These rates have been explained by occurrence of true double sensitization or cross-reactivity, the latter accounting for 70%–80% of double sensitization in the case of these two species [65].

Component-resolved diagnosis based on the use of defined, properly characterized and purified recombinant allergens has emerged as a powerful tool for circumventing problems in venom allergy diagnosis [66,67]. Commercial recombinant forms of *Api m* 1 from HBV and *Ves v* 5, *Ves v* 1 from *V. vulgaris* venom are now available and have been successfully used for differentiation of true double sensitization from cross-reactivity cases [19,20] during diagnosis. Studies performed mainly with non-glycosylated variants of these recombinant allergens have provided the proper identification of the culprit venom. In brief, by using non-glycosylated recombinant forms of *Api m* 1, *Api m* 2, and *Ves v* 5, expressed in *E. coli*, it was possible to distinguish bee and/or wasp sensitization in 29 allergic patients previously diagnosed with double positivity when venom extracts were used as the allergenic material [68]. A similar study, performed using r*Ves v* 5 and r*Api m* 1 and sera from 20 patients previously diagnosed as positive for sIgE presence for both bee and wasp venom, showed a rate of double positivity/double negativity/single positivity for r*Api m* 1 and r*Ves v 5* of 12/1/9 patients on sIgE test. Thereafter, a double-positivity/double-negativity/single-positivity ratio of 6/2/14 was obtained in the case of BAT [19]. Müller *et al.* found that only 47% of 76 patients previously identified as double positive for whole venom extract from honeybee and wasp reacted with r*Ves v 1*, r*Ves v 5*, and *rApi m 1* during sIgE detection [69]. In addition, 100% of the patients previously detected as wasp venom single positive reacted to r*Ves v* 1 and/or r*Ves v* 5, and 78.3% of single HBV allergic patients had sIgE for r*Api m* 1. Recombinant forms of *Ves v* 1 and *Ves v* 5 expressed on insect cells were able to identify 16/20 patients previously identified as double positive on sIgE detection assays [65]. Recently, natural and recombinant forms of antigen 5 and phospholipase A1 from *Vespula vulgaris* and *Polistes dominula* (*Pol d* 1 and *Pol d* 5) proved to be necessary for proper identification of culprit venom during diagnosis of allergy to the venom of these species[70].

As seen, detection of sIgE to r*Ves v* 1, r*Ves v* 5, and r*Api m* 1 represents a very reliable strategy in order to recognize double sensitization from cross-reactivity during diagnosis of honeybee and/or YJV allergy. However, it is important to note that *Api m* 1 has a limited diagnostic sensitivity (60.0% to 72.2%), restricting its usefulness for sIgE detection as a unique relevant allergen included during HBV allergy diagnosis [21]. As an alternative, it was proposed that the inclusion of other honeybee allergens in panels for CRD might assist in solving the problem related to the low sensibility. In fact, by using a set of recombinant *A. mellifera* venom allergens (*Api m* 1, *Api m* 2, *Api m* 3, *Api m* 4, *Api m* 5, *Api m* 10), it was possible to detect sIgE reactivity for at least one allergenic protein in 94.4% of the patients' sera [25]. Therefore, the inclusion of other recombinant forms of HBV allergens that differ from r*Api m* 1, and which are now available, may improve the results of venom allergy diagnosis by raising the levels of sIgE detection. Interestingly, to date, more than 60 Hymenoptera allergenic proteins have been identified, cloned, and recombinantly expressed [14], and most of them could be exploited as candidates to be used in clinical practices for improving venom allergy diagnosis.

4. Venom Immunotherapy: Trends and Prospects for the Use of Recombinant Allergens

There are two major approaches in order to treat systemic allergic reactions caused by Hymenoptera stings. Acute management mainly comprises the use of self-injectable epinephrine, while long-term management is related to SIT intervention, which is the only disease-specific approach currently used. It involves the administration of increasing amounts of crude venom extract, typically beginning with 0.1–1.0 μg, in order to induce tolerance in allergic patients. SIT has proved to be highly effective. In fact, for wasp venom allergy, more than 90%–95% of the patients did not show any systemic reactions after being re-stung, while more than 80% did not develop reactions in the case of honeybee [4,10,71]. However, local and severe adverse reactions may occur during treatment, with higher rates observed for patients receiving bee venom compared to wasp venom [72,73]. It has been reported that 20%−40% of the patients suffered systemic side effects during SIT with HBV, while 5%−10% suffered in the case of wasp venoms [72]. In addition, in a recent systemic review, Boyle *et al.* published an interesting meta-analysis of one quasi-randomized controlled and six randomized trials of Hymenoptera venom SIT [74]. Trials included ant, wasp, and bee immunotherapy in children and adults, using a sublingual (1) or subcutaneous (6) route, accounting for a total of 392 participants. Meta-analysis showed that only 3/113 (2.7%) participants receiving SIT experienced a subsequent systemic allergic reaction to a sting challenge, compared with 37/93 (39.8%) of the untreated participants (risk ratio (RR) 0.10, 95% confidence interval (CI) 0.03 to 0.28). It is important to note that no differences were identified between patient groups or modes of treatment, showing that the sublingual could be considered a feasible administration route for SIT, which could also improve patients' compliance since it is also a less invasive route for allergen administration. Authors also found a significant risk of systemic adverse reaction outcomes. In summary, the meta-analysis showed that SIT using venom extracts is a highly effective strategy to treat Hymenoptera venom allergy, but it is related to a significant risk of severe adverse reaction occurrence. Therefore, novel strategies are required in order to reduce the incidence of severe adverse reactions, thus improving the SIT safety profile.

To date, immunotherapy schedules remain highly heterogeneous as they vary in the nature of the allergenic material used, dose amount, administration route and the time necessary to reach

the protective and maintenance dose. For the latter, protocols could be divided into *slow protocols*, requiring several weeks to attain protective and maintenance doses, *rush* (also *semi-rush*) *protocols* requiring several days (4–7 days), and finally, *ultra-rush* protocols requiring only hours to 1–2 days [75]. In general, it has been stated that *slow*, *semi-rush*, and *rush* protocols are safer than *ultra-rush*. A comparative study between *ultra-rush* and *semi-rush* buildup with jack jumper ant (*Myrmecia pilosula*) venom found high percentages of occurrence of side effects in patients included on an *ultra-rush* schedule [76]. However, some authors have reported similar rates of adverse reactions during immunotherapy using *ultra-rush*, *rush*, and *slow* schedules [77,78]. Then, the safety profile of strategies, with respect to the time used to reach the protective or maintenance doses, probably relies more on the type of insect causing the allergy than on the procedure used to induce tolerance.

Subcutaneous is the main administration route currently used on SIT procedures for HVA. However, different approaches, such as painless routes for allergen application, are needed in order to improve patient compliance. Sublingual immunotherapy (SLIT) has been successfully used on allergic rhinitis treatment and provides an interesting, alternative method of allergen administration for venom allergy immunotherapy [79,80]. More than 60 randomized double-blind, placebo-controlled trials performed with SLIT have demonstrated its efficacy and safety profile for the treatment of different allergic diseases [81]. To date, only one study has evaluated the effect of SLIT on Hymenoptera venom allergy. A proof-of-concept clinical trial was conducted in humans using HBV and patients with a history of large local reactions (LLRs) after an insect sting [82]. Significant reduction in the diameter of LLRs (20.5 to 8.5 cm) was observed for the active patients, while no effects were detected in the placebo group. However, further studies are required in order to establish the efficacy and safety of SLIT as a more compliant and suitable procedure to treat HVA.

To date, mainly crude and commercially standardized venom extracts are used as allergenic materials in SIT. However, as noted, their uses are associated with the occurrence of nonspecific sensitization and severe adverse reactions during SIT [74], with higher effects when the culprit insect is not properly identified. It is important to point out that crude venom extracts present variations on allergenic compound composition and stability, leading to considerable variations in the outcomes of SIT. Recombinant allergenic proteins emerge as an interesting option in order to face these disadvantages [67].

Thus far, recombinant allergens from Hymenoptera venom have been tested mainly for diagnosis improvement. In fact, to our knowledge, only one study has evaluated the *in vivo* induction of tolerance to insect venom during SIT by using a recombinant Hymenoptera allergen [83]. In their work, the authors developed a murine model of *Vespula vulgaris* venom allergy and found lower rates of toxic effects during immunotherapy with the recombinant form of *Ves v* 5 than with the crude venom extract. Authors also found high levels of tolerance induction when the recombinant allergen was used as allergenic material.

To date, no study has been conducted in humans in order to evaluate recombinant allergens as an alternative material to be use in Hymenoptera venom immunotherapy. However, results obtained with recombinant allergens for other allergic diseases [84,85] show the potential of applying a similar approach on HVA specific treatment. In theory, heterologous expression could lead to the development of personalized panels of safer unmodified or modified recombinant allergens that may reduce the incidence of severe adverse reactions and nonspecific sensitization during Hymenoptera venom SIT. However, it would be necessary to reduce economic costs and improve the safety and efficacy profile in

order to position this technology as a competitive commercial alternative to the use of crude venom extracts, for the development of a novel commercially licensed product.

5. Production of Recombinant Allergens: From Gene to Proper Expression Systems

As previously discussed, recombinant DNA technology represents a powerful tool to overcome the remaining pitfalls in the diagnosis and immunotherapy of Hymenoptera venoms. Large amounts of standardized, highly purified, non-modified recombinant allergens with a similar physiochemical and immunologic profile as the natural variants can be produced by using this technology. Modified hypoallergenic and chimeric proteins can also be obtained in order to improve the efficacy and safety of SIT. In this regard, recombinant allergens engineered to have a reduced specific IgE reactivity while conserving T-cell epitopes have been successfully tested, as they induce tolerance without occurrence of undesirable side effects [86,87]. All these features are moving the scenario of recombinant allergens for some allergic diseases towards the development of CRD and future patient-tailored therapy schemes [88]. Future progress in the scenario of recombinant Hymenoptera venom allergen production highly depends on the selection of a proper system for its heterologous expression. As noted, more than 60 Hymenoptera venom allergenic proteins have been cloned and expressed using bacteria, yeast, and the mammalian cell system [14]. *E. coli* remains a highly feasible system for allergenic protein expression, as it enables the production of non-glycosylated molecules aiding to solve CCD-mediated cross-reactivity [68], therefore allowing the proper identification of the culprit venom. However, the use of the prokaryotic system for allergen expression is compromised in several cases, since most Hymenoptera allergens currently identified have conformational IgE epitopes and carbohydrate motifs. Proper folding and glycosylation steps are crucial for heterologous production of molecules with a physiochemical and immunologic profile similar to the natural variants. Attempts to express Hymenoptera allergens using the *E. coli* system have rendered different results in terms of structural and immunological similarities of heterologous proteins in comparison with the natural counterpart. A recombinant non-glycosylated form of *Api m* 1 expressed in *E. coli* showed an identical structural and immunological profile in relation to the natural enzyme, indicating a native-like folding of the protein [89]. In that case, glycosylation was not critical for the allergenicity of the molecule. In contrast, heterologous expression of *Api m* 2 using *E. coli* yielded a protein with lower enzymatic activity and sIgE binding capacity compared to those of the natural (n) form of *Api m* 2 and r*Api m* 2 expressed in baculovirus-infected insect cells [90]. Interestingly, Skov *et al.* reported that a recombinant form of *Vespula vulgaris* hyaluronidase (*Ves v* 2) obtained by using the *E. coli* system had similar levels of enzymatic activity as n*Ves v* 2 after *in vitro* refolding and purification steps [91]. The purified r*Ves v* 2 was crystallized and analyzed using sequence alignment and structural comparison, showing a high level of 3D structure homology with natural *Api m* 2 from HBV. In addition to this, Justo Jacomini *et al.* reported that the recombinant hyaluronidase obtained from the venom of the endemic Brazilian wasp *P. paulista* (*Poly p* 2) using the *E. coli* system was recognized by allergic patient sera at higher levels than n*Poly p* 2 [39]. In this case, heterologous protein was expressed in an insoluble form, and was then solubilized but not re-natured. The study proposed that allergen recognition was mediated by linear rather than conformational B-cell epitopes previously described on n*Pol p* 2 by combining proteomic procedures [37,38] and bioinformatics tools. Hyaluronidase variants from venom of queen and worker

individuals of *Solenopsis invicta* (*Sol i* 2) have been successfully cloned and expressed in *E. coli* [92]. High levels of soluble r*Sol i* 2 was produced at 16 °C, which was recognized by human sIgE from allergic patients' sera. The authors pointed out that, by using *E. coli*, an inexpensive and time-saving protocol for functional allergen production was developed, which is a common objective when heterologous protein expression procedures are designed. As observed, heterologous expression using *E. coli* remains a feasible strategy to obtain large amounts of functional allergens that could be used in diagnosis and treatment of HVA. However, it is important to highlight that optimal expression conditions should be explored for each allergen, and that, in several cases, bacteria may not be used for heterologous expression due to the inability to achieve the proper protein folding.

Limitations associated with recombinant expression of Hymenoptera venom allergens in prokaryotes could be tackled by using eukaryotic expression systems currently available, namely yeast, baculovirus-infected insect cells, plants and mammalian cells. Recently, *Spodoptera frugiperda* Sf9 insect cells have received particular interest because high levels of N-glycosylated lacking α 1,3-core fucosylation allergens, properly folded and displaying all sIgE epitopes profile, could be obtained using this system [14,65]. Commercial forms of r*Api m* 1 (ImmunoCAP i208; Thermo Fisher Scientific, Uppsala, Sweden), r*Ves* v 1, and r*Ves v* 5 produced in baculovirus-infected insect cell line Sf9 are now available for specific diagnosis of *A. mellifera* or/and *V. vulgaris* venom allergy [20,63,93]. n*Ves v* 1 and n*Ves v* 5 are non-glycosylated proteins that share non-sIgE epitopes with n*Api m* 1; therefore, recombinant variants of these allergens have been successfully used to identify culprit venom in individuals previously diagnosed with double sensitization. However, some concerns have emerged when using this system for expression of allergens that are glycosylated in their natural form, as recombinant, non-fucosylated (CCD-free) allergens obtained in this system have, in general, lower diagnostic sensitivity than those displayed by natural allergens or crude venom extracts. As noted above, r*Api m* 1 expressed in Sf9 cells lacking α-1,3-linked fucose and N-linked glycosylation site was described as a low-sensitivity recombinant variant for HBV allergy diagnosis [21,94]. A study conducted by Jakob *et al.* showed that this variant was able to detect 72% of patients diagnosed with HBV allergy [95], which is similar to previous reports for other r*Api m* 1 variants, but lower than levels obtained in diagnosis with n*Api m* 1 (80%). Further analysis showed that those sensitivity differences are mainly associated with CCD recognition on natural material by sIgE in patients' sera. Even when the authors concluded that benefits associated with avoidance of cross-reactivity and false double sensitization justified the use of this non-glycosylated recombinant form of r*Api m* 1 during diagnosis, these results support the idea that novel strategies are required in order to increase the sensitivity of diagnosis when using this r*Api m* 1 variant. One of these strategies is the use in CRD of recombinant forms of recently identified HBV allergens (*i.e.*, r*Api m* 10, r*Api m* 5, r*Api m* 6) that are under-represented in crude venom extracts, but are still recognized by sIgE in allergic patients. In summary, considering structural and immunological features, the insect cells, especially Sf9, represent a highly efficient system for heterologous expression of Hymenoptera allergens, as they ensure proper protein folding and avoidance of cross-reactivity by producing α-1,3-linked fucose-lacking molecules.

The methylotrophic yeast *Pichia pastoris* is a system widely used for expression of recombinant proteins, including several allergens. Features like high yields, genetic simplicity, capability to perform post-translational modifications, and the ability to ensure proper protein folding makes it an attractive

system for protein production in the biotechnological and pharmaceutical industries. To date, however, a limited number of Hymenoptera venom allergens have been expressed using this yeast. For instance, high levels of a non-enzymatically active form of *Ves v* 1 were produced in *P. pastoris* [96] after introducing a point mutation at the active site of the allergen. The modified r*Ves v* 1 form was produced as a secreted soluble protein facilitating the subsequent purification steps. The allergens recognized sIgE from allergic patients, inhibited binding of sIgE to crude venom extract, and induced *in vitro* histamine release in sensitive basophils. Similarly, a recombinant form of the hypoallergenic antigen 5 from *Polybia scutellaris*—a South American wasp—was obtained using the *P. pastoris* system [97]. The allergen was engineered to avoid non-native glycosylation and was expressed at high levels (27 mg/L) in a scale-up schedule for optimal methanol concentration determination. Comparative immunological assays showed a similar IgE epitope profile of the recombinant molecule in relation to the natural variant, showing its potential as a candidate for the improvement of component-resolved diagnosis of *P. scutellaris* venom allergy. Considering these results and general features related to protein expression, *P. pastoris* appears as an inexpensive and suitable option for immunologically active allergen production, especially for research and academic use. Determination of optimal expression conditions, and rational design of molecules to avoid cross-reactivity incidence and assure high yields of the active protein production, remain important issues to tackle in order to improve allergen expression using this system.

To the best of our knowledge, few or no works have been published for heterologous production of Hymenoptera venom allergens using plant and mammalian cell-based systems. However, in the case of plant cells, this situation could change in the next few years, given the increasing interest in developing "*molecular farming*" as a cost-effective alternative for production of pharmaceutical proteins. Plant cell-based systems are easy to scale up for large amounts of low-cost biomass production, having lower risk of protein contamination with human pathogens compared to bacteria, yeast, or mammalian cells [98]. In addition, plant cells are able to perform full post-translational modifications in the expressed molecule, ensuring proper folding and biological activity [99]. Meanwhile, although 50%–80% of the recombinant biopharmaceuticals approved each year are produced in mammalian cells, no commercially available Hymenoptera venom allergens have been obtained using this system. Expression of heterologous protein using mammalian cells is expensive, and the required post-translational modifications for proper allergen folding can be achieved using more cost-effective systems such as yeast and insects cells.

The success of CRD relies on the proper selection of protocols and systems used for recombinant allergen expression. In turn, the selection of the expression system depends highly on the structural and immunological features of each allergen. To date, the bacteria *E. coli* remains the simplest and most inexpensive system for heterologous protein production, being commonly used for academic and research purposes. In the case of Hymenoptera venom allergens, the use of *E. coli* also helps to avoid the presence of CCDs, as no glycosylation is produced in these cells, therefore decreasing the incidence of cross-reactivity. However, this feature can also limit its use due to the inability to perform post-translational modifications or to allow the formation of disulfide bonds, often leading to improper protein folding and loss of conformational IgE epitopes. Thus, a refined balance among proper folding, conservation of IgE conformational epitopes, and avoidance of CCDs is required in order to preserve allergenicity and prevent the incidence of cross-reactivity.

In this context, Hymenoptera allergens engineered to express as non-glycosylated, CCD-free proteins in insect cells have proven to be a highly efficient alternative and are now commercially available.

6. Concluding Remarks

Recent progress on identification and molecular characterization of Hymenoptera venom allergens enables the production of modified and non-modified recombinant allergens. By using different "omics" branches allied high-throughput screening techniques, we are now exploring whole "venome" [14], generating a large amount of biological data and hence detecting novel allergens that can be used for designing more specific and accurate venom allergy diagnosis strategies. The inclusion of novel, clinically relevant, commonly non-glycosylated and highly purified allergens in component-resolved diagnosis (CRD) proved to drastically reduce cross-reactivity during allergy diagnosis. Consequently, a significant improvement of specific immunotherapy (SIT) has been achieved. Furthermore, the ability to produce homogeneous panels of allergens with a defined physiochemical profile enhances the possibilities of their application in SIT schemes and, even more so, in the development of a personalized therapy. In theory, this approach could lead to a decrease in the incidence of severe systemic side effects during treatment. However, several challenges remain to be addressed in order to license the use of recombinant allergens for therapeutic interventions. Moreover, the production of hypoallergenic recombinant variants, or T-cell epitope peptides, along with the search for a less invasive administration route, could improve the therapy's safety profile, the patient's quality of life and the success in facing Hymenoptera venom allergy (HVA).

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Author Contributions

Amilcar Perez-Riverol wrote the first draft of the manuscript, performed the bibliography searches and conceptualized the review; Débora Lais Justo-Jacomini and Ricardo de Lima Zollner provided valuable corrections to the drafts. Marcia Regina Brochetto-Braga revised all versions of the manuscript, submitted it and with Amilcar Perez-Riverol provided the answers to reviewer's comments.

Conflicts of Interest

The authors declare no conflict of interest.

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4. CHAPTER 2

Molecular cloning, expression and IgE-immunoreactivity of phospholipase A1, a major allergen from *Polybia paulista* (Hymenoptera: Vespidae) venom. **Perez-Riverol, A**; Campos-Pereira, F.D., Musacchio-Lasa, A.; Romani Fernandes, L.G.; dos Santos-Pinto, J.R.A.; Justo-Jacomini, D.L.; Oliveira de Azevedo, G.; Bazón, M.L.; Palma, M.S.; Zollner R.L. and Brochetto-Braga, M.R. (**Toxicon** 2016, 124, 44-52).

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Molecular cloning, expression and IgE-immunoreactivity of phospholipase A1, a major allergen from *Polybia paulista* (Hymenoptera: Vespidae) venom

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abstract

Polybia paulista (Hymenoptera: Vespidae) is a clinically relevant social wasp that frequently causes stinging accidents in southeast Brazil. To date, diagnosis and specific immunotherapy (SIT) of allergy are based on the use of crude venom extracts. Production of recombinant forms of major allergens from *P. paulista* venom will improve diagnosis and SIT of allergic patients by reducing the incidence of cross- reactivity and non-specific sensitization. Here, we describe the molecular cloning, heterologous expression, purification and IgE-mediated immunodetection of phospholipase A1 (*Poly p* 1), a major allergen from *P. paulista* venom. The cDNA of *Poly p* 1 was extracted from venom glands and then cloned, and further expression of the recombinant allergen (r*Poly p* 1) was achieved in *Escherichia coli* BL21 (DE3) cells. Purification of r*Poly p* 1 was performed using immobilized Ni^{2+} metal affinity chromatography. Also, a single-step chromatographic method allowed the purification of native *Poly p* 1 (n*Poly p* 1) from the wasp's venom glands. We used western blotting to evaluate IgE-reactivity of the sera from 10 *paulista* venom-allergic patients to r*Poly p* 1 and n*Poly p* 1. High levels of insoluble r*Poly p* 1 were obtained during heterologous expression. After solubilization of inclusion bodies and purification of the recombinant protein, a unique band of ~34 kDa was detected in SDS-PAGE analysis. Allergen-specific IgE

Abbreviations: r*Poly p* 1, recombinant phospholipase A1 from *P. paulista* venom; n*Poly p* 1, native phospholipase A1 from *P. paulista* venom; sIgE, specific IgE; PLA1, phospholipase A1; vPLA1s, vespid phospholipases A1; HVA, Hymenoptera venom allergy; SIT, specific immunotherapy; CRD, component-resolved diagnosis.

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(sIgE) from allergic patients' sera recognized r*Poly p* 1, n*Poly p* 1 and crude venom extract to a similar extent. Our results showed that r*Poly p* 1 could be used for development of component-resolved diagnosis (CRD) and molecular-defined SIT of *P. paulista* venom allergy.

1. Introduction

Hymenoptera (Apoidea, Vespidae, Formicidae) venom allergy is one of the most common causes of anaphylaxis worldwide, accounting for 1.5%-34.1% of the cases (Bilò, 2011). Because of the close coexistence of social Hymenoptera with human populations, an estimated 56%-94% of adults worldwide have been stung at least once in their lifetime (Jennings et al., 2010). After being stung, allergic patients experience local and/or systemic reactions, including life threatening anaphylaxis. The estimated prevalence of systemic reactions associated with Hymenoptera venom allergy (HVA) is 0.3%-8.9%, of those cases, anaphylaxis occurs in 0.3%e 42.8% (Bilò and Bonifazi, 2009).

In Brazil, despite the great number of clinically relevant social Hymenoptera and stinging accidents, specific diagnosis and immunotherapy rely on the use of whole venom extracts. These allergenic materials are associated with the occurrence of IgE-mediated cross-reactivity during diagnosis, which could lead to non-specific sensitization and undesired side effects along the SIT (Ollert and Blank, 2015).

Several works have stated that CRD based on the use of panels of recombinant allergens significantly improves specific identification of culprit venom in patients diagnosed with double sensitization (Spillner et al., 2014; Perez-Riverol et al., 2015). To date, mainly recombinant forms of phospholipase A2 (*Api m* 1) and hyaluronidase (*Api m* 2) from *Apis mellifera,* along with phospholipase A1 (*Ves v* 1) and antigen 5 (*Ves v* 5) from *Vespula vulgaris,* have been used to distinguish bee and/or wasp sensitization in allergic patients. Additionally, other bee venom allergens such as phosphatase (*Api m* 3) and icarapin (*Api m* 10) have recently been shown to improve detection of honeybee venom (HBV) sensitization in allergic patients (Frick et al., 2015). Müller et al. (2012) reported that only 47% of 76 patients previously diagnosed as double positive for whole venom extract of *A. mellifera* and of *V. vulgaris* reacted with r*Ves v* 1, r*Ves v* 5, and r*Api m* 1 during specific IgE (sIgE) detection. The combined use of natural and recombinant forms of *Ves v* 1, *Ves v* 5, phospholipase A1 (*Pol d* 1) and antigen 5 (*Pol d* 5) from *Polistes dominula* venom led to the identification of single sensitization in 69% of 25 patients previously diagnosed as double positive to wasp venoms (Monsalve et al., 2012). Interestingly, Korošec et al. (2012) were able to identified 92% (184/200) of patients previously diagnosed with *Vespula* venom allergy, using r*Ves v* 1 and r*Ves v* 5.

Phospholipase A1 is a major allergen widely distribute in social Hymenoptera venoms (Santos et al., 2011; Hou et al., 2016). Vespid phospholipases A1 (vPLA1s) are known to hydrolyze ester bonds of 1,2-diacyl-3-sn glycerophospholipids at the *sn-1* position, converting these substrates into their corresponding lyso compounds and releasing fatty acids (Santos et al., 2007). During the envenoming process, vPLA1s are able to disrupt the phospholipid packing of biological membranes, causing severe hemolysis and leading to cardiac dysfunction and death in animals (Hou et al., 2016). The allergen showed direct hemolytic action against washed red blood cells (Santos et al., 2007). Molecular characterization and recombinant production of vPLA1s will improve strategies to reduce the toxic effects and immunologic reactions caused by this allergen during envenoming.

Similar to most wasps, proteomic and immunologic analyses of *P. paulista* venom led to identification of three major allergens: phospholipase A1 (*Poly p* 1), hyaluronidase (*Poly p* 2) and antigen 5 (*Poly p* 5) (Santos et al., 2010). Purification, molecular and functional characterization of n*Poly p* 1 showed that this allergen is a non-glycosylated protein of ~34 kDa, identified as a lipase of the GX class (Santos et al., 2007). Native *Poly p* 1 is 53-82% identical to the phospholipases of wasp species from the Northern Hemisphere. The protein structure has the a/b fold common to many lipases: a core consisting of a tightly packed β-sheet composed of a six-stranded parallel and one anti-parallel b-strand, surrounded by four a-helices. A proteomic study from Santos et al. (2011) suggested that n*Poly p* 1 exists as a mix of multiple forms that show different levels of reactivity with sera of *P. paulista* venom-allergic patients.

To date, only the *Poly p* 2 has been cloned and expressed, using the prokaryotic system *E. coli* BL21 (DE3) (Justo-Jacomini et al., 2014). Recombinant *Poly p* 2 was produced as an insoluble protein, solubilized and later purified. High levels of IgE-reactivity were detected when sera from patients previously diagnosed with *P. paulista* venom allergy were tested against this protein.

Here, we describe the heterologous expression, purification and sIgE-mediated immunodetection of r*Poly p* 1. IgE-reactivity of sera from sensitized patients to the recombinant allergen was compared with the recognition profile of its native counterpart (n*Poly p* 1) and crude venom extract. Our results show that r*Poly p* 1 represents a good candidate for improvement of *P. paulista* venom allergy-specific diagnosis.

2. Materials and methods

2.1. Allergic patients' sera

Sera from 10 *P. paulista* venom-sensitized patients, regardless of sex or age, were obtained from the Ambulatório de Anafilaxia of the Hospital das Clínicas, Faculdade de Ciências Médicas, Universidade Estadual de Campinas-UNICAMP. The study was approved by the Ethics Committee of FCM-UNICAMP under nº 187/2006. Informed consent was obtained in written form from all participants of the study, and participation was voluntary. Upon confirmation of positive intradermal reaction to *P. paulista* venom extract, the sera samples were collected and store at -80 ºC until use. Five sera from non-sensitized patients were used as negative controls.

2.2. Polybia paulista venom

P. paulista nests were captured around or within the campus of Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Rio Claro, SP, Brazil. The collected wasps were immediately anesthetized at low temperature (-80 ºC), and their venom glands were extracted using sterile tweezers. For n*Poly p* 1 purification, the glands (4000) were washed with sterile water and suspended in a protease inhibitor mix (2 mM Leupeptine and 1 mM phenylmethylsulfonyl fluoride; SIGMA, USA). The gland suspensions were then macerated, washed three times with the protease inhibitor mix and centrifuged at 10,000 g for 15 min at 4 ºC. The supernatant

was collected, lyophilized and stored at −80 °C until use.

2.3. RNA extraction and cDNA synthesis

For total RNA purification, 200 venom glands were washed in milli-Q sterile water, suspended in 200 mL of Trizol® (Life Technologies, USA), macerated, and then stored for 7 days at 80 °C. After RNA extraction, the first-strand cDNA synthesis was per- formed using 1 mg of total RNA, the oligo dT-primer 5'- GGC CAC GCG TCG ACT AC(T)$_{17}$-3' adapter (Gibco-Life Technologies, USA), and the ImProm-II Reverse Transcription kit (Promega,USA) according to manufacturer's instructions.

2.4. Poly p 1 coding sequence amplification and cloning

Primers for *Poly p* 1 coding sequence amplification were designed using data provided by Santos et al. (2007) (GenBank; access numbers: EF101736.1 and AM491805.1) and synthesized by Sigma-Aldrich (USA). Restriction sites (underlined) for NdeI and EcoRI were included in the forward (5'CTTA CATATG CTG ATT CCG GAA TGC CCG TTT AAC 3') and reverse (5'AGT G GAATTC TTA AAG TTT AAT GCC CTC GTT ATG ACA ATA AG 3') primers, respectively. PCR was performed as described by Justo-Jacomini et al. (2013), with minor modifications. Using an Eppendorf Mastercycler (Eppendorf, Germany), the following conditions were applied: initial denaturation at 95 °C for 5 min; 40 cycles of denaturation at 95 °C for 1 min; annealing at 57.5 °C for 1 min and extension at 72 °C for 3 min; and a final extension at 72 °C for 15 min. PCR products were checked by electrophoresis in agarose 2%, purified using IlustraTM GFXTM PCR DNA and Gel Band Purification (GE Healthcare, USA), and further cloned into pCR®8/GW/TOPO® vector from the pCR®8/GW/TOPO® Cloning Kit (Invitrogen, USA) as per the manufacturer's instructions.

Immediately, One Shot®Mach1TMT1R chemically competent *E. coli* cells were reared in SOC Medium (2.0% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$,10 mM $MgSO_4$, and 20 mM glucose) and transformed by thermal shock. Transformed cells were plated on Luria-Bertani agar (1.0% Tryptone, 0.5% yeast extract and 1.0% NaCl, pH 7.0) containing 100 mg/mLspectinomycin and incubated overnight at 37 °C. Vector preparations containing *Poly p* 1 coding sequence were obtained from a 5-mL culture of transformed clones, using the QIAprep®Spin miniprep kit (Qiagen, Germany) following the manufacturer's protocol, and were further analyzed by restriction digestion with *Eco* RI enzyme (Promega, USA).

2.5. Gene sequencing

Sequencing reactions were performed using the gene-specific primers described above (2.4) as well as 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). Sequencing was conducted 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. Sequences were analyzed and edited using the DNASTAR® Lasergene Sequence Analysis software (http://www.dnastar.com/t-allproducts.aspx).

2.6. Heterologous expression and solubilization

Plasmid constructs containing the *Poly p* 1 coding sequence were double-digested with *Eco RI* and *Nde I* restriction enzymes and sub-cloned into a pET-28a vector. The sequence was cloned in frame with the N-terminal 6xHis tag of the commercial vector to facilitate r*Poly p* 1 purification through immobilized metal affinity chromatography. The pET-28a_*poly p* 1 plasmid construct was then used to transform *E. coli* XL1 Blue {recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F proAB lacIqZDM15 Tn10 (Tetr)]} (Stra- tagene, USA). The obtained vector preparations were used to transform the *E. coli* BL21 (DE3) chemically competent cells [F- ompThsdSB (r-B mB-) gal demD(srl-recA) 306:Tn10(DE3)] (Nova- gen, USA).

Heterologous expression was performed using the protocol described by Justo-Jacomini et al. (2014), with minor modifications. Briefly, 5 mL of LB medium supplemented with kanamycin (30 mg/ mL) was inoculated with transformed clones and grown overnight at 37 °C and 200 rpm (Orbital Shaker Tecnal, Model TE-420). The pre-inoculums were then used to inoculate 50 mL of fresh medium. Once the culture reached a DO_{600} = 0.4-0.6, expression was induced by adding isopropyl β-D-1- thiogalactopyranoside (IPTG, Invitrogen, EUA) to a final concentration of 1 mM. Induced cultures were incubated at different temperatures (37, 30, 25 and 20 °C). For each temperature, samples of 2.0 mL were collected at several times post-induction (5, 6, 7 8, 9, 10, and 24 h). Cells were then collected, suspended in 200 mL of lysis buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 20 mM imidazole) and lysed by sonication in ice (six pulses of 1 min, with 1 min between each pulse). Cell lysates were then centrifuged (8000 g, 15 min, 4 °C), and the pellet and supernatant were analyzed by SDS-PAGE to determine the best expression conditions and levels of soluble and insoluble expression.

For large-scale protein production, 5 mL of a pre-inoculum was added to 100 mL of LB medium. After induction with 1 mM IPTG, bacterial culture was incubated at 20 °C, 250 rpm for 10 h. Then, cells were collected by centrifugation (4000 g, 15 min and 4 °C), suspended in 15 mL of lysis buffer and lysed; the insoluble fraction was collected by centrifugation (8000 g, 15 min, 4 °C). The pellet was further solubilized overnight at 4 °C, with gentle agitation using 10 mL of a solubilization buffer (50 mM Tris-HCl 0.5 M NaCl, 20 mM imidazole and 8 M urea, pH 8). Soluble fractions were then collected after centrifugation (7500 g, 15 min, 4 °C), analyzed by SDS-PAGE and stored at 4 °C until use.

2.7. Recombinant allergen purification

For r*Poly p* 1 purification, 10 mL of the soluble fraction were filtered with 0.22 μm sterile filter and applied to a commercial prepacked column, HisTrap HP™ (Ni^{2+} Sepharose™ High Performance; GE Healthcare, Sweden), coupled with a peristaltic pump (Pharmaceutical Biotech, Sweden). The column was then washed with 10 mL (10X-column volume) of each washing buffer (50 mM Tris-HCl pH 8, containing 0.5 M NaCl, 8 M urea, and with increasing 50, 75 and 100 mM imidazole). Elution of His-tagged r*Poly p* 1 was performed with 10 mL of elution buffer (50 mM Tris-HCl 0.5 M NaCl, 8 M urea, 200 mM imidazole; pH 8). Five fractions of 2 mL were collected at a flow rate of 1 ml/min. All fractions collected during the purification process were further analyzed by (15%) SDS-PAGE.

2.8. Purification of native phospholipase A1 from P.paulista venom

The freeze-dried venom (23 mg of total protein) was solubilized in 50 mM sodium acetate buffer pH 5.2 and submitted to a cation-exchange chromatography in a Hiprep FF CM column (16X10 mm, 20 mL; GE Healthcare) previously equilibrated with the same buffer and coupled with an Akta-FPLC system. Elution was accomplished by a linear gradient of 0-1 M NaCl. Fractions of 5 mL were collected at a flow rate of 2 mL/min, and the elution was monitored by measuring the absorbance at 280 nm.

2.9. SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed according to Laemmli (1970), using a Mini-Protean® Tetra Cell System (BioRad). A prestained standard High-Range Rainbow Molecular Weight Marker (GE Healthcare, USA) and a PageRuler Prestained Protein Ladder (Thermo Scientific, USA) were loaded for SDS-PAGE and western blot analyses, respectively. After running, the gels were stained with either Coomassie Brilliant Blue R-250 (CBB) or silver stain (Fermentas, Germany).

2.10. Protein quantification

Protein fractions and purified native or recombinant allergens were quantified using the modified Bradford method and bovine serum albumin (Sigma, USA) as a standard (Sedmak and Grossberg, 1977).

2.11. Enzymatic activity of phospholipase A1

Phospholipase A1 enzymatic assays were performed using the EnzChek® phospholipase A1 assay kit (Invitrogen, USA) following the manufacturer's instructions. The measurement of all fractions collected after the cation-exchange chromatography was performed in triplicate.

2.12. IgE-binding analyses

Proteins submitted to SDS-PAGE gels were transferred to a 0.22 mm nitrocellulose membrane using a semi-dry system (Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell, Bio-Rad, USA), and the transfer efficiency was assessed by staining the gel with Coomassie Blue G-250 (Sigma, USA). Prior to the addition of patients' sera, membranes were blocked with 20 mM Tris-HCl 150 mM NaCl pH 7.4, with 0.5% Tween-20 (Sigma-Aldrich, USA) (TBS-T wash solution) and 3% non-fat dried milk (block solution). The membrane-blocking procedure was performed for 1 h of incubation at room temperature under slow agitation on a Rocker II™ platform mixer (Boekel Scientific, USA). After the wash (3 times with TBS-T), the membranes were transferred to a mini PROTEAN® II multi-screen apparatus (Bio-Rad, USA). Four hundred microliters of each patient's sera (diluted at 1:50 in a solution of TBS-T with 1% non-fat dried milk) were transferred to the individuals' channels. The system containing the membrane and the sera samples was incubated overnight at room temperature under slow agitation on a Rocker II™ platform mixer. Immunodetection was performed using anti-human IgE (ε-chain specific) peroxidase conjugate antibody (Sigma-Aldrich, USA) diluted at 1:5000 (TBS-T and 1% non-fat dried milk), and the bands were visualized in Image Quant 400 (GE Healthcare, Sweden) using the chemiluminescent substrate Luminata™ Forte Western HRP substrate (Millipore, USA).

3. Results

3.1. Recombinant Poly p 1 coding sequence

A unique 958 pb consensus cDNA sequence was obtained and annotated (GenBank GI: HQ023233.1) after the sequencing of 10 *poly p* 1_pCR®8/GW/TOPO® positive clones. Analyses of the generated sequence, using the Blastn tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), showed the highest similarity with the phospholipase A1 coding sequence from *Polistes dominula* (84%) and *Polistes annularis* (82%). The putative primary sequence of r*Poly p* 1(ADT89774.1) (Fig. 1) showed 302 amino acids with 13 Cys residues, 12 of them potentially involved in disulfide-bridge formation (Cys6-Cys89, Cys181- Cys176, Cys224-Cys219, Cys295- Cys242, Cys270-Cys241, Cys268-Cys263), as suggested by Santos et al. (2007). Analyses of the primary sequence r*Poly p* 1, using the Compute pI/MW tool

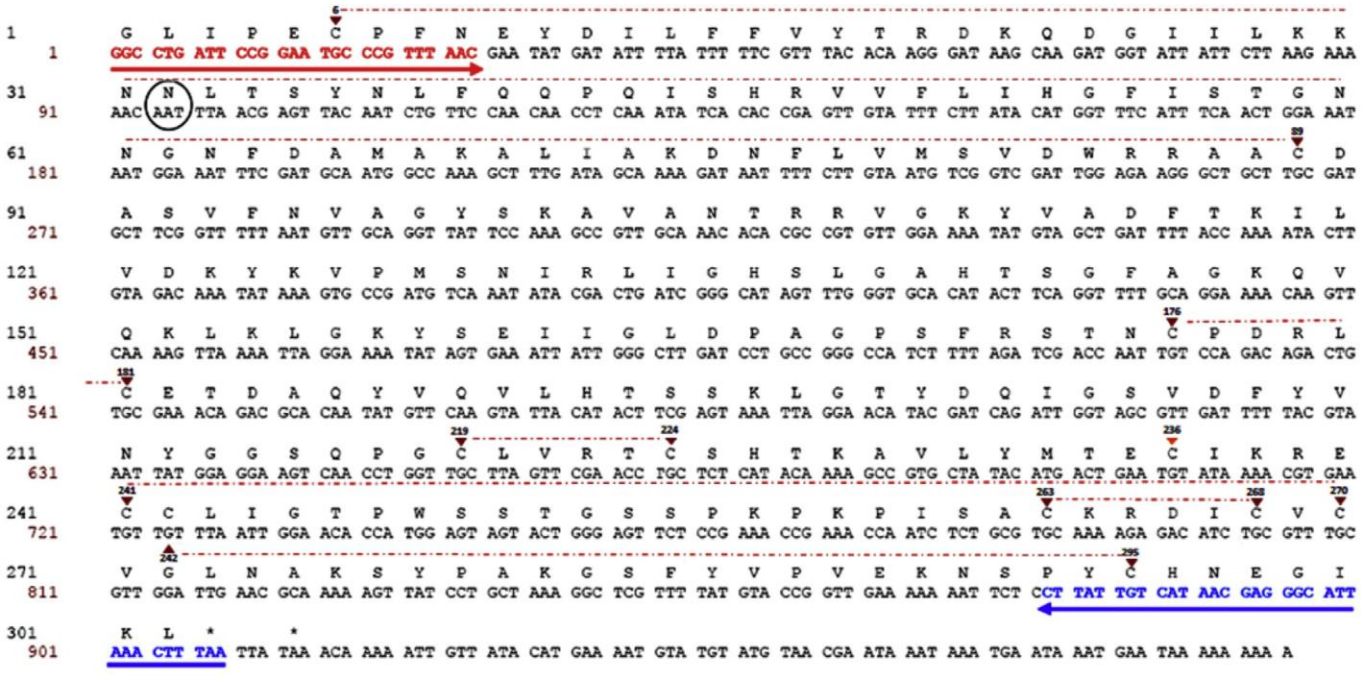

Fig. 1. cDNA and predicted amino acid sequence of the mature r*Poly p* 1 (Gen Bank: ADT89774.1). Forward (red arrow) and reverse (blue arrow) primers used for gene specific amplification are indicated. Black oval mark shows putative N-glycosylation site. Disulfide bridges and the cysteine residues involved in their formation are marked with discontinuous red line and red triangles, respectively. Cysteine residue (236) not involved in disulfide-bridge formation is indicated with an orange triangle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(http://web.expasy.org/compute_pi/), showed a theorical pI value of 9.12 and a predicted molecular weight of 33.33 KDa.
Sequence alignment of r*Poly p* 1 (Fig. 2) showed low levels of identity with the venom phospholipases A1 of endemic wasps from the Northern Hemisphere -*P. annularis* (*Pol a* 1), GenBank: AAD52615.1 (71.9%); *V. vulgaris* (*Ves v* 1), GenBank: AAB48072.1 (47.4%); and *V. germanica* (*Ves g* 1), GenBank: CAJ28931.1 (53.8%)- and with the ant venom PLA1 from *Solenopsis invicta* (*Sol i* 1), GI: NP_001291510.1 (27.2%).

3.2. Heterologous expression and solubilization of rPoly p 1 inclusion bodies

Recombinant *Poly p* 1 was produced in *E. coli* BL21 (DE3) cells using pET-28a vector. Analysis of expression conditions showed that r*Poly p* 1 could be detected as soon as 5 h after induction with 1 mM IPTG, growing at 20 ºC with agitation at 220 rpm (Fig. 3). The highest level of the recombinant allergen (706 mg/L) was obtained 10 h post-induction using these growth conditions. In SDS-PAGE (8e20%), r*Poly p* 1 appears as a band of ~34 kDa, corresponding to the 34.1 kDa theoretical molecular weight including the 6XHis tag, and similar to the 33.9 kDa molecular weight of mature n*Poly p* 1, as determined by Santos et al. (2007). No heterologous expression was detected at 37 ºC, 30 ºC or 25 ºC, and no increase in the expression levels was observed after 24 h post-induction (not shown).
SDS-PAGE analysis of soluble and insoluble fractions of *E. coli* BL21 (DE3) cells after lysis showed that r*Poly p* 1 is detected exclusively in the insoluble fraction (Fig. 4). No recombinant protein was detected in the supernatant of lysis.

3.3. rPoly p 1 purification

Recombinant *Poly p 1* detected in the insoluble fraction after cell disruption was partially solubilized (30%) with 50 mM Tris-HCl 0.5 M NaCl, 20 mM imidazole and 8 M urea, pH 8. As r*Poly p* 1 contains a 6XHis tag, the HisTrap HP™ (GE Healthcare, Sweden) commercial prepacked column was used for recombinant allergen purification. Analyses of the collected fractions during purification showed that almost 100% of solubilized r*Poly p* 1 bonded to the Ni^{2+} Sepharose™ High Performance column (Fig. 5). The heterologous protein was eluted with 200 mM imidazole. In SDS-PAGE analysis, a unique band with the expected MW (~34 kDa) was detected after elution. Further analysis of the SDS-PAGE results using the ImageJ (https://imagej.nih.gov/ij/) confirmed that r*Poly p* 1 was obtained with 95-99% purity. Protein quantification showed that purified r*Poly p* 1 was obtained at a concentration of 212 mg/L.

3.4. nPoly p 1 purification and enzymatic activity

The chromatographic profile of the crude venom extract (23 mg) obtained from *Polybia paulista* (Fig. 6) was similar to the profile described by Justo-Jacomini et al. (2013), showing 6 peaks designated A through F. The major difference resided in the absence of the peak related to hyaluronidase activity. Low levels of *Poly p* 2 were further confirmed by SDS-PAGE and enzymatic activity analysis (data not shown).
The n*Poly p* 1 was detected in peaks E (fractions 18e21) and F (fractions 26-29). Determination of PLA1 activity of pooled fractions from each peak, using the commercial system EnzChek® phospholipase A1 assay kit, showed 257 U/mL for peak E and 462

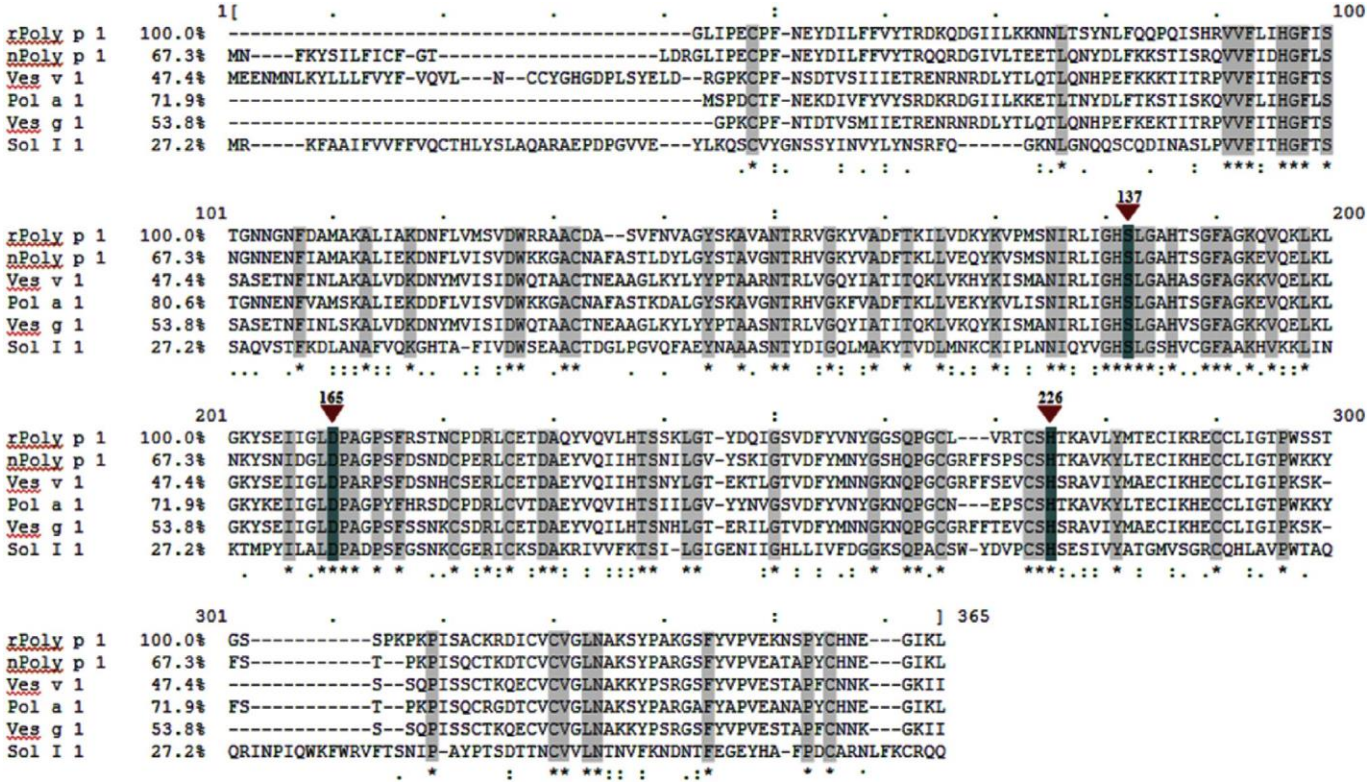

Fig. 2. Sequence alignment and identity of the predicted r*Poly p* 1 protein (GenBank: ADT89774.1) with the primary sequences of n*Poly p* 1, other vPLA1s (*Pol a* 1, *Ves v* 1, *Ves g* 1) and the ant PLA1 from *Solenopsis invicta* venom (*Sol i* 1). In green are the amino acid residues of the catalytic triad (serine 137, asparagine 165 and histidine 226). Red triangles show the correspondent positions in the mature r*Poly p* 1. In grey are the identical residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

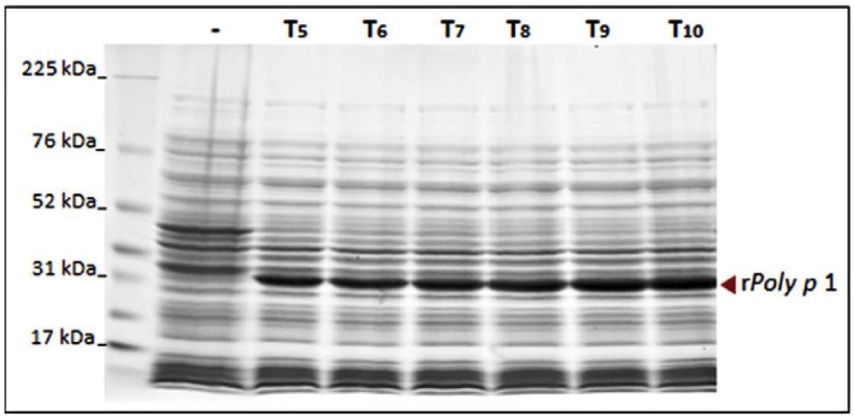

Fig. 3. SDS-PAGE (8-20%) analysis showing the expression profile of r*Poly p* 1 in *E. coli* BL21 (DE3) cells at different times after induction (20 °C, 1 mM IPTG). T_5–T_{10} represent hours after induction. (–) corresponds to a negative control (non-induced clone at T_{10}).

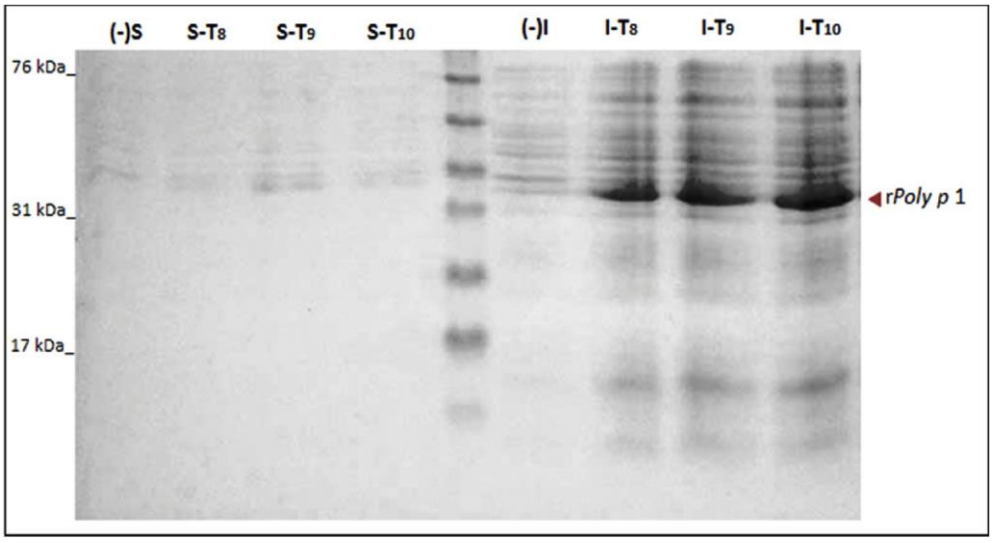

Fig. 4. SDS-PAGE (12%) analysis of r*Poly p* 1 *E. coli* BL21 (DE3) cell lysate. (S) soluble and (I) insoluble fraction after cell disruption and centrifugation. T_8–T_{10} represent hours after induction. (–) corresponds to a non-induced r*Poly* p 1 clone at T_{10}, used as a negative control.

U/mL for peak F, accounting for a phospholipase A1 activity recovery of 92%. In SDS-PAGE analysis, purified n*Poly p* 1 from different fractions appeared as a unique band with the expected molecular weight and with a slight difference to r*Poly p* 1 due to the presence of the 6xHis tag in the recombinant form of the allergen (Fig. 7).

3.5. Specific IgE-reactivity to recombinant and native Poly p 1

Specific IgE-mediated immunodetection was analyzed against r*Poly p* 1, n*Poly p* 1 and crude venom extract, using sera from 10 *P. paulista* venom-sensitized patients. Western blot assays showed similar reactions from the crude venom extract (Fig. 8A), n*Poly p* 1 (Fig. 8B) and r*Poly p* 1 (Fig. 8C) to the sera of patients previously diagnosed with *P. paulista* venom allergy using the ImmunoCAP system. No reaction occurred when *E. coli* BL21 (DE3) cell extracts were incubated with positive sera (not shown), nor when venom, native and recombinant allergen forms were incubated with sera from non-sensitized patients (negative controls) (Fig. 8 A,B,C).

4. Discussion

Venoms from social Hymenoptera are complex mixtures of low molecular weight compounds (biogenic amines, lipids and carbohydrates, cationic peptides) and high molecular weight proteins, some with allergenic activity. Several venom allergens are glycosylated proteins and contain the so-called cross-reactive carbohydrate determinants (CCDs). Consequently, the use of crude extracts in allergy diagnosis is associated to cross-reactivity, miss-identification of the culprit venom, and finally, with non-specific sensitization of allergic patients submitted to SIT. Efforts to develop a molecular diagnosis of HVA based on the use of a panel of recombinant allergens obtained via different expression systems will improve allergy diagnosis and the SIT safety profile.

E. coli remains the most popular platform for recombinant protein expression and has been previously used for the production of several Hymenoptera venom allergens (Rosano and Ceccarelli, 2014; Spillner et al., 2014). In this work, we obtained high levels of r*Poly p* 1 expressed in *E. coli* BL21 (DE3) cells. The concentration of recombinant protein (212 mg/L), determined after purification with immobilized Ni^{2+} metal affinity chromatography, was similar to and even higher than those reported for other allergens

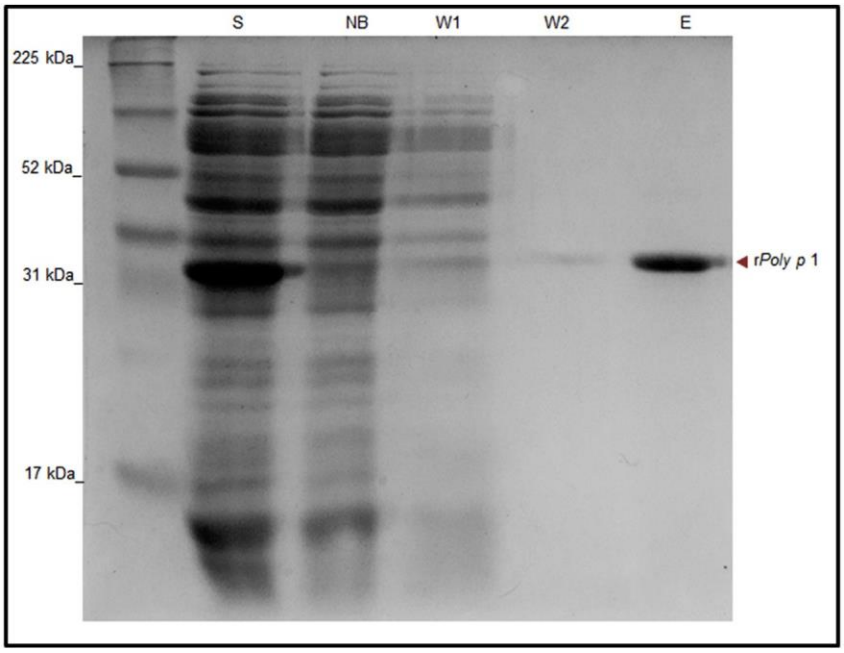

Fig. 5. SDS-PAGE (12%) analysis of fractions obtained during r*Poly p* 1 purification process. Samples: S = Solubilized proteins (8M urea); NB = Protein not bound to the column; W1–W2 = fractions corresponding to column-washing steps with 75 and 100 mM imidazole; E = fraction obtained after elution with 200 mM imidazole.

expressed in *E. coli* (King et al., 1997; Skov et al., 2006; Lockwood et al., 2012) and other systems (Vinzón et al., 2010; Borodina et al., 2011). This agrees with the feasibility of the cell system and the purification procedure employed to express r*Poly p* 1 at high rates.

However, r*Poly p* 1 was produced exclusively in inclusion bodies. Heterologous protein expression in *E. coli* results in production of non-glycosylated molecules and usually prevents the formation of a disulfide bridge. Both factors may lead to protein misfolding, inactivation and aggregation. n*Poly p* 1 has been described as a non-glycosylated protein with six predicted disulfide bridges stabilizing its 3D structure (Santos et al., 2007). Analysis of the predicted r*Poly p* 1 primary sequence also showed the presence of 13 cysteine residues, 12 of which are potentially involved in the formation of six disulfide bridges (Fig. 1). The fast growth kinetics of *E. coli*, along with the referred high levels of r*Poly p* 1 expression and the possible failure to form the six predicted disulfide bridges, resulted in the production of an insoluble recombinant allergen. Further conditions during cultivation dlower temperature, inductor concentration and aeration ratesdand co-expression with solubility-enhancement tags (Correa and Oppezzo, 2015) could be tested in order to obtain r*Poly p* 1 as a soluble protein using *E. coli* systems. Others authors have overcome the insolubility problem during recombinant protein expression by simply lowering the cultivation

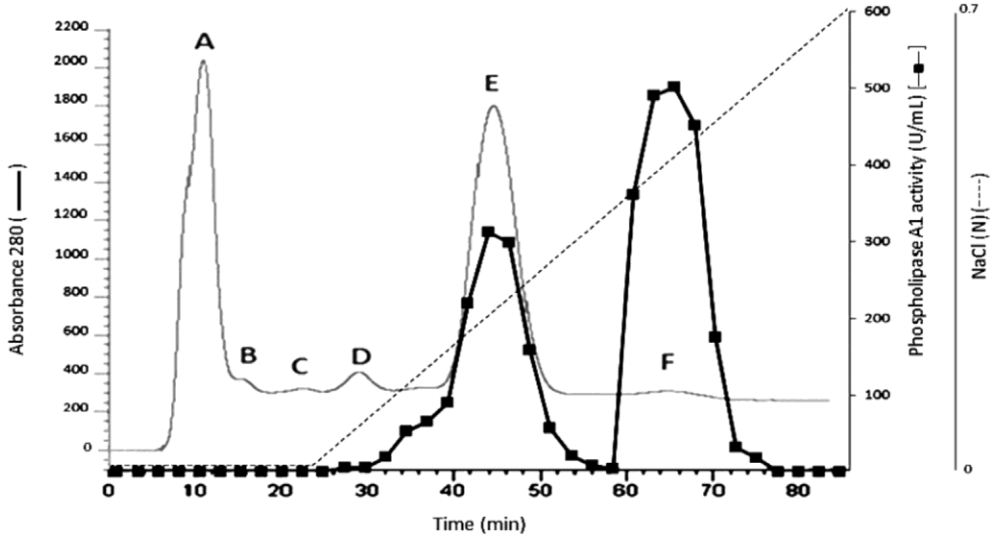

Fig. 6. Cation-exchange chromatography profile of *P. paulista* crude venom extract (23 mg), obtained in a Hiprep FF CM column (16 × 10 mm, 20 mL; GE Healthcare) coupled to an AKTA-FPLC system. The column was washed with an isocratic buffer of 50 mM sodium acetate pH 5.2. The elution was performed under a linear gradient from 0 to 1 M NaCl in the same buffer, at a flow rate of 1 mL/min, collecting fractions of 5 mL. The protein was monitored by measuring the absorbance at 280 nm, represented by a continuous line (___). Phospholipase A1 enzymatic activity is represented with a shifting line-block (—■—).

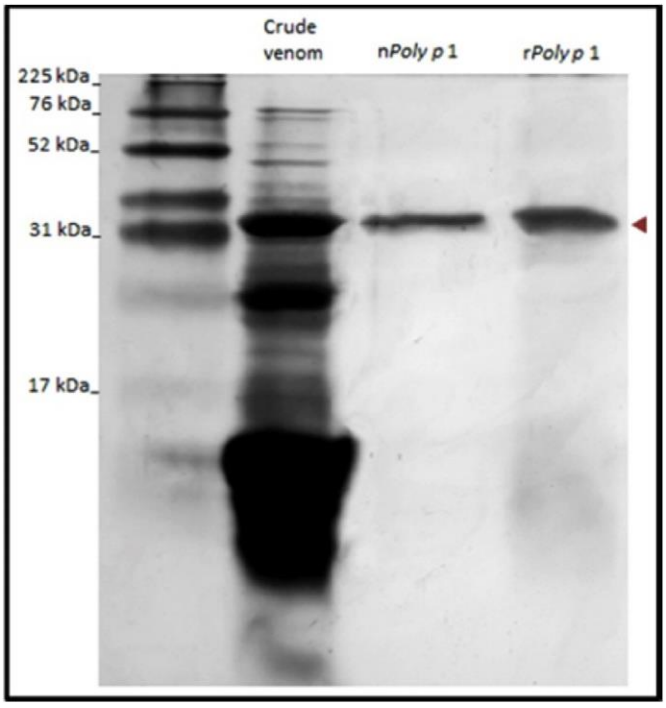

Fig. 7. SDS-PAGE (12%) analysis of crude venom extract, n*Poly p* 1 and r*Poly p* 1. Gel was staining with silver-nitrate.

temperature (6-10 ºC) (Song et al., 2012) or by the combination of different factors (San-Miguel et al., 2013; Vu et al., 2014; Sina et al., 2015). Finally, other expression platforms could be used to obtain r*Poly p* 1 as a soluble protein. For instance, the expression of *Ves v* 5 on yeast (Borodina et al., 2011) and insect cells (Seismann et al., 2010) circumvented insolubility problems of heterologous allergen production.

Previous attempts to obtain PLA1s from the venom of social Hymenoptera in *E. coli* also resulted in their expression as insoluble proteins. Obtained recombinant venom PLA1s showed lower binding to specific antibodies elicited by the native form of the allergens (King et al., 1997). These results were mainly associated with physicochemical as well as immunological differences between the renatured recombinant allergens and their native counterparts. In our case, similar levels of sIgE-reactivity were detected in solubilized, but not refolded, r*Poly p* 1 and in denatured n*Poly p* 1 through western blot analysis (Fig. 8B). These findings showed that linear IgE epitopes in the primary structure of n*Poly p* 1 are involved in eliciting an allergic response in sensitized patients and are recognized in r*Poly p* 1. Similar behavior has been reported for either refolded or denatured recombinant allergens from dust mites (Tanyaratsrisakul et al., 2009; Floch et al., 2012) and social Hymenoptera venom (Skov et al., 2006; Justo-Jacomini et al., 2014), expressed on inclusion bodies in *E. coli*. Comparison of the recognition levels of r*Poly p* 1 with those of n*Poly p* 1 and crude venom extract showed that solubilized r*Poly p* 1 could be used for the development of CRD of *P. paulista* venom allergy.

The one-step cation-exchange chromatographic procedure used in this work allowed complete purification of n*Poly p* 1. This procedure diverged from the methodology described by Santos et al. (2007), in which the venom extract was initially submitted to a gel filtration chromatography, and then the fractions with phospholipase activity were pooled and submitted to cation-exchange chromatography. Interestingly, this work shows n*Poly p* 1 appearing in two peaks, and the fractions of each peak were analyzed with SDS-PAGE and with a highly specific fluorescence assay for phospholipase A1 enzymatic activity quantification. These results may be related to the heterogeneity of the venom materialdobtained from individuals of different nestsdused for chromatographic separation. Also, as was previously shown (Santos et al., 2011), n*Poly p* 1 exists as a mixture of multiple forms in *P. paulista* venom, which could be eluting at different NaCl concentrations during purification. Nevertheless, in SDS-PAGE analysis, all fractions with high levels of PLA1 activity showed a single band corresponding to n*Poly p* 1 molecular weight (~34 kDa) (not shown). Further analysis of SDS-PAGE loaded with n*Poly p* 1 pooled fractions 18e21 from peak E and fractions 26-29 from peak F, and staining with silver nitrate confirmed purity rates of 95-99% (Fig. 7). Consider these results, a novel, efficient and economical purification process could be used for the purification of active n*Poly p* 1.

Recombinant PLA1s obtained from the venom of various social Hymenoptera species have been widely used in molecular allergy diagnosis for differentiation of true double sensitization from cross-reactivity (Müller et al., 2012; Monsalve et al., 2012). The use of PLA1 has two remarkable advantages: as non-glycosylated proteins, they lack CCDs and thus reduce the incidence of cross-reactivity. Furthermore, these major allergens are found in the venoms of wasps and ants, but not of honeybees; therefore, venom PLA1s represent useful markers to distinguish honeybee from wasp

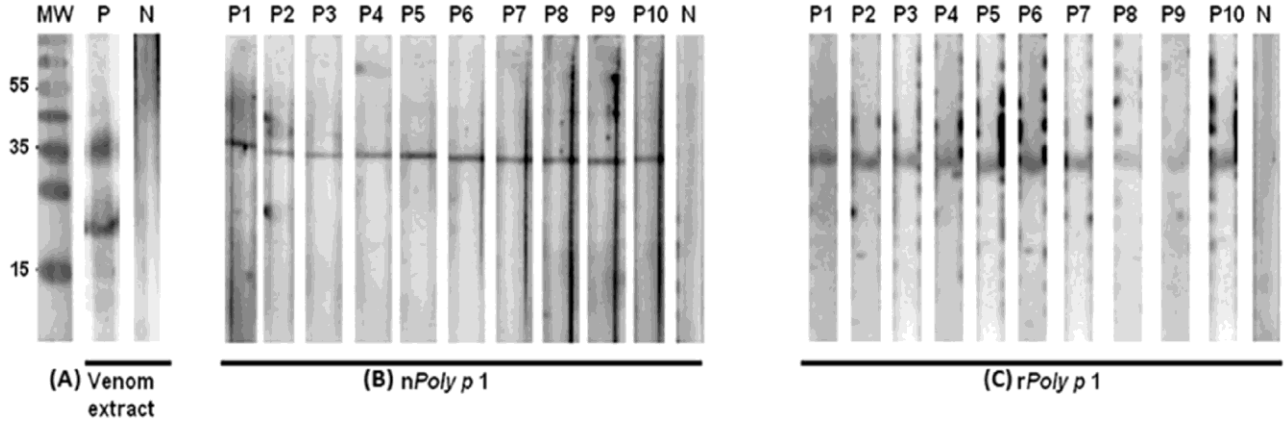

Fig. 8. (A, B, C). Antigenicity of *Poly p* 1 protein evaluated using a Western blot. Recognition of (A) *Poly p* 1 in crude venom, (B) native-purified (n*Poly p* 1) and (C) recombinant (r*Poly p* 1) by serum IgE in *P. paulista* venom-sensitized patients. Serum samples: P1-P10= individual sera of *P. paulista* venom-sensitized patients; P= pool of sera; N= serum from non-sensitized patient, as a negative control. The molecular weight marker (kDa) is indicated.

and/or ant venom allergy. In this context, and given the fact that only one (*Poly p* 2) of the three major allergens from *P. paulista* venom has thus far been cloned and evaluated as a candidate for the development of component-resolved diagnosis (Justo-Jacomini et al., 2014), we have cloned, expressed and analyzed the immunological activity of r*Poly p* 1 in the present study. As 100% of allergic patients' sera specifically recognized the r*Poly p* 1 to a similar extent as the purified n*Poly p* 1, the recombinant allergen appears to be a good candidate for the improvement of molecular diagnosis of *P. paulista* venom allergy. Recombinant *Poly* p 1 could be used in combination with r*Poly p* 2 in order to detect patients that could be sensitized by either n*Poly p* 1 or n*Poly p* 2. The production of panels of major allergens from *P. paulista* venom including *Poly p* 5, currently in expression process will significantly reduce miss-identification of culprit venom during diagnosis and enhance the possibility of developing more effective, safe and less invasive strategies of SIT, aiding to improve the quality of life of *P. paulista* venom-allergic patients.

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5. CHAPTER 3

Improved production of the recombinant phospholipase A1 from *P. paulista* wasp venom expressed in *E.coli*. **Perez-Riverol, A**; Musacchio-Lasa, A.; Romani Fernandes, L.G.; dos Santos-Pinto, J.R.A.; Esteves, F.G.; Bazón, M.L.; Zollner R.L; Palma, M.S. and Brochetto-Braga, M.R. (Submitted to **Molecular Biotechnology**)

Improved production of the recombinant phospholipase A1 from *P. paulista* wasp venom expressed in *E.coli*

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Abstract:

Phospholipase A1 is one of the three major allergens identified in the venom of *P. paulista* (Hymenoptera: Vespidae), a clinically relevant wasp from Brazil Southeast. The recombinant form of this allergen (rPoly p 1) could be used for the development of molecular diagnosis of venom allergy. Early attempts to produce rPoly p 1 using *Escherichia coli* BL21 (DE3) cells rendered high yields of the insoluble rPoly p 1 with low levels of solubilized protein recovery (30%). Here, we aimed to improve the production of rPoly p 1 in *E. coli* by testing different expression conditions. The downstream solubilization of rPoly p 1 inclusion bodies was optimized to increase recovery. The venom allergen was also cloned in pPICZαA vector and tested for soluble expression as a secreted protein in *Pichia pastoris* X-33 cells. Expression in *P. pastoris* was extremely low hindering the subsequent molecular and structural characterization of the allergen. A significantly increase in the expression levels and recovery of the rPoly p 1 produced *in E. coli* was obtained after the optimization of

the culture and solubilization conditions. Solubilized rPoly p 1 was purified to homogeneity (99%) and sequenced by mass spectrometry (NanoLC-ESI-CID). The sIgE-mediated recognition of the produced allergen by sera of allergic patients and the sensitivity associated to the use of rPoly p 1 was evaluated in immunoblotting analyses. Overall, a sevenfold increase of the solubilized rPoly p 1 yield (1.5 g/L of fermentation broth) was obtained compared to previously informed protocol. The improvement of the strategy for rPoly p 1 production described here will provide the amounts of allergen necessary for the subsequent steps including protein refolding, immunological characterization and ultimately, for the development of molecular diagnosis of *P. paulista* venom allergy.

Keywords: *Polybia paulista*, recombinant phospholipase A1, recovery, solubilization, component-resolved diagnosis.

Highlights:

.rPoly p 1 expression yields were increased after induction at 16°C with 0.1 mM ITPG
. rPoly p 1 was completely solubilized with 6M urea in citrate-phosphate buffer (pH 2.6)
. A sevenfold increase in solubilized and purified rPoly p 1 yields (1.5 g/L) was obtained
. The production of rPoly p 1 in yeast cells rendered extremely low expression levels
. rPoly p 1 showed high levels of sensitivity (95%) in patients with specific IgE to wasp venoms

1 INTRODUCTION

Escherichia coli remains the most popular platform for expression of recombinant proteins (FERRER-MIRALLES et al., 2015). The success in the use of this prokaryotic system is related to the high production yields, its fast growth kinetic and high genetic plasticity along with the relative simplicity of the media and conditions required for cell growing. However, heterologous expression in *E. coli* often results in protein overproduction, aggregation and inclusion bodies formation, finally causing protein misfolding and insolubility (CORREA; OPPEZZO, 2015)**.** These pitfalls must be avoided as they compromise the downstream use of the heterologous target protein. To date, several strategies including co-expression with molecular chaperones (DE MARCO et al., 2007; VOULGARIDOU et al., 2013), the use of

fusion-tags (YOUNG; BRITTON; ROBINSON, 2012), evaluation of optimal culture conditions and optimization of solubilization process (PATRA et al., 2000; VEMULA et al., 2015) have been used to improve the soluble production of heterologous proteins in this cell system.

E. coli have been extensively used for expression of several Hymenoptera venom allergens (SPILLNER; BLANK; JAKOB, 2014; PEREZ-RIVEROL et al., 2016). The expression in *E.coli* has rendered confronted results leading either to production of active, native-like recombinant allergens or misfolded molecules with reduced immunogenicity (DUDLER et al., 1992; SOLDATOVA et al., 1998; SKOV et al., 2006; PEREZ-RIVEROL et al., 2016). However, *E. coli* represents a suitable alternative to produce high levels of recombinant allergens as non-glycosylated molecules. It has been well documented that several native allergens present the cross-reactive carbohydrates determinants (CCDs) which in the case of insects are defined by an α1,3-linked fucose residue at the innermost N-acetylglucosamine of the carbohydrate core structure (OLLERT; BLANK, 2015).

The IgE-mediated recognition of CCDs epitopes is responsible for high levels of cross-reactivity during HVA diagnosis (MÜLLER et al., 2009), thus compromising the successful and safety of the venom immunotherapy. The use of recombinant allergens expressed in *E. coli* as CCD-depleted proteins has improved the allergy diagnosis by preventing the occurrence of CCD-mediated cross-reactivity (SPILLNER; BLANK; JAKOB, 2014; PEREZ-RIVEROL et al., 2015). Furthermore, *E. coli* represents a suitable system for production of venom allergens such as phospholipase A1 which are difficult to express in eukaryotic cells (SEISMANN et al., 2010a; BORODINA et al., 2011) .

Phospholipase A1 (PLA1) is one of the three major allergens identified in the venom of *P. paulista* (SANTOS et al., 2007), a clinically relevant Brazilian wasp. Combined with honeybee, this wasp is responsible for up to 15 000 sting accidents and a high number of anaphylaxis events with potential fatal outcomes (PEREZ-RIVEROL et al., 2017). The development of reliable systems for allergy diagnosis is a mandatory

prerequisite for starting venom immunotherapy on patients allergic to *P. paulista* venom. Similar to other insect PLA1s (SEISMANN et al., 2010a; KOROŠEC et al., 2012; MONSALVE et al., 2012), the production of a native-like recombinant form of Poly p 1 could result in the rational use of this venom component in allergy diagnosis. The native form of the allergen (nPoly p 1) is a non-glycosylated protein with no homologue toxins in honeybee venom (OLLERT; BLANK, 2015) turning it a potential feasible marker to differentiate true double sensitization to HBV and wasp venom from cross-reactivity incidence during identification of the primary sensitizing insect.

The nPoly p 1 is a ~34 kDa protein belonging to the lipase GX class. The tridimensional model of the allergen shown an α/β fold common to many lipases: a core consisting of a tightly packed β-sheet constituted of six-stranded parallel and one anti-parallel β-strand, surrounded by four α-helices (SANTOS et al., 2007). The analysis of the Poly p 1 primary sequence revealed the presence of 13 cysteine residues with 12 of them potentially involved in disulfide-bonds formation (SANTOS et al., 2007; PEREZ-RIVEROL et al., 2016). The presence of these disulfide bridges in Poly p 1 structure could be critical for protein proper folding and soluble production of the allergen in bacterial cells. In a previous attempt to produce Poly p 1 in *E. coli*, we obtained high expression levels with the protein exclusively found in the insoluble fraction after bacterial cells lysis (PEREZ-RIVEROL et al., 2016). The subsequent solubilization step resulted in low recovery (30%) of the solubilized recombinant allergen hampering the production of the levels of protein required for downstream refolding, molecular and immunological analyses.

In the present study, we aimed to obtain soluble rPoly p 1 in *E.coli* by lowering the culture temperature and testing different concentrations of the expression inductor (IPTG). Also, to maximize the recovery of the solubilized rPoly p 1, we optimized the downstream solubilization process. Finally, as an alternative to produce rPoly p 1 as a soluble protein, we tested the expression in the eukaryotic system *P. pastoris*. Previously, an enzymatically inactive form of the PLA1 from *V. vulgaris* venom (Ves v 1) has been successfully expressed using this eukaryotic system (BORODINA et al., 2011). Unfortunately, the recombinant expression of rPoly p 1 in this eukaryotic

system rendered almost undetectable levels of the heterologous protein. In contrast, a sevenfold increase in the yields of the solubilized rPoly p 1, expressed in *E. coli,* was obtained. The improved strategy for the recombinant production of rPoly p 1 described here will replace the time and resources-expensive procedure currently used to obtain the amounts of rPoly p 1 required in the downstream analyses for its molecular and immunological characterization.

2 Materials and Methods

2.1 Allergic patients' sera

Sera from patients (n=40) with a history of allergic reactions to insect stings and previously diagnosed with sIgE to *P. paulista* venom were obtained from the Ambulatório de Anafilaxia of the Hospital das Clínicas, Faculdade de Ciências Médicas, Universidade Estadual de Campinas-UNICAMP. Five sera from non-sensitized healthy volunteers were used as negative controls. The study was approved by the Ethics Committee of FCM-UNICAMP under n° 187/2006. Informed consent was obtained in written form from all participants of the study and participation was voluntary.

2.2 Bacterial strain and solubilization buffers

All expression analyses were conducted using a clone of *E. coli* BL21 (DE3) cells [F-ompThsdSB (r-B mB-) gal demD(srl-recA) 306:Tn10(DE3)] (Novagen, USA) transformed with the construction pET-28a_*poly p 1*, previously obtained in our laboratory (PEREZ-RIVEROL et al., 2016). For solubilization experiments, the following buffers (CHANDRA MOHAN, 2003) were prepared: Citrate-Phosphate Buffer (0.1 M citric acid, 0.2 M dibasic sodium phosphate, 0.15M NaCl; pH 2.6); Tris-HCl Buffer (0.02 M Tris (hydroxymethyl)aminomethane, 0.02 M Hydrochloric acid, 0.15M NaCl; pH 7.4); Phosphate Buffer (0.1 M sodium phosphate monobasic, 0.1 M sodium phosphate dibasic, 0.15M NaCl; pH 8.0) and Carbonate-Bicarbonate Buffer (0.1 M sodium carbonate , 0.1 M sodium bicarbonate, 0.15M NaCl; pH 10.2).

2.3 Evaluation of recombinant Poly p 1 soluble expression

Experiments for rPoly p 1 soluble expression in *E. coli* were conducted using the protocol described previously (PEREZ-RIVEROL et al., 2016) with variations in the induction conditions. The expression was performed at 6 ^{o}C, 10 ^{o}C and 16 ^{o}C by adding IPTG (Invitrogen, Brazil) to final concentrations of 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 mM. For each combination of temperature and IPTG concentrations, samples of 2.0 mL were collected at 10h and 24h post-induction. Cells were centrifuged at 4 ^{o}C, 10 000 x g for 10 min and the resulted pellets were suspended in 200 µL of 20 mM Tris-HCl 150 mM NaCl; pH=7.4. Cell lysis was performed by sonication on ice (six pulses of 1 min, with 1 min between each pulse). Cell lysates were then centrifuged (10000X g, 15 min, 4 ^{o}C), and the pellet and supernatant were analyzed by (12%) SDS-PAGE.

2.4 Protein solubilization

For optimization of the downstream solubilization step, the insoluble fraction of the cell lysate was incubated individually with the buffer solutions described in Section 2.2, containing 8 M urea. Fractions of 1 g (wet weight) of the *E. coli* cell lysate pellet were suspended in 5, 10 or 15 mL of each buffer and incubated with gentle agitation either at 4 °C overnight or at 25°C / 37°C for 2 h. After incubation, the solutions were centrifuged at 10 000 x g for 20 min. The soluble and insoluble fractions obtained after centrifugation were analyzed by (12%) SDS-PAGE. Once the best solubilization conditions were determined, the minimum urea concentration (2-6 M) required for optimal recovery of the recombinant protein, was evaluated.

2.5 Expression in the methylotrophic yeast *P. pastoris*

The rPoly p 1 coding sequence (Gen Bank: HQ023233.1) was chemically synthetized after codon optimization (http://gcua.schoedl.de/) for expression in *P. pastoris*. A single mutation (H226A) was introduced in the predicted active site of the protein to avoid the potential deleterious effects of phospholipase activity in yeast cells growth (BORODINA et al., 2011). The synthetized gene was then cloned into the pPICZαA vector that provide the α-mating factor for secreted production of rPoly p 1 and the N-terminal 6xHis tag for protein purification. The construction was used to transform *P.*

pastoris X-33 strain. All the procedures described above were conducted by Genscript (Piscataway, US). The transformants were then selected using YPD-agar plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) supplemented with 100 µg/mL of Zeocine (Invitrogen, US).

For allergen expression, a single colony was used to inoculate in 25 mL of BMGY (buffered minimal glycerol medium, recipe from Invitrogen). The pre-inoculum was incubated overnight at 28ºC, with shaking at 250 rpm. The OD_{600} of the culture was measured and a suitable volume was centrifuged at 3000 x g, 4 ºC for 10 min to suspended in 100mL of BMMY (buffered minimal methanol medium) at a final OD_{600}=1. The BMMY medium was transferred to a 1L baffled shake flask and incubated for 96 hours with daily addition of (1%) methanol (final concentration). Expression was conducted at 25 ºC or 28 ºC, and with shaking at 200 rpm or 250 rpm to test different temperature and aeration conditions. Aliquots of 2.0 mL were collected daily and further evaluated in (12%) SDS-PAGE.

2.6 Recombinant Poly p 1 purification

The purification of the target protein expressed in *E. coli* was conducted using a protocol described previously (PEREZ-RIVEROL et al., 2016), with minor modifications. Briefly, the supernatant obtained after the solubilization was filtered (0.22µm o 0.45µm) and placed on an Amicon Ultra-15 Centrifugal Filter (Merck Millipore, Ireland) for buffer exchange. Sequential steps for increasing the pH of the solubilization buffer (citrate-phosphate buffer, containing 0.15 M NaCl, 6 M urea) from pH=2.6 to 7 were conducted. Then, a final exchange using the equilibration buffer (20 mM Tris-HCl, 0.15 M NaCl, 6 M urea, 20 mM Imidazole; pH 7.4) was performed. The resulting solution (5 mL) was applied to a commercial prepacked HisTrap HP™ column (Ni^{2+} Sepharose™ High Performance; GE Healthcare, Sweden). The column was then washed with 10 column volumes of buffer containing 20 mM Tris-HCl, 0.15 M NaCl, 6 M urea, and 75 mM imidazole; pH 7.4. The elution of the N-terminal 6xHis-tagged rPoly p 1 was performed with 10 mL of elution buffer (20 mM Tris-HCl, 0.15 M NaCl, 6 M urea, 200 mM imidazole; pH 7.4). Two milliliters fractions were collected for analysis in (12%) SDS-PAGE.

2.7 Mass spectrometry analysis

Gel pieces containing the purified allergen were excised and destained twice for 30 min at 25 °C with 50 mM ammonium bicarbonate/50% (v/v) acetonitrile, dehydrated in acetonitrile, dried and treated with four proteolytic enzyme: 20 ng/mL of trypsin (Promega, US); 50 ng/mL of chymotrypsin (Promega, US), 40 µg/µL of Glu-C/V8 protease (Sigma, US) and 40 ng/µL of Asp-N protease (Promega, Madison, USA). Peptide extraction was performed with 0.5% formic acid (FA) and 0.5% FA in 30% acetonitrile (ACN). The extracted peptides were pooled, desalted and cleaned with PerfectPure C18 pipet tips (Eppendorf, Hamburg, Germany). Mass spectrometry analysis was performed by NanoLC-ESI-CID (Amazon ETD- Bruker Daltonics, Bremen, Germany). MS data was acquired in the m/z range of 350 to 3500, and MS/MS spectra in information-dependent data acquisition over the mass range of m/z 100-3500. MS/MS spectra were interpreted and peak lists were generated by DataAnalysis 4.1 (Bruker Daltonics). For protein identification searches were performed using the MASCOT 2.2.06 (Matrix Science, London, UK) against updated Hymenoptera database.

2.8 SDS-Polyacrilamide gel electrophoresis and protein quantification

SDS-PAGE were performed according to Laemmli (1970) (LAEMMLI, 1970), using a Mini-Protean® Tetra Cell System (BioRad). After running, the gels were stained with Coomassie Brilliant Blue R-250 (CBB). The protein concentration of the samples was determined by BCA method (SMITH, 1989), with a bovine serum albumin (Sigma, US) as a standard. Densitometric analyses of the samples were performed using the TotalLabTM Quant v.12.3 (UK) software.

2.9 Immunoblotting

The Anti-His Antibody Selector Kit (Qiagen, US) and a rat anti-mouse IgG (subclass IgG1) (Sigma, US) conjugate to horseradish peroxidase (1:500) were used for recognition of the 6xHis tag of the rPoly p 1 expressed in *E. coli* and *P. pastoris.* The immunoblotting analyses were conducted following the manufacturer instructions. The IgE-mediated immunodetection of rPoly p 1 by sera of allergic patients was performed according with the protocol described previously (PEREZ-RIVEROL et al.,

2016). In all immunoblotting analyses the bands were visualized in an Image Quant 400 (GE Healthcare, Sweden) using the chemiluminescent Luminata™ ForteWestern HRP substrate (Millipore, US). For the preliminary analysis of the sensitivity related to the use of rPoly p 1, sera from patients (n=40) previously diagnosed with sIgE to wasp venom were analyzed for immunodetection of the recombinant allergen.

3. RESULTS

3.1 Evaluation of soluble expression in *E. coli*

Previous attempts to express rPoly p 1 in *E. coli* cells failed to produce the recombinant allergen as a soluble protein (PEREZ-RIVEROL et al., 2016). To overcome the insolubility problem we evaluated the expression profile of rPoly p 1 under the combined effects of induction with IPTG concentrations ranging from (0.005-1 mM) and cell growth at low temperature (6°C, 10°C, 16°C). The (12%) SDS-PAGE analysis of soluble fractions obtained after bacterial cell lysis showed that rPoly p 1 was not detected in any of the combinations tested, after 10h (Data S1 Figure 1) or 24h post-induction. Also, no rPoly p1 was detected in these soluble fractions after immunoblotting analysis conducted using the anti-His antibody (data not shown).

Interestingly, although we were no able to obtain the recombinant allergen as a soluble protein, the expression at 16°C and 0.1 mM IPTG (Figure 1, Lane 6) significantly improved the production of the rPoly p 1 compared with the levels previously reported (PEREZ-RIVEROL et al., 2016) (Figure 1, Lane 3). The analysis of the insoluble fraction after bacterial cells disruption revealed that rPoly p 1 represent 30% of total proteins content which is superior (2.5 times) to the levels (12%) obtained with the previously informed conditions (20°C,1 mM IPTG). The amounts of the recombinant allergen in the insoluble fraction do not increase after 10h post-induction (data not shown). Expression at temperatures under 16°C or induction with IPTG concentrations lower than 0.1 mM does not resulted in soluble production of rPoly p 1 and significantly decreased the overall protein yields. The Table 1 summarizes the results obtained during rPoly p 1 production under each combination of temperature and IPTG concentrations tested.

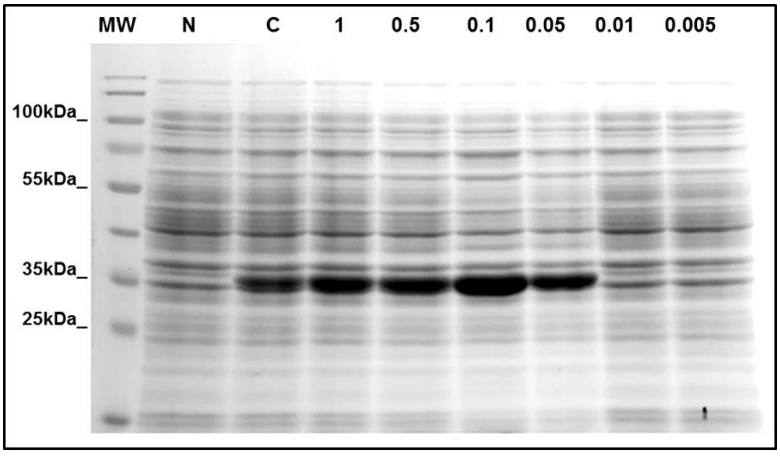

Figure 1. SDS-PAGE (12%) analyses of the cell lysate insoluble fractions from *E. coli* BL21 (DE3) obtained after rPoly p 1 expression (16°C, 10 h post-induction). Samples: N= negative control (non-transformed *E. coli* BL21 (DE3) induced with 1 mM IPTG), C= positive control (rPoly p 1 expression under previously condition tested (PEREZ-RIVEROL et al., 2016)) and 1-0.005= transformed *E. coli* BL21 (DE3) cells after induction with 1, 0.5, 0.1, 0.05, 0.01 and 0.005 mM IPTG.

Table 1: Recombinant Poly p 1 levels (percentage of total proteins content) on pellet of bacterial cell lysate after expression under different growing conditions. The results indicated the mean values with standard deviation of three replicates of the experiment.

	Recombinant Poly p 1 levels [%]						
Temperature	**IPTG concentrations**						**Control[a]**
	1 mM	**0.5 mM**	**0.1 mM**	**0.05 mM**	**0.01 mM**	**0.005 mM**	
16°C	19±0.5	21±0.6	30±1.2	27.6±2.7	NE	NE	
10°C	9±0.3	10±0.9	11±1.1	6±0.4	NE	NE	12±0.6
6°C	3±0.6	4±0.8	4±0.1	4±0.4	NE	NE	

[a] Positive control (PEREZ-RIVEROL et al., 2016), expression at 20 °C induced with 1 mM IPTG, NE: No expression of rPoly p 1 detected

The results indicated the mean values of three experimental replicates.

3.2 Solubilization of recombinant Poly p 1

Considering the low recovery values (30%) informed for rPoly p 1 solubilization (PEREZ-RIVEROL et al., 2016), we evaluated different parameters to increase the soluble allergen recovery in the downstream solubilization process. The optimization

includes evaluation of different buffer compositions, pH values and total protein concentrations in the starting sample (50, 100 and 200 mg/L). Also, we evaluated the effects on solubilization efficiency caused by incubation at different temperatures.

The analyses showed that incubation of rPoly p 1 inclusion bodies (0.5 - 1 mg/mL protein concentration) in citrate-phosphate buffer (pH=2.6) containing 8M urea at 25°C for 2h resulted in completely solubilization (98%) of the allergen (Figure 2). Incubation with the other buffers described in the section 2.2 resulted in a reduced recovery of solubilized rPoly p 1 with lower values accounting for solutions with pH closer to 9.1, the theorical pI of rPoly p 1. We also found that incubation at 4°C for 16h and 37°C for 2h, or with higher protein concentration (200 mg/L), slightly decrease the efficiency of the solubilization process, regardless buffer composition. Table 3 summarized the results obtained in the solubilization analyses.

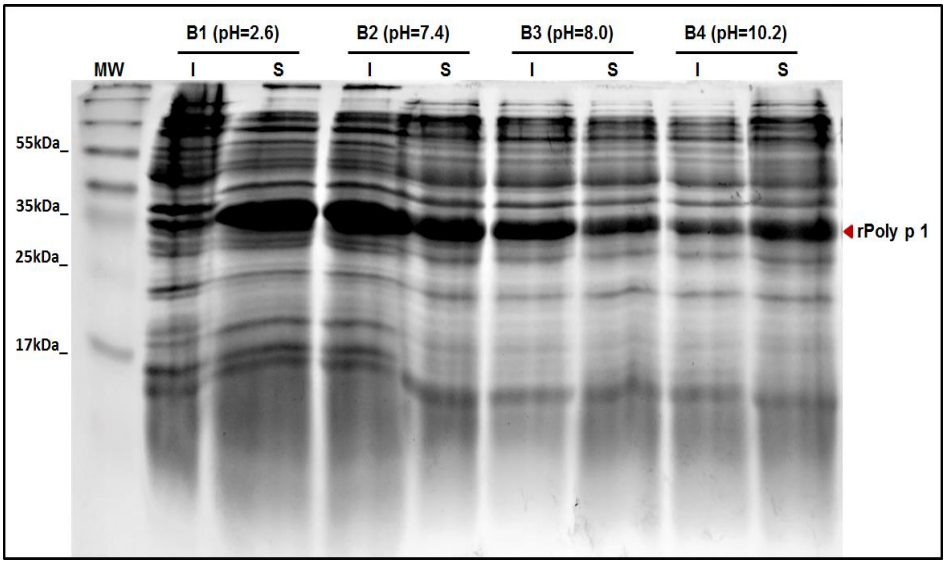

Figure 2. SDS-PAGE (12%) of the insoluble (I) and soluble (S) fractions obtained after protein solubilization of *E. coli* cell lysate pellets in presence of 8M urea, at 25 °C. Buffers: B1= Citrate-phosphate, B2= Tris-HCl, B3= Phosphate and B4= Carbonate-bicarbonate.

Once we determined the optimal solubilization conditions, the effects of urea concentration in rPoly p 1 inclusion bodies were evaluated. We found that solubilization with citrate-phosphate buffer containing 6M urea at 25°C, also resulted in complete recovery (98%) of rPoly p 1 in the soluble fraction. Lower concentrations of urea caused a decrease in the efficiency of solubilization process (Figure 3).

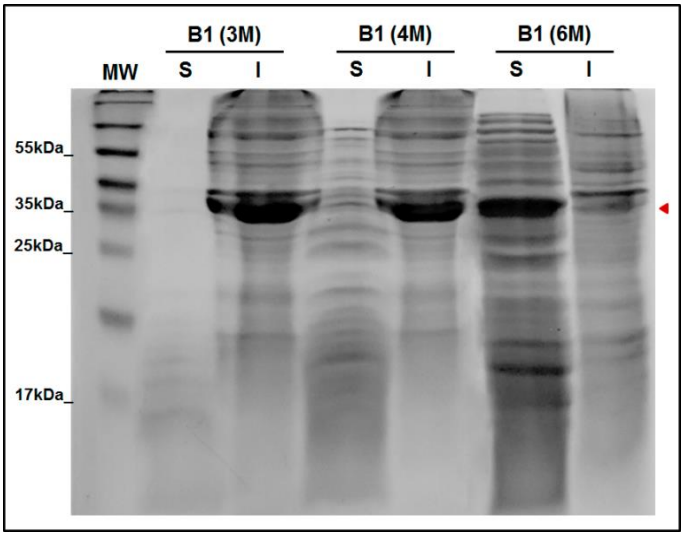

Figure 3**.** SDS-PAGE (12%) of the insoluble (I) and soluble (S) fractions obtained after protein solubilization of *E. coli* cell lysate pellets in citrate-phosphate buffer pH 2.6, containing 3 M, 4 M and 6 M urea, at 25 °C. Molecular weight marker is indicated.

Table 2: Summary of the solubilization efficiency of rPoly p 1 after incubation under different conditions in the presence of 8M urea.

Buffer [a]/pH	**Concentration [mg/L]**	**Solubilization [%] [b]**		
		4 °C	**25 °C**	**37 °C**
Citrate-phosphate / 2.6	200	85 ± 4.8	96 ± 1.3	93 ± 2.8
	100	87 ± 1.8	98 ± 2.3	95 ± 5.1
	50	87 ± 3.5	98 ± 4.1	95 ± 3.4
Tris-HCl / 7.4	200	27 ± 1.8	38 ± 1.8	33 ± 2.9
	100	28 ± 1.8	40 ± 1.8	35 ± 3.9
	50	27 ± 0.9	40 ± 2.8	33 ± 2.6
Phosphate / 8.0	200	12 ± 0.6	28 ± 1.9	20 ± 1.5
	100	15 ± 0.9	30 ± 1.8	22 ± 1.5
	50	15 ± 0.8	30 ± 1.4	22 ± 1.8
Carbonate-bicarbonate /10.2	200	52 ± 1.6	55 ± 2.8	61 ± 3.9
	100	55 ± 2.4	58 ± 0.8	65 ± 4.6
	50	55 ± 1.6	57 ± 0.7	65 ± 3.1

[a] All the buffers in presence of 8M urea.

[b] Total protein concentration of samples.

The results indicate the mean values with standard deviation (SD) of three experimental replicates.

3.3 Recombinant Poly p 1 purification and sequencing

After optimization of solubilization process, 10 mL of the solution contained rPoly p 1 were loaded in an Amicon Ultra-15 Centrifugal Filter (Merck Millipore, Ireland) for buffer exchange and protein concentration. The resulted 5 mL solution (20 mM Tris-HCl 0.15 M NaCl, 6 M urea; pH 7.0) was used for protein purification. A unique band of ~34 kDa, similar the native counterpart (nPoly p 1) was detected after elution and analysis in a (12%) SDS-PAGE (Figure 4a). Further densitometric analysis of the gel (TotalLab^{TM} Quant v12.3 software) confirmed that the recombinant allergen was obtained to homogeneity (97-99%). The overall yield of the purified protein was 1.5 g/L of bacterial culture broth which is sevenfold higher than the values obtained (212 mg/L) in the preliminary study (PEREZ-RIVEROL et al., 2016). The analysis with an anti-His antibody showed a unique band of the expected molecular weight confirming the identity of the produced protein (Figure 4b)

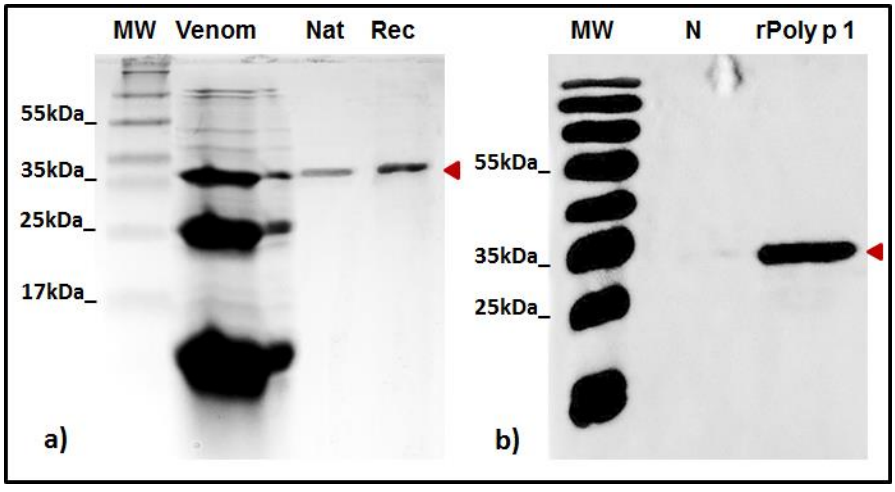

Figure 4. SDS-PAGE (12%) (a) and 6XHis-mediated immunodetection (b) of the purified rPoly p 1. Samples: Nat= nPoly p 1, Rec= rPoly p 1, N=negative control (pellet from a non-induced clone)

The purified rPoly p1 was sequenced for the first time using a gel-based proteomic approach similar to the procedure described for Poly p 5 (DOS SANTOS-PINTO et al., 2014). The amino acid sequence and a representation of the mass spectra from the 44 peptide fragments produced after the enzymatic digestion of the recombinant

allergen are provided in the Supporting Information (Data S1 Table 1 and Figure 2). Using this information, the primary sequence of rPoly p 1 (Figure 5) was obtained covering around 94.7% of the informed sequence for the mature form of the native allergen (GenBank: A2VBC4). The rPoly p 1 sequence showed different levels of homology with other venom PLA1s from clinically relevant insects such as *P. annularis* (71.3%), *V. germanica* (Ves g 1) (53.4%), *S. invicta* (27.8%) and *V. vulgaris* (52.7%).

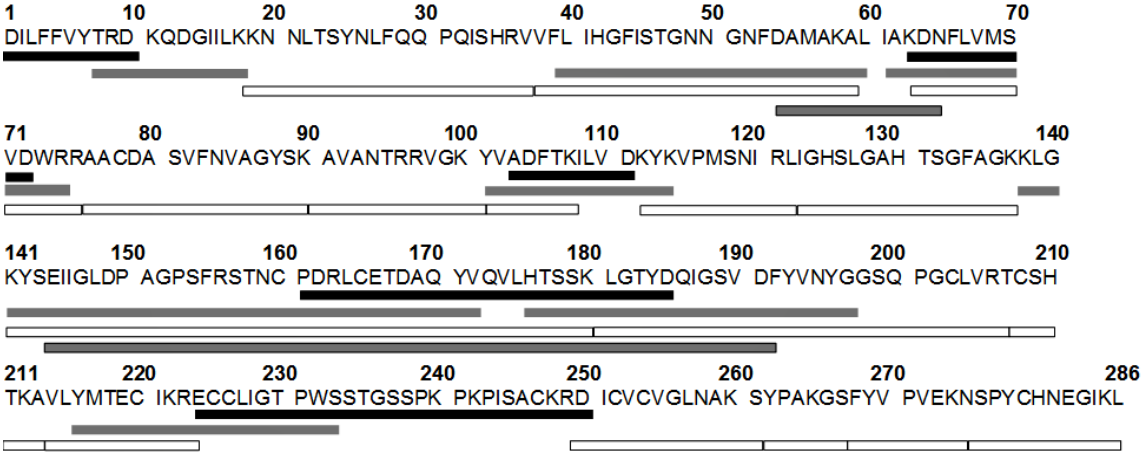

Figure 5. Sequence of the recombinant form of *P. paulista* venom phospholipase A1 obtained from the interpretation of the CID spectra of the peptides generated by digestion with Asp-N protease ( ), trypsin ( ), chymotrypsin ( ) and protease Glu-C/V8 ( ).

3.4 Expression in *Pichia pastoris*

Considering that none of the culture conditions tested in *E.coli* resulted in soluble expression of rPoly p 1, we aimed to produce the allergen as a soluble recombinant protein in *P. pastoris*. The Poly p 1 coding sequence containing the mutation H226A to avoid the potential deleterious effect of the phospholipase A1 activity in yeast cells was successfully cloned in pPICZαA vector. Four positive clones were obtained after transformation of *P. pastoris* X-33 cells with the pPICZαA_*poly p 1* (H226A) construction (Figure 6a). Immediately, the transformants were tested under different conditions for production of rPoly p 1 as a soluble secreted protein.

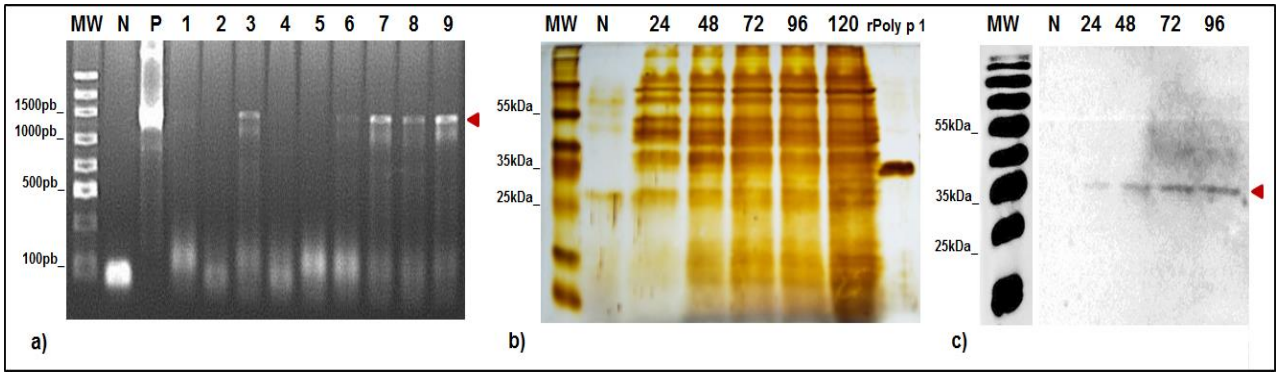

Figure 6. Recombinant Poly p 1 expression in yeast. (a) Positive *P. pastoris* transformants detecting by PCR. (b) (12%) SDS-PAGE and (c) anti-His immunoblotting analyses of the supernatant-collected fractions at 24, 48, 72, 96, 120 h post induction. Samples: P=pPICZαA_poly p 1 construct, N= *P. pastoris* transformed with empty pPICZαA; 1-9 clones. Molecular weight markers are indicated.

The (12%) SDS-PAGE analyses of the supernatant fractions collected daily after induction at 28 °C with shaking at 250rpm resulted in no detectable levels of yeast-expressed rPoly p 1. In order to increase sensitivity of the detection, all gels were stained with silver nitrate (Fermentas, Germany) (Figure 6b), also with negative results. Finally, the fractions collected (24h-96h) were analyzed with the anti-His antibody for detection of the target protein. Almost undetectable bands were visualized on the immunorecognition after settled the Image Quant 400 in ultrasensitive capture (Figure 6c), suggesting that the expression yields of rPoly p 1 in *P. pastoris* are significantly low (nanograms/liter of fermentation broth). Variations in culture conditions including lower aeration (200 rpm) and growth temperature (25 °C) to avoid protein degradation do not resulted in higher expression of the recombinant allergen. We tested the cell pellets for intracellular expression of rPoly p 1 also with no detectable levels of the target protein (data not shown).

3.5 Immunodetection

Sera from patients previously diagnosed with sIgE to wasp venoms recognized the rPoly p 1 produced in *E.coli*, after optimization of the expression and solubilization process. Similar to previous report (PEREZ-RIVEROL et al., 2016), a single band with the expected molecular weight (~34 kDa) was detected for each individual

serum (Figure 7). The levels of recognition were similar to those obtained with the native form of the allergen.

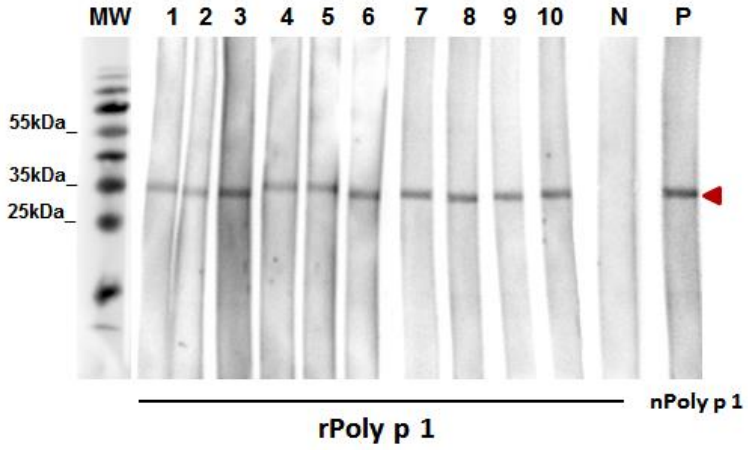

Figure 7. IgE-mediated immunodetection of rPoly p 1 expressed in *E.coli* by sera of allergic patients sensitized to wasp venom. Samples: 1-10= individual serum, N= randomly selected serum from a non-allergic healthy volunteer; P= Pool of sera (5) from sensitized individuals.

As the evaluation of the sensitivity is a major issue in the immunological characterization of recombinant allergens, we conducted a preliminary analysis of the sensitivity of rPoly p 1 using the sera from patients (40) previously diagnosed with sIgE to wasp venoms. Up to 38 sera (95%) reacted with rPoly p 1 in immunoblotting assays. The results obtained in the analysis are summarized in the Table 3.

Table 3. Summary of the results obtained in the sensitivity analysis performed by immunoblotting using rPoly p 1 and sera from patients (40) previously diagnosed with sIgE to wasp venoms.

Patient	Immunoblotting rPoly p 1	Patient	Immunoblotting rPoly p 1
1	+++	21	+++
2	++	22	+++
3	+++	23	++
4	++	24	+++
5	+++	25	+++
6	+++	26	+++

7	+++	27	+++
8	+++	28	++
9	+++	29	-
10	++	30	+++
11	++	31	+
12	+++	32	+++
13	+++	33	+
14	+	34	+++
15	+++	35	+++
16	+++	36	+++
17	+++	37	+++
18	+++	38	+++
19	-	39	+++
20	++	40	+++

Intensity of reaction (classified by a visual method):
(+++) high, (++) moderate, (+): limited, (-): no reaction

4. Discussion

Overexpression, lack of posttranslational modifications and improper disulfide-bonds formation usually results in protein misfolding, insolubility and aggregation during heterologous expression in *E.coli*. Considering that insoluble production of recombinant proteins is a major challenge for their biotechnological, clinical or pharmaceutical potential uses, several strategies have been proposed to overcome insolubility problems. These strategies include shifting of the expression conditions, co-expression with molecular chaperones and the use of solubility improving tags (CORREA; OPPEZZO, 2015). In particular, the analyses of culture conditions for solubility improvement often include the expression at low temperatures (VERA et al., 2007; SAN-MIGUEL; PÉREZ-BERMÚDEZ; GAVIDIA, 2013) combined with low concentration of inductor and optimization of the media formulation (HORTSCH; WEUSTER-BOTZ, 2011).

Most of the Hymenoptera venom allergens identified to date are soluble, high molecular weight enzymes with a variable number of disulfide-bonds stabilizing their structures (MÜLLER, 2011). Some of them, such as venom hyaluronidases (Api m 2, Ves v 2, Poly p 2) (KUBELKA; ALTMANN; MÄRZ, 1995; PINTO et al., 2012) and dipeptidyl peptidases (Api m 5, Ves v 3) (BLANK et al., 2010) are glycosylated and could present the clinically relevant CCDs. These characteristics add several difficulties their recombinant production in *E.coli*. In the case of Poly p 1, a major allergen of *P. paulista* venom that could be used as a reliable marker of *P. paulista* sensitization, the early attempts for its recombinant expression in *E. coli* resulted in protein overexpression and insolubility (PEREZ-RIVEROL et al., 2016). Further efforts to solubilize the recombinant allergen showed low protein recovery (30%). The poor levels of solubilized protein recovery hampered the downstream refolding, structural and immunological analyses as large quantities of the protein are require on these subsequent steps. Thus, we aimed to improve the production of soluble rPoly p 1 by modifying the expression conditions or by optimizing the downstream solubilization process. Also, we tested the expression of the rPoly p 1 as a secreted protein in the metylotrophic yeast *P. pastoris*.

To cope with rPoly p1 overexpression that could be causing allergen missfold and insolubility we tested the effects of cultivation at low temperatures and induction with low IPTG concentrations. In addition to reduce protein production kinetic, growth at low temperatures have a positive effect over protein solubility by reducing hydrophobic interactions (FERRER-MIRALLES et al., 2015). None of the combination of low temperature/IPTG concentrations tested resulted in the expression of the recombinant allergen as a soluble protein (Data S1 Figure 1). Interestingly, although rPoly p 1 expression levels significantly drop after induction at 6°C and 10°C/0.5-0.05 mM, no soluble expression was detected. These findings contrast with those informed by several authors in which induction at low temperature resulted on improved solubility of the recombinant protein (MIN et al., 2012).

Min et al, 2012 (MIN et al., 2012) circumvented insolubility problems related to recombinant expression of three different target proteins by lowering the growth

temperature to 6°C and 10°C. Cultivation at 10°C combined with induction with low lactose concentration resulted in partial solubilization of the humanized monoclonal anti-TNF-α scFv (GST-hD2) expressed in *E.coli* (SINA; FARAJZADEH; DASTMALCHI, 2015). A reduction in growth temperature also helped to obtain the soluble form of α-chain of the human neonatal Fc receptor (FcRn) in *E.coli* (NG; LIM; LAI, 2016). Soluble yield of the anticancer agent TRAIL expressed in *E. coli* was improved by expression with low IPTG concentrations (0.1 mM) at 28°C (LI; GU; WU, 2016). Recently, Tsai et al, 2016 (TSAI et al., 2017) reported that soluble expression of the recombinant form of Man i 1, a major allergen identified in *Manguifera indica* is promoted under cultivation at 16°C and induction with IPTG 0.1mM.

The discrepancy of ours results with those mentioned above suggested that saturation of cell folding machinery caused by overexpression is not the unique factor promoting recombinant allergen insolubility. As noted, a significant drop in the target allergen expression yields do not resulted in soluble production of rPoly p 1 (Table 1). The native Poly p 1 is a 34 kDa protein with six potential disulfide bridges involved in allergen 3-D structure stabilization (SANTOS et al., 2007; PEREZ-RIVEROL et al., 2016). It has been well documented that disulfide-bonds formation during protein expression in *E.coli* is compromised by the reducing conditions of bacterial cytoplasm (BERKMEN, 2012). Considering this, the cytoplasmic expression of the allergen could by promoting cysteine reduction, preventing adequate disulfide-bonds formation in rPoly p 1, finally causing rPoly p 1 misfolding and insolubility. The use of expression vectors that enable the periplasmic production of the protein represent an alternative to ensure proper formation of disulfide bonds (DE MARCO, 2009; SOCKOLOSKY; SZOKA, 2013) resulting in soluble expression of rPoly p 1.

Incubation of the allergen inclusion bodies with citrate-phosphate buffer, pH 2.6 containing 8M urea, at 25 °C for 2h, completely solubilized (98%) rPoly p 1. Further analysis showed that incubation with 6M urea also resulted in completely solubilization of the recombinant allergen. The recovery of rPoly p 1 was lower for buffer solutions with pH closer to the predicted theorical pI= 9.12 of rPoly p 1 (Figure 2). Overall, these findings suggested that pH has a crucial role in promote inclusion

bodies disaggregation and rPoly p 1 solubilization. Patra et al, (2000) (PATRA et al., 2000) informed a similar result after optimization of solubilization process for soluble recovery of the recombinant human growth hormone (r-hGH). Our findings also agree with those informed by Vemula et al, 2015 (VEMULA et al., 2015) in which recovery of a recombinant human granulocyte colony stimulating factor (rhG-CSF) from inclusion bodies was significantly increased by moving away the pH of solubilization buffer from the theorical pI of the target protein.

We failed to produce significant levels of rPoly p 1 as a soluble protein in the methylotrophic yeast *P. pastoris*. The expression in this eukaryotic system usually renders large quantities of correctly processed target protein in the fermentation broth (LOOSER et al., 2015). Our results contrast with a previous report related to the expression of an enzymatically inactive form of the PLA1 from venom of *V. vulgaris* (Ves v 1) using this system (BORODINA et al., 2011). Based on that study, we also evaluated the expression of a mutated variant of the rPoly p 1 (H226A) gene to avoid the potential deleterious effect of phospholipase A1 activity in yeast cells growth. Due to the extremely low levels of expression obtained (nanograms) for the different conditions tested, rPoly p 1 could not be detected by (12%) SDS-PAGE analyses (Figure 6b). The recombinant allergen was detected exclusively by immunoblotting using a highly sensitive chemiluminescent system (Figure 6c).

Considering that the mutation (H226A) and the yeast strain used here were similar to those used during expression of Ves v 1, we propose that the differences of our results could be related mainly to the structural differences between these venom PLA1s. As noted, the alignment of rPoly p 1 and Ves v1 sequences showed only a 52.7% of homology. Unfortunately, there is limited information about the production of venom PLA1s in eukaryotic expression systems (BORODINA et al., 2011; SEISMANN et al., 2010a). However, the comparison of the production yields informed here for solubilized and purified rPoly p 1 expressed in *E.coli* with those obtained for soluble Ves v 1 in *P.* pastoris (BORODINA et al., 2011) suggest that even when a downstream refolding procedure is required, the bacterial system represents the most feasible option for heterologous expression of the allergen. An

optimized refolding procedure could be used to ensure high recovery of native-like rPoly p 1.

Finally, the sensitivity analysis showed high levels (95%) of immunodetection of the recombinant allergen by a cohort of patients (40) previously identified with sIgE to *P. paulista* venom (Table 3). Fail in the detection of one single patient could result in a fatal outcome after a sting accident (OLLERT; BLANK, 2015). Low diagnostic sensitivity is a common problem related to the use of recombinant allergens, especially those produced as non-glycosylated proteins. It has been informed that only 57% (100/175) of allergic patients previously diagnosed with IgE to HBV reacted with a refolded form of rApi m 1 expressed in *E.coli*, while 91% (153/169) recognize nApi m 1 (KOROŠEC et al., 2011). Similar data related to low diagnostic sensitivity of a unique allergen have been informed in other studies not only for rApi m 1 (KÖHLER et al., 2014) but also for recombinant forms of the major allergens from *P. dominula* (MONSALVE et al., 2012) and *V. vulgaris* venoms (STURM et al., 2010). In the case of venom PLA1s at least one study has reported a sensitivity of 54% in individuals unequivocally diagnosed with allergy to *V. vulgaris* venom (EBO et al., 2012). The higher percentage of sensitivity (95%) found in our analysis with rPoly p 1 could be due to variations in the clinical relevance of these PLA1s on each venom, to the lower number of sera analyzed and mainly, to the different semiquantitave approach used in our study. A re-analysis using BAT and including an increased cohort of sera will complements these preliminary results. However, the levels of sensitivity obtained in this preliminary analysis suggest that rPoly p 1 is a feasible marker of *P. paulista* allergy that could be used in combination with other *P. paulista* venom allergens for development of molecular diagnosis.

5. Concluding remarks

Overall, the optimization of the recombinant expression and solubilization steps reported here resulted in a sevenfold increase on the yields (1.5 g/L bacterial culture) of the solubilized rPoly p 1, expressed in *E. coli*. A time and resources saving strategy is now available for production of large quantities of solubilized rPoly p1, which is a mandatory prerequisite for performed protein refolding, downstream

molecular and immunologic analyses and ultimately, for the introduction of the allergen in routine diagnosis. The rPoly p 1 showed high levels of sensitivity which is a critical issue regarding its potential use in clinical practice. The development of molecular diagnosis based in the use of rPoly p 1 could provide a valuable tool to increase to reliability of the procedures currently used for identification of culprit venom in Brazilian allergic individuals. Consequently, a significant improvement in the outcomes of immunotherapy and in the patients' quality of life could be achieved.

6. Acknowledgments

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[a]All the references cited in the manuscript are included in the References section of this Thesis

[b]The Supplementary Data (S) related to this manuscript is provided in the Appendix section of the Thesis

6. CHAPTER 4

Venoms from several clinically relevant Neotropical wasps are devoid of cross-reacted carbohydrate determinants. **Perez-Riverol, A**; Miehe, M.; Jabs F.; Zollner R.L.; Spillner, E. and Brochetto-Braga, M.R. (To be submitted on **Journal of Allergy and Clinical Immunology**)

Cross-reactivity analyses of the recombinant phospholipase A1 from *Polybia paulista* wasp venom. **Perez-Riverol, A**; Romani Fernandes, L.G.; Musacchio-Lasa, A.; dos Santos-Pinto, J.R.A.; Moitinho Abraham, D.; Izuka-Moraes, G.H.; Jabs F.; Miehe, M.; Palma, M.S.; Zollner R.L.; Spillner, E and Brochetto-Braga M.R. (To be submitted in **Molecular Immunology**)

Venoms from several clinically relevant Neotropical wasps are devoid of cross-reacted carbohydrate determinants

Short communication

Hypersensitivity to insect venoms represents an important human health problem in Neotropical regions. In Latin America, Hymenoptera stings is one of the three major causes (14%) of allergic reactions and anaphylaxis (SOLE et al., 2011). While venoms and allergens from European and North American species have been extensively characterized, little is known about the allergenic arsenal from Neotropical species. Despite the wide diversity of clinically relevant Hymenoptera identified in this geographical zone, no individual component obtained from endemic species are available for molecular diagnosis (PEREZ-RIVEROL et al., 2017). Consequently, venom allergy diagnosis is based on the use of extracts from either endemic or European species (PEREZ-RIVEROL et al., 2016). The use of these venom extracts is often related to high levels of cross-reactivity mainly due to the presence of the cross-reactive carbohydrate determinants (CCDs) (OLLERT; BLANK, 2015).

Insect CCDs are defined by an alpha 1,3-linked fucose residue at the innermost N-acetylglucosamine of the carbohydrate core structure (ALTMANN, 2007). In honeybee venom (HBV), several major allergens have been identified as CCD-carrying proteins. These include the phospholipase A2, hyaluronidase, icarapin and vitollegenin (HELBLING; MÜLLER, 2013). *Vespula* spp. hyaluronidases, dipeptidyl peptidases (Ves v 3) and vitellogenins also present these type of N-glycosylation (HELBLING; MÜLLER, 2013). In contrast, *P. dominula* venom is devoid of CCDs (BLANK et al., 2013a). Cross-reactivity related to clinically irrelevant sIgE that recognize CCDs are known to cause up to 69%-75% of double positive results to HBV and yellow jacket venom (YJV) (MÜLLER et al., 2009; SEISMANN et al., 2010b). Fail in the identification of culprit venom due to CCD-related cross-reactivity often lead to the inclusion of multiple venoms in the immunotherapy which results on increasing risks of side effects and *the novo* sensitizations (SPILLNER; BLANK; JAKOB, 2014).

The molecular basis for the incidence of cross-reactivity during diagnosis of venom allergy caused by Neotropical insects remains unclear. The limited number of venomic and allergomic studies conducted with clinically relevant Hymenoptera from these regions hampers the understanding of the causes underlying cross-reactivity. In the case of *P. paulista*, and *P. scutellaris* which are highly aggressive Neotropical wasps, several allergens are known to be glycosylated (DOS SANTOS et al., 2010; VINZÓN et al., 2012; DOS SANTOS-PINTO et al.,2014;). However, none of these allergens have been analyzed for the presence of CCDs. The results derived from this analysis could significantly modified the procedures used for diagnosis of allergy in Neotropical regions and could lead to rational selection of the allergens to be include in molecular diagnosis.

Here, we addressed the CCD-related reactivity of six clinically relevant Neotropical wasps. First, the quality of the collected venoms was analyzed in a (12%) SDS-PAGE stained with Coomasie blue R-250 (Figure 1). The general presence of N-linked glycans was evaluated using the biotinylated Concanavalin A (Vector Laboratories, US) which recognizes α-linked mannose modifying protein structure. The specific detection of the α 1,3-core fucosylation typical from insect CCDs was conducted using a rabbit anti–horseradish peroxidase (HRP) serum. The HBV (i1), *V. vulgaris* (i3), *P. dominula* (i4) and Ves v 3 were used as controls. All the protocols used in these assays are described on the Supplementary Data.

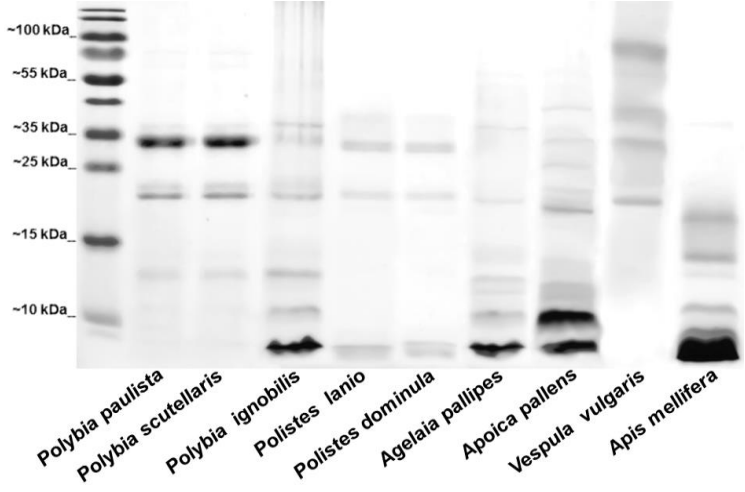

Figure 1**.** (12%) SDS-PAGE analysis of the venoms from the Brazilian wasps and from the European clinically relevant insects (controls).

Immunoblotting analyses revealed the presence of several glycosylated proteins in all the venoms tested (Figure 2). In contrast, none of the extracts were recognized by the anti-HRP antibodies indicating that the venom components of these wasps are CCD-lacking (Figure 2). No reaction was detected also in ELISA analyses performed with the rabbit anti-HRP serum (Figure 3A). The honey bee (HBV) as well as YJV and Ves v 3 showed a significant positive response while no recognition was detected with *P. dominula* venom (negative control).

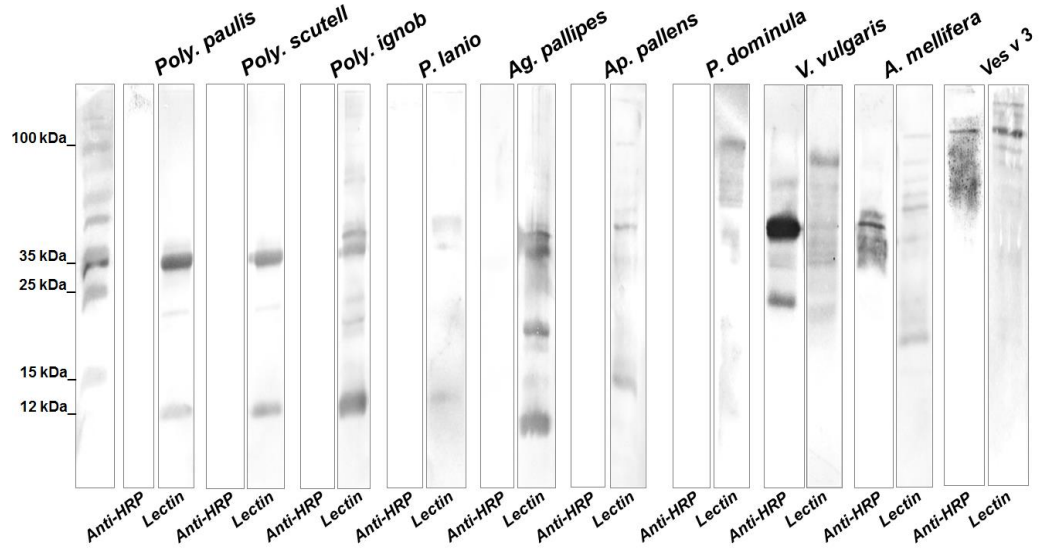

Figure 2. Glycosylation pattern of the Neotropical wasp venoms analyzed by Western blot. The general presence of glycosylation is detected with the lectin Concanavalin A while the CCDs were detected with anti-HRP antibodies

In addition, we tested the sIgE-mediated recognition of the wasp venoms by sera (n=2) from patients with reactivity to insect CCDs which are known to lack CCD-independent allergen recognition. As shown in Figure 3, none of the venoms were recognized by the patients' sera in ELISA while a significant signal was detected in the positive controls.

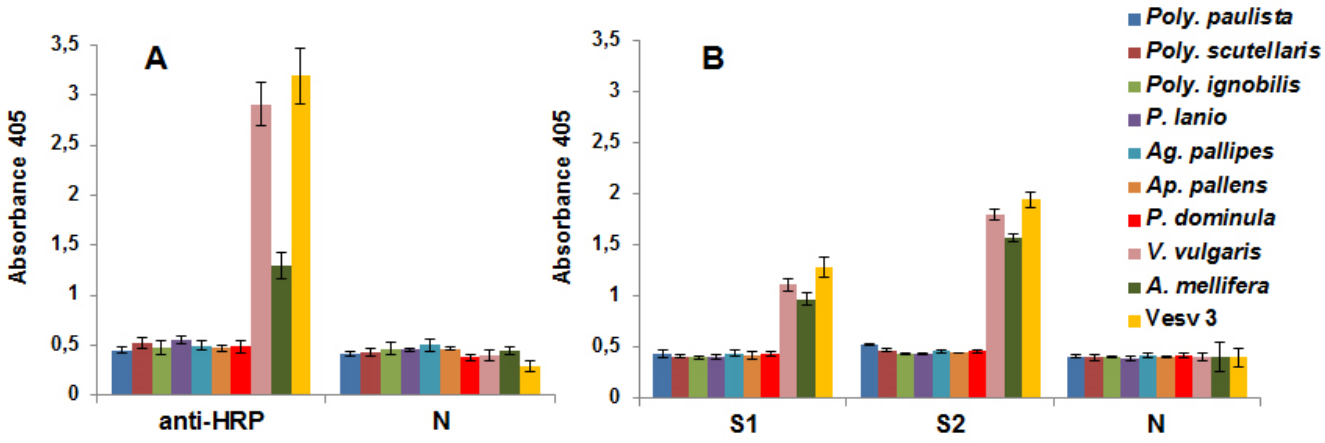

Figure 3. Venom immunodetection by anti-HRP (A) and CCD-reactive sIgE in sera from allergic patients (B)

The findings informed here are consistent with previously published data that reported a limited cross-reactivity among *Polistes* and *Vespula* venoms, potentially due to the lack of the α 1,3-core fucosylation in the carbohydrates modifying the Polistes allergens (REISMAN et al., 1982). A recent contribution extended those results and showed that the venoms from six Polistes species are devoid of CCDs (BLANK et al., 2013a). The lack of insect CCDs in the venom from the wasps analyzed here has major implications for the procedures currently used for allergy diagnosis in Neotropical region, particularly in Brazil. The crude venoms from these species could be used for identification of CCD-independent sensitizations. The sIgE detection during diagnosis will indicate a true sensitization or the occurrence of cross-reactivity related to the presence of common B-cell epitopes on homologues allergens (OLLERT; BLANK, 2015). Consequently, the rational design of strategies for differential allergy diagnosis should be based in the use of species-specific allergens and/or proteins with non-cross reactive epitopes.

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[a] All the references cited in the manuscript are included in the References section of this Thesis

[b] The Supplementary data (Data S1) related to this manuscript is provided in the Appendix section of the Thesis

Cross-reactivity analyses of the recombinant phospholipase A1 from *Polybia paulista* wasp venom

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ABSTRACT

Polybia paulista (Hymenoptera: Vespidae) is a clinically relevant wasp from South America. The venom of this insect can cause life threatening anaphylaxis in untreated allergic individuals. Recently, an immunologically active recombinant form of the phospholipase A1 from *P. paulista* venom (rPoly p 1) was produced in *E.coli*. In this work, we performed an immunological characterization of rPoly p 1 envisioning its use for the development of molecular diagnosis of allergy. Cross-reactivity incidence was assessed using sera from patients previously diagnosed with single sensitization to bee, ant or wasp venoms. The venom phospholipases A1-based cross-reactivity among insects from Neotropical and temperate regions was also evaluated. The recombinant allergen was not recognized by specific IgE (sIgE) in the sera from patients monosensitized to fire ant or honey bee venoms. The sera from rPoly p 1-sensitized mice cross reacted with venoms from other clinically relevant wasps regardless the geographical regions of origin. The levels of PLA1-based cross-reactivity correlates with the homology among the 3-D models of the venom PLA1s from the species tested. The gradual behavior of the cross-reactivity described here emphasize the limitations but also the potential of rPoly p 1 and, in general, wasp PLA1s for differential specie-specific diagnosis of insect venom allergy.

Keywords: *Polybia paulista*, phospholipase A1, cross-reactivity, sensitization, molecular diagnosis

Highlights:

. Intradermic administration of rPoly p 1 resulted in allergen specific sensitization of mice

.Sera from rPoly p 1-sensitized mice showed no cross-reactivity with honeybee/fire ant venoms

.First report on venom PLA1-based cross-reactivity among wasps from Neotropical and temperate regions

. Cross-reactivity on wasp PLA1 correlates with the homology in the primary and 3-D structures

. rPoly p 1 could allow the differentiation of true wasp/bee and wasp/ant sensitizations from cross-reactivity

1 INTRODUCTION

Hypersensitivity to Hymenoptera venoms can cause a wide range of clinical symptoms including severe anaphylactic reactions with potentially fatal outcome (BILÒ, 2011). Identification of the culprit insect is critical for the success and safety profile of the allergy treatment. Immunotherapy using the proper venom is often well tolerated and reduce the risk of subsequent hypersensitivity reactions in up to 98% of the patients treated (OLLERT; BLANK, 2015). However, the identification of the primary sensitizing venom is hampered by several factors. These include (i) the low sensitivity due to the underrepresentation of relevant allergens in the extracts used for diagnosis, (ii) the high rates of asymptomatic sensitizations and (iii) the incidence of cross-reactivity. In developing countries, the lack of allergenic materials obtained from endemic species also hinders the precise distinction of the allergy-causative insect.

Brazil hosted a wide range of clinically relevant insects including wasps (DOS SANTOS et al., 2010; BAPTISTA-SAIDEMBERG; SAIDEMBERG; PALMA, 2011;), bees (GUIMARÃES, 2009) and ants (DOS SANTOS PINTO et al., 2012) (Figure 1). It has been estimated that 10 to 15 thousands of bee and/or wasp- related accidents occur annually in the country (GUIMARÃES, 2009; SERVIÇO DE VIGILÂNCIA SANITÁRIA (SVS), 2016), most of them caused by the Neotropical wasp *Polybia paulista* (GUIMARÃES, 2009; PEREZ-RIVEROL et al., 2017). *Polybia paulista* is a highly aggressive insect that is related with dozens of obits annually and is a major cause of morbidity in Argentina, Brazil Southeast and Paraguay (GUIMARÃES, 2009; SERVIÇO DE VIGILÂNCIA SANITÁRIA (SVS), 2016). Unfortunately, the true number of sting accidents, anaphylaxis and fatalities caused by this social wasp is underestimated due to the lack of epidemiological studies (GUIMARÃES, 2009; PEREZ-RIVEROL et al., 2017). Due to its clinical relevance and abundance, *P. paulista* became a model for became a model for proteomic, peptidomic and allergomic studies and its venom allergens are currently under evaluation for development of CRD.

The venom of *P. paulista* comprises three major allergens including phospholipase A1 (Poly p 1) (SANTOS et al., 2007), hyaluronidase (Poly p 2) (PINTO et al., 2012; JUSTO JACOMINI et al., 2013, 2014) and antigen 5 (Poly p 5) (DOS SANTOS et al., 2010; DOS SANTOS-PINTO et al., 2014). To date, no individual component from *P. paulista* or any other Neotropical insect is available for allergy diagnosis. The use of crude venoms or allergens from species of temperate regions in diagnosis of Brazilian patients is often associated to high levels of cross-reactivity and low sensitivity. Cross-reactivity can be caused by the presence of cross-reactive carbohydrates determinants (CCDs) or common epitopes on the structures of some native allergens comprised in the venom (SPILLNER; BLANK; JAKOB, 2014). Moreover, the immunotherapy with venom extracts could be associated to the occurrence of undesirable side-effects and potentially non-specific sensitizations (INCORVAIA et al., 2011).

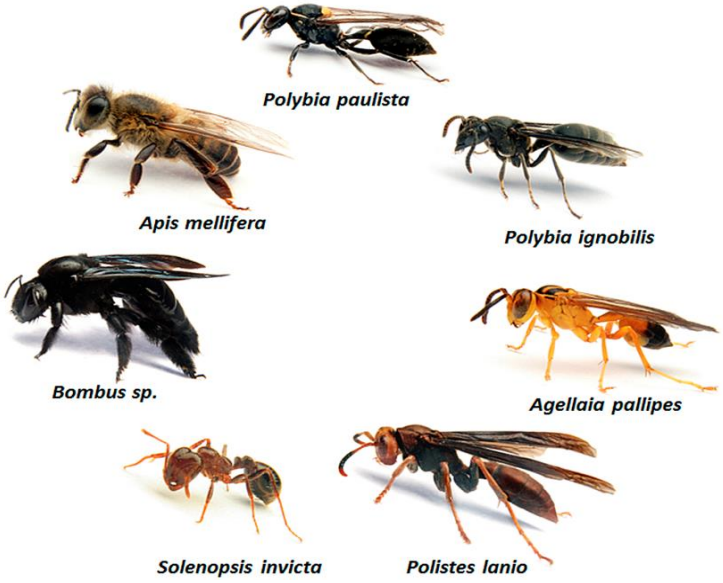

Figure 1. Clinically relevant species of social Hymenoptera from Brazil. (**Photos provided by Professor M.S. Palma**)

Recombinant allergens represent a valuable alternative for specific immunodetection of venom-elicited IgE in allergic patients (SPILLNER; BLANK; JAKOB, 2014). Unlike procedures based on the use of whole venoms, this alternative allowed the diagnosis using defined panels of CCD-depleted allergens thus limiting the incidence of cross-reactivity (EBERLEIN et al., 2012; MÜLLER et al., 2012). To date, dozens of Hymenoptera venom allergens, most from species of temperate regions, have been identified, heterologously expressed and are under evaluation for molecular diagnosis of allergy (SPILLNER; BLANK; JAKOB, 2014). In contrast, significantly limited information related to the expression and molecular characterization of allergens from Neotropical insects is currently available, hampering the specific diagnosis of allergy to endemic species (PEREZ-RIVEROL et al., 2017).

Phospholipases A1 (PLA1s) are major allergens identified in several wasp and ant venoms (HOFFMAN, 1985; KING et al., 1996; HOFFMAN; SAKELL; SCHMIDT, 2005; SANTOS et al., 2007; SUKPRASERT et al., 2013; HOU et al., 2016). Insect venom PLA1s are important marker for diagnosis and have been extensively used to distinguish true double sensitizations to wasp and honey bee venoms from cross-reactivity (EBO et al., 2012; MONSALVE et al., 2012; SEISMANN et al., 2010a; STURM et al., 2010). The native phospholipase A1 from *P. paulista* venom (nPoly p 1) is a non-glycosylated protein of ~34 kDa that showed direct hemolytic activity against red blood-cells (SANTOS et al., 2007). Recently, a recombinant form of the allergen was produced in *E. coli* cells (PEREZ-RIVEROL et al., 2016)**.** The heterologous allergen reacted with sIgE in the sera from allergic patients. Further analyses of rPoly p 1 are needed envisioning the development of allergen-based molecular diagnosis.

Here, we analyzed the cross-reactivity incidence associated to the use of rPoly p1 as an important prerequisite for development of CRD of *P. paulista* venom allergy. To the best of our knowledge, the venom PLA1s-based cross-reactivity among several species from Neotropical and temperate regions is reported for the first time. Structural analyses suggest that the cross-reactivity correlates with the levels of

homology on primary and 3-D structures of the wasp PLA1s. Moreover, the use of rPoly p 1 prevented the incidence of cross-reactivity with sera from individuals with sIgE to *S. invicta* (fire ants) and *A. mellifera* (honey bee) venoms. The results obtained here showed that rPoly p 1 a feasible marker for differential detection of sIgE to wasp venoms. In contrast, rPoly p 1 could be efficiently used for differentiation of wasp from ant/honey bee sensitizations during molecular diagnosis of P. paulista venom allergy.

2 Materials and Methods

2.1 Allergic patients' sera

Sera from allergic patients with sIgE to *P. paulista* (n=4), *A. mellifera* (n=12) or *S.invicta* (n=4) venoms previously diagnosed by ImmunoCAP 250 (Phadia) or ELISA. regardless sex or age, were obtained from the Ambulatório de Anafilaxia of the Hospital das Clínicas, Faculdade de Ciências Médicas, Universidade Estadual de Campinas-UNICAMP. Two sera from non-sensitized healthy volunteers were used as negative controls. The study was approved by the Ethics Committee of FCM-UNICAMP under n° 187/2006. Informed consent was obtained in written form from all participants of the study, and participation was voluntary.

2.2 Insect venoms and allergens

Nests from wasp species (*P. paulista, P. ignobilis, P. scutellaris, P. lanio, A. pallens and A. pallipes*) were captured around or within the campus of Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Rio Claro, SP, Brazil (Licensed by Sisbio, No. 58500). *A. mellifera* individuals were obtained from colonies kept in the vivarium of UNESP's Rio Claro Institute of Biosciences. The venom extracts along with the native and recombinant forms of Poly p 1 were obtained using the protocol described previously (PEREZ-RIVEROL et al., 2016). Commercial forms of the native phospholipase A2 (Api m 1) and mellitin (Api m 4) (Sigma, US) were used in cross-reactivity analyses. The venom extract from *S. invicta* was kindly provided by Prof. R.L. Zollner from the stock at the Laboratory of Translational Immunology (UNICAMP). The venoms and PLA1s from the European species *P. dominula* (Pol d 1) and *V. vulgaris* (Ves v 1) were kindly provided by Euroimmun (Lübeck, Germany).

2.3 Mice sensitization

Five Balb/c mice (female, 2 weeks) were immunized intradermically with six doses of 20 µg of rPoly p 1 in PBS/Al(OH)$_3$ weekly. Three mice immunized with 20 µg of solubilized proteins from *E. coli* BL21 (DE3) transformed with an empty pET-28a vector served as negative controls.

2.4 Immunoblotting and ELISA

The sIgG and sIgE-mediated recognition of venoms and allergens by patients' sera and/or by sera from rPoly p 1-sensitized mice were assessed by immunoblotting and/or ELISA using protocols previously described (PEREZ-RIVEROL et al., 2016; SCHIENER et al., 2017). A detailed description of these protocols is provided in the Supplementary data (Data S1). The data analysis and the standard deviation calculation relied on Microsoft Excel, version 14.0 (Microsoft, US).

2.5 Molecular modelling

The 3-D models of vespid PLA1s were generated by MODELLER (MARTÍ-RENOM et al., 2000) using the crystal structure of the venom phospholipase A1 from *Vespa basalis* (PDB ID:4QNN) as general template. The model was subjected to energy minimization using YASARA software (KRIEGER et al., 2009) and then validated using MolProbity (CHEN et al., 2010) The ribbon diagram was prepared with PyMOL (http://www.pymol.org) using the homology models as input.

3 RESULTS

3.1 Recombinant Poly p 1- mediated sensitization of mice

The capability of rPoly p 1 to induce *in vivo* sensitization of mice (n=5) after intradermic immunization was tested by Western blot. The analyses for detection of sIgE showed that all sera reacted with nPoly p 1 (Figure 2a) and with *P. paulista* venom (Figure 2b). The reaction was allergen-specific as a unique band corresponding to the predicted molecular weight of Poly p 1 (~34 kDa) was obtained on immunoblotting with purified nPoly p 1 and with the positive control (rPoly p 1) (Figure 2d). In the case of IgE-mediated immudetection of the crude venom, a

second and less-intense band (~25 kDa) was observed (Figure 2b). As this molecular weight is similar to that reported for Poly p 5 (DOS SANTOS-PINTO et al., 2014), another major allergen from *P. paulista* venom, we tested a pool of sera from rPoly p 1-sensitized mice against the purified native Poly p 5 (50 µg). No reaction was observed (Figure 2c).

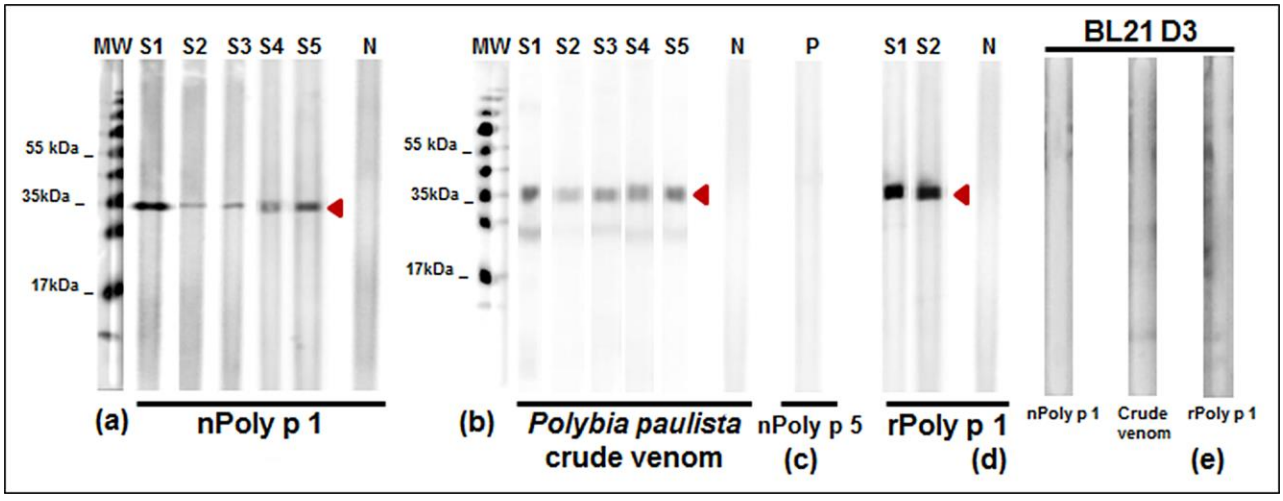

Figure 2. IgE-mediated immune reactivity of sera from rPoly p 1-sensitized mice against (a) nPoly p 1, (b) *P. paulista* crude venom, (c) rPoly p 5 (c) and rPoly p 1 (d). Samples: S1-S5= mouse individual serum; P= pool of mice sera (n=5); N=pool of sera from mice (n=3) immunized with *E.coli* BL21 (DE3) cells lysate (negative control). The molecular weight marker (kDa) is indicated.

3.2 Cross-reactivity of sera from rPoly p 1-sensitized mice

The sera from sensitized mice did not cross-react neither with allergens (Api m 1, Api m 4) nor crude venom from *A. mellifera* (Figura 3a). Also, no reaction was observed with crude venom of *S. invicta* (Figure 3b). The ELISA analyses for detection of cross-reactive sIgE/sIgG to these allergenic materials also rendered negative results (Figure 5).

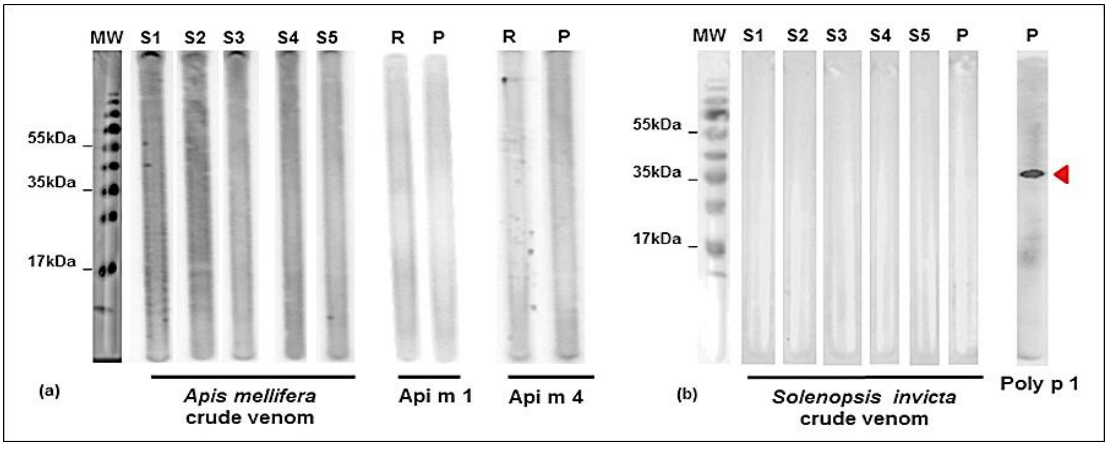

Figure 3. Analysis of IgE-mediated cross-reactivity of sera from rPoly p 1-sensitized mice with *A. mellifera* venom/Api m 1, Api m 4 and *S. invicta* crude venom (b). Samples: S1-S5= mouse individual serum; P= pool of mice sera (n=5); R=randomly selected serum. The molecular weight marker (kDa) is indicated.

In contrast, the IgE and IgG elicited in mice after rPoly p 1-mediated sensitization showed different levels of cross-reactivity among venoms of clinically relevant wasps from Neotropical (Figure 4a) and temperate regions (Figure 4b). The highest levels of cross-reactivity were obtained with the venoms of the more taxonomically related species *P. scutellaris* and *P. ignobilis*. A significant reaction was also detected with venoms from Neotropical species of different genera such as *P. lanio*, *A. pallipes*, *A. pallens* and with venom from *P. dominula,* a clinically relevant wasp from Northern Hemisphere (*P. dominula*) (Figure 4b). In contrast, no reaction was detected with *V. vulgaris* venom.

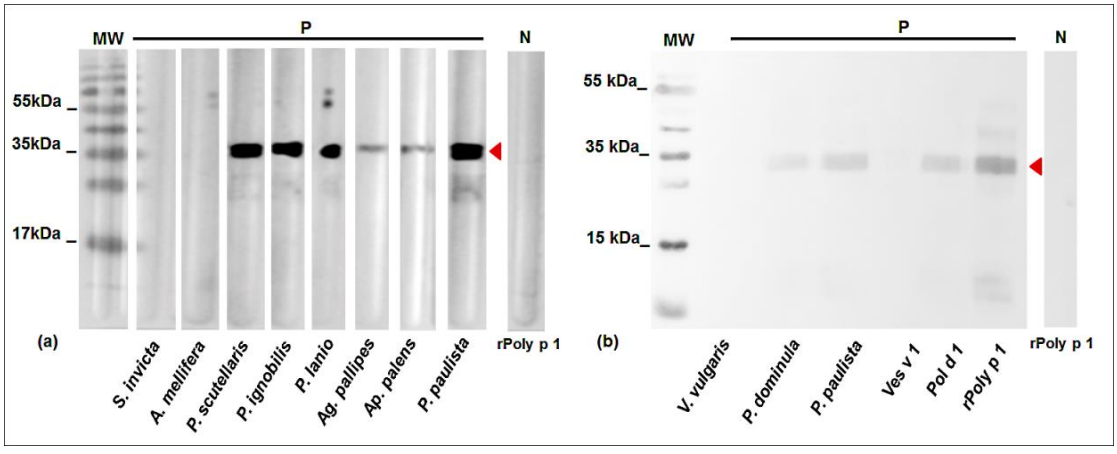

Figure 4. Venom phospholipases A1 based cross-reactivity among Hymenoptera from Neotropical (a) and temperate regions (b) analyzed by Western blot. Samples: P= pool of

sera from rPoly p 1-sensitized mice (n=5). N=pool of sera from mice (n=3) immunized with *E.coli* BL21 (DE3) cells lysate (negative control).

ELISA analyses also showed the incidence of cross-reactivity among venoms from Neotropical and temperate regions (Figure 5). The results were similar to those obtained in the immunoblotting with the higher signals corresponding to the close-related species *P. scutellaris* and *P. ignobilis*. Interestingly, high levels of cross-reactivity were also detected for *Polistes* venoms, with *P. dominula* showing a slightly higher signal than *P. lanio* among all individual serum. Also matching with the immublotting analysis, no reaction was detected with *A. mellifera, S. invicta, V. vulgaris* venoms or rVes v 1. The Figure 5 showed the results for cross-reactive sIgE and IgG detection from a representative mouse serum. The complete set of results with all individual serum is provided in Supplementary Data (S2, Figures 1 and 2).

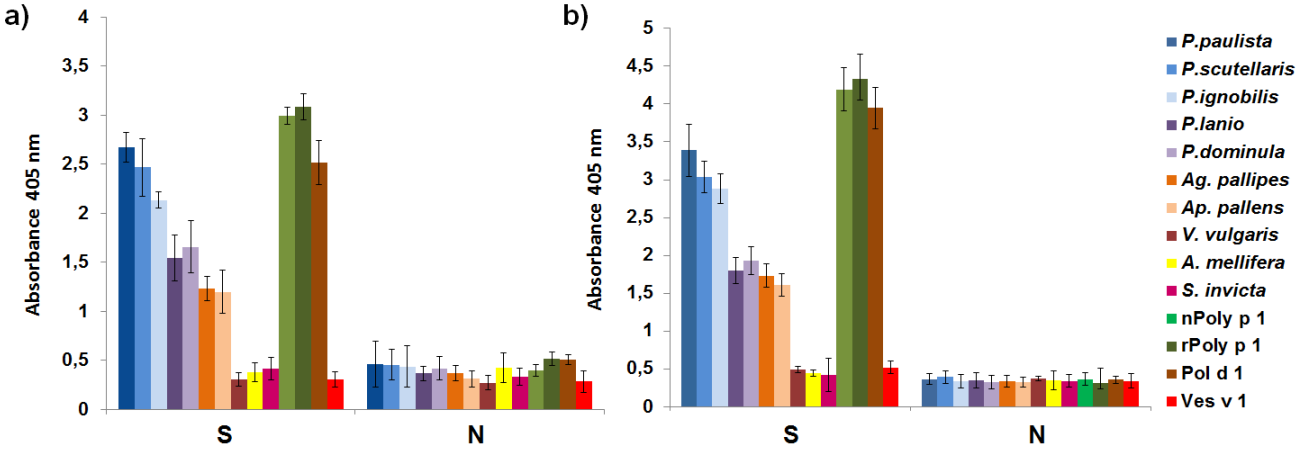

Figure 5. Detection of cross-reactive sIgE (a) and sIgG (b) in the sera from rPoly p 1-sensitized mice measured by ELISA. S: mouse serum with the highest levels of anti-rPoly p1 antibodies. N= negative control (serum from mouse immunized with a cell lysate of *E.coli* BL21 (DE3) transformed with an empty pET-28a plasmid). The results represent the media of three replicates of the experiment.

Wasp venom phospholipases A1 are non-glycosylated and thus CCD-free allergens (SEISMANN et al., 2010a). Consequently, the PLA1-based cross-reactivity detected here should be related to the presence of common epitopes among the different variants of the allergen evaluated. To address this possibility and similar to a previous report with wasp venom antigens 5 (SCHIENER et al., 2017), we analyzed

the identity on the primary sequence and 3-D models of the venoms PLA1s from some of the species included in our study.

As no crystal structures from the PLA1s evaluated here are available, the 3-D models of rPoly p 1 (GenBank ID: ADT89774.1), nPoly p 1 (GenBank ID: A2VBC4.1), Pol d 1 (GenBank ID: AAS67041.1), Ves v 1 (GenBank ID: AAB48072.1) and Sol i 1 (GenBank ID: NP_001291510) were built using the deposited pdb (PDB ID: 4QNN) of the venom PLA1 from the hornet *Vespula basalis*. The identity of the rPoly p 1 primary sequence with these PLA1s ranged from 36% (Sol i 1) to 74% (Pol d 1) (Figure 6). Meanwhile, in the structural analysis, the root-mean square deviation (RMSD) of the atomic positions of the 3-D models varied from 0.172 to 0.083, with Sol i 1 and Pol d 1 showing the lowest and highest identity, respectively. The results obtained in the multiple sequences alignment and the overlay of the 3-D models (Figure 6) matched with the results of the immunoblotting and ELISA analyses. Higher values of sequence and structural identities are associated with stronger IgE and IgG-mediated recognition of the corresponding PLA1s in the insect venoms. Unfortunately, there is no information available in DNA/protein databases related to PLA1s from *P. scutellaris*, *P. ignobilis*, *P. lanio*, *A. pallipes* and *A. pallens* venoms to conduct similar structural evaluations. The results obtained in the immunoblotting, ELISA and structural analyses are summarized in Table 1.

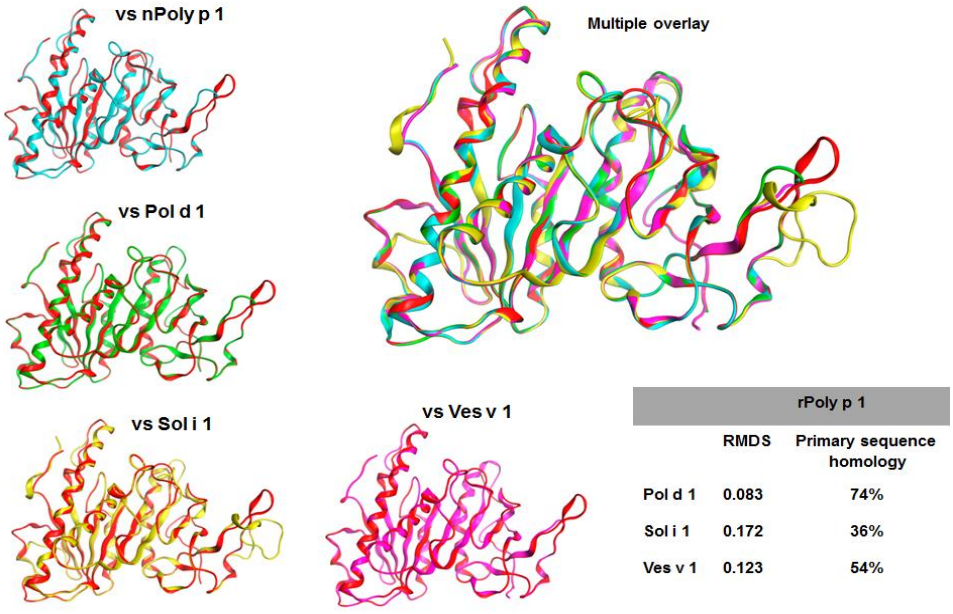

Figure 6. Overlapping of the 3-D models of some venom PLA1s from some of the species included in the study with the rPoly p 1. Samples: rPoly p 1 (red), nPol y p 1 (blue), Pol d 1 (green), Sol i 1 (yellow), Ves v 1 (purple). The primary sequence identity and the RMSD of atomic positions of the 3-D models from the venom PLA1s analyzed compared to rPoly p 1 is shown.

Table 1: Summary of the results obtained on the structural, immunonoblotting and ELISA analyses with PLA1s from venom of other clinically relevant wasps

	rPoly p 1			
Vespid PLA1s	**Sequence identity**	**RMSD**	**Relative intensity on immublotting reaction**	**ELISA (DO_{405}nm)[a]**
P. paulista	71%	0.080	+++	2.55
P. scutellaris	**n.a**		+++	2.41
P.ignobilis	**n.a**		+++	2.24
P. lanio	**n.a**		++	1.53
P.dominula (Pol d 1)	74%	0.083	++	1.79
A. pallipes	**n.a**		+	1.20
A. pallens	**n.a**		+	1.17
V. vulgaris (Ves v 1)	54%	0.123	-	0.65
S. invicta (Sol i 1)	36%	0.172	-	0.44
A. mellifera	**no venom PLA1**		-	0.39

n.a: no available in nucleotide/protein databases, (-): negative

[a] Data from the mouse serum with the highest sIgE levels to rPoly p 1

3.3 Cross-reactivity of sera from allergic patients

To reassess the ability of the rPoly p 1 to distinguish double sensitization to wasp/bee or wasp/ fire ant venoms from cross-reactivity, we evaluated the IgE-mediated recognition of rPoly p 1 and *P. paulista* crude venom by sera from patients monosensitized to HBV (12) or fire ants (6). All the sera from allergic patients cross-reacted with *P. paulista* crude venom (Figure 7a) while no recognition was observed with rPoly p 1 (Figure 7b).

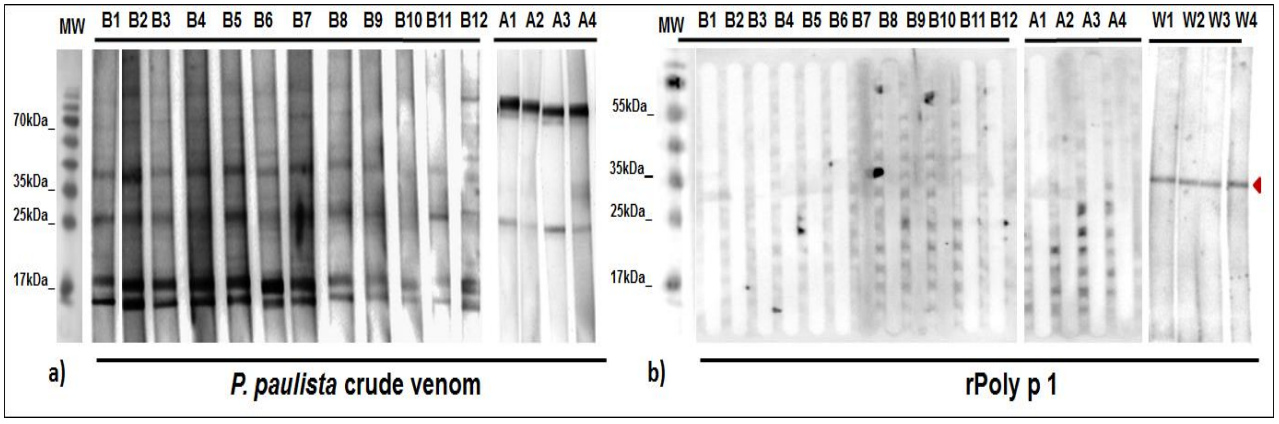

Figure 7. Cross-reactivity analyses of sera from Brazilian allergic patients previously diagnosed by ImmunoCAP 250. Samples: sera from patients with sIgE to honeybee venom (B1-B12), ant (A1- A4) or wasp (Control: W1-W4) venoms. The molecular weight marker (kDa) is indicated.

In the case of the immunorecognition of *P. paulista* venom by sera from patients previously diagnosed with single sIgE to HBV several bands were observed (Figure 6a). Four bands with a molecular weight of ~10kDa, ~17kDa, ~25kDa and ~40 kDa showed the highest intensity. The IgE-mediated immunodetection using sera from patients diagnosed with sIgE to fire ant venom showed a different pattern with two major bands (~25 kDa and ~100 kDa) detected. In summary, all sera from patients monosensitized to HBV or ant venoms cross reacted with *P. paulista* crude venom while none of the sera recognized rPoly p 1.

4 Discussion

Usually, positive results in skin tests or *in vitro* sIgE detection during venom allergy diagnosis do not reflect a clinically relevant sensitization (OLLERT; BLANK, 2015). Cross-reactivity associated to the presence of CCDs (; SEISMANN et al., 2010b; BREHLER; GRUNDMANN; STOCKER, 2013) or common epitopes from homologue allergens such as hyaluronidases (JIN et al., 2010), dipeptidyl peptidases (BLANK et al., 2010) and vitellogenins (BLANK et al., 2013b), often results in misidentification of the primary sensitizing insect. Wasp venom phospholipases A1 are naturally CCD-lacking allergens with no homologues in HBV (SEISMANN et al., 2010a). These features prevent the incidence of CCD-associated cross-reactivity turning this group of allergens in a feasible marker for differentiation of wasp and/or HBV allergy. However, little is known about the venom PLA1s-based cross-reactivity related to the presence of common epitopes among clinically relevant wasps or ants.

To date, a limited number of venom PLA1s have been expressed in prokaryotic and eukaryotic systems (SPILLNER; BLANK; JAKOB, 2014) with only Ves v 1 and Pol d 1 being used in routine diagnosis (OLLERT; BLANK, 2015). Insect venom PLA1s are often produced as insoluble proteins in *E. coli* (PEREZ-RIVEROL et al., 2016) and showed low levels of expression in eukaryotic cells systems (SEISMANN et al., 2010a; BORODINA et al., 2011). The difficulties in producing large amounts of soluble insect venom PLA1s partially hinder its uses in molecular allergy. However, considering the benefits of using this group of allergens as a reliable marker to avoid cross-reactivity in molecular diagnosis, we performed an extend cross-reactivity analysis of a recombinant form of the PLA1 from the *P. paulista* venom. To the best of our knowledge, rPoly p 1 is the only venom PLA1 from a Neotropical wasp obtained as a recombinant protein that is currently undergoing immunological evaluation (SANTOS ET AL., 2007; PEREZ-RIVEROL ET AL., 2017) envisioning its use in molecular diagnosis of allergy.

As an initial step to address the allergenicity and the extent of the rPoly p 1-based cross-reactivity, we immunized mice (5) with the recombinant allergen. Intradermic administration of rPoly p 1 resulted in the allergen-specific mice sensitization.

A predominant band with the expected molecular weight (~34kDa) was obtained for each individual serum incubated with the nPoly p 1 and the venom extract (Figure 2a, b). This recognition indicates that the recombinant protein retains the relevant IgE-epitopes from the native form of the allergen. The allergen-specific recognition has particular interest for the goal of develop molecular diagnosis and immunotherapy of *P. paulista* allergy as it could prevent the incidence of cross-reactivity and the inclusion of clinically irrelevant venoms in treatment.

A second band (~25kDa), significantly less intense, was observed in the immunodetection of *P. paulista* crude venom with sera from rPoly p 1-sensitized mice (Figure 2b). This molecular weight corresponds with that repored for the nPoly p 5 (DOS SANTOS-PINTO et al., 2014), one of the three major allergens of *P. paulista* venom. Poly p 1 and Poly p 5 have non-structural homology and are CCD-lacking proteins preventing the occurrence of common epitopes and/or CCD-related cross-reactivity. Nonetheless, to exclude the possibility of non-specific recognition of the allergen, we tested a pool of sera from rPoly p 1-sensitized mice against nPoly p 5. As expected, no reaction occurred (Figure 2c). It has been informed that the PLA1 from *P. paulista* venom occurs as a mix of different molecular forms with at least one having a molecular weight of ~25 kDa (DOS SANTOS et al., 2010, 2011). The finding of a second band in the immunoblotting analyses could be related to the recognition of this less predominant form of the PLA1 comprised in the *P. paulista* crude venom.

The sera from rPoly p1-sensitized mice did not cross-react with honey bee (Figure 3a) and fire ant venoms (Figure 3b). Also, no reaction occurred with two of the HBV major allergens (Api m 1 and Api m 4). These results reinforce the hypothesis that rPoly p 1 could be useful to avoid wasp/ant and wasp/bee cross-reactivity during diagnosis of allergy and consequently to asses true *P. paulista* venom sensitization (PEREZ-RIVEROL et al., 2016). Previously, venom PLA1s from wasp of temperate regions have proved to be useful for specific identification of culprit venom. The combined use of CCD-lacking variants of Ves v 1 and Ves v 5 expressed in insect cells enabled the reliable assignment of sensitization to yellow jacket venom in more than 90% of patients previously diagnosed with double sensitization to wasp and

HBV (SEISMANN et al., 2010a). Similar results were obtained in a study conducted using the recombinant forms of Pol d 1/Ves v 1 in combination with Pol d 5/Ves v 5 for identification of culprit venom in patients diagnosed with double sensitization to *P. dominula* and *V. vulgaris* (MONSALVE et al., 2012).

For the first time, we showed the incidence of cross-reactivity among the venom phospholipases A1 from insects of Neotropical and with species of temperate regions. The immunoblotting (Figure 4) and ELISA analyses (Figure 5) showed different levels of cross-reactivity that correlates with the homology in the primary and 3-D structures of the PLA1s from some of the species included in the study (Figure 6 and Table 1). While no PLA1 has been identified in HBV, Sol i 1 and Ves v 1 presented the lowest values of structural homologies with Poly p 1 and resulted in no recognition by mice sera. In contrast, the high levels of identity detected in Pol d 1 (71%) were associated with a high signal on cross-reactive sIgE and IgG immunodetection analyses (Figure 4b and 5). Unfortunately, despite being clinically relevant wasps, no nucleotide or protein sequences of venom PLA1s from the Neotropical species tested are currently available, hampering the analysis of the structural homologies with rPoly p 1.

Several venom PLA1s from *Vespula* spp. showed up to 95% of homology (MONSALVE et al., 2012). As suggested for this group, the extensive cross-reactivity obtained for *Polybia* species could be due to high levels of identity on primary sequences and 3-D structures. At least for *P. scutellaris*, it has been shown that the sequence of antigen 5 (Poly s 5) has a 99 % of identity with its counterpart in *P. paulista* venom (VINZÓN et al., 2010, 2012). A similar behavior could be expected for PLA1 from *P. scutellaris* and *P. ignobilis*. The alignment of the primary sequence and 3-D model of the venom PLA1 from *P. dominula*, which is the major European counterpart of the Neotropical wasp *P. lanio* showed a high identity with Poly p 1 (Figure 5). Interestingly, the levels of cross-reactivity with mice sera were similar for *P. dominula* and its Brazilian counterpart, suggesting that venom PLA1 from *P. lanio* could also have high levels of identity with Poly p 1, and retains homologues linear and conformational IgE-epitopes present in Poly p 1 and Pol d 1.

Similar to our results, a recently published analysis (SCHIENER et al., 2017) informed about the extensive antigen 5-based IgE cross-reactivity in seven clinically relevant Hymenoptera species. Considering that antigen 5 is also a CCD-free allergen, the authors also proposed that the high levels of cross-reactivity detected are related to the presence of shared B-cell epitopes in the homologue venom antigens 5. As in the case of antigens 5, our results showed that wasp PLA1s represent a reliable alternative to differentiates wasp/bee and wasp/ant allergy but should not be used as a unique marker to identify the wasp-specific specie responsible for the primary sensitization. However, the gradual behavior of the wasp venom PLA1-based cross-reactivity detected here suggests that this allergen could be successfully used to facilitated specie-specific sensitization in allergic patients.

Diagnosis based in the use of venom extracts is a common practice in Latin America and is often related with a misidentification of culprit insect. On immunoblotting analysis we showed that all sera from bee (12) and fire ant (4) monosensitized patients cross-reacted with *P. paulista* (Figure 7a).These results agree with the previous reports that has associated the use of crude venom with the occurrence of high levels of cross-reactivity during diagnosis (CARBALLADA et al., 2010; SEVERINO et al., 2010; BREHLER; GRUNDMANN; STOCKER, 2013;). Interestingly, no IgE-mediated immunodetection occurred in the similar analysis conducted with rPoly p 1 (Figure 7b). A unique band of ~34kDa was detected only in the immunoblotting conducted with sera from wasp monosensitized patients. These results reinforced the suggestion that rPoly p 1 represents a feasible marker for differentiation of clinically relevant wasp sensitizations from CCDs/common peptides-mediated cross-reactivity with bee and ant venoms in Brazilian allergic patients. However, it is important to note that further analyses such as basophil activation test (BAT) (BALZER et al., 2014) involving a large number of sera are require in order to extends these preliminary results and finally develop rPoly p 1-based molecular diagnosis of *P. paulista* allergy.

5 Concluding remarks

To date, no recombinant allergens from venom of *P. paulista* or any other Neotropical wasp are available for molecular diagnosis of allergy. To overcome this situation and improves the specific identification of allergic patients we conducted an immunological characterization of a recombinant form Poly p 1, a major allergen from *P. paulista* venom. We assessed the incidence of cross-reactivity associated to the use of the recombinant allergen. For the first time we showed the incidence of extensive venom PLA1-related cross-reactivity among wasps from Neotropical and temperate regions. The levels of cross-reactivity correlate with the homology on the primary sequence and 3-D models thus suggesting the presence of common epitopes among venom PLA1s in *Polybia* species and wasp from different Genera and geographical regions. These results could be significantly important for the design of molecular diagnosis to elucidate sensitization to wasp venoms. In contrast to the results obtained with wasps, rPoly p 1 prevents the incidence of cross-reactivity with sera from *A. mellifera* and *S. invicta* monosensitized individuals. Thus, rPoly p 1 is a valuable tool for discriminate the occurrence of clinically relevant wasp/bee and wasp/ant sensitizations from cross-reactivity. Overall, the uses rPoly p1 could result in a remarkable improvement in diagnosis of Brazilian allergic patients finally allowing the selection of the appropriate venom for specific immunotherapy.

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[a] All the references cited in the manuscript are included in the References section of this Thesis

[b] The Supplementary data (Data S1) related to this manuscript is provided in the Appendix section of the Thesis

7. CONCLUDING REMARKS

The specific diagnosis of *P. paulista* venom allergy, a clinically relevant from Brazil, is hampered by the absence of recombinant allergens obtained from this wasp. No individual allergenic proteins are currently available for CRD. To overcome this, the phospholipase A1 from *P. paulista* venom (Poly p 1) was cloned and successfully produced in *E. coli* cells (1.5 g/L). The recombinant protein retains sIgE epitopes from the native form (nPoly p 1) of the allergen, suggesting that it is a potential candidate for development of molecular diagnosis. The comprenhesive immunological characterization performed in this work showed that the rPoly p 1 enables the differentiation of wasp/bee and wasp/ant venoms double sensitization from cross-reactivity and could be used for specie-specific identification of the culprit insect. The incidence of venom phospholipases A1-based cross-reactivity among Neotropical wasps and with wasps of the Northern Hemisphere was showed for the first time. This result could have major implications in the design of strategies for specie-specific identification during molecular diagnosis of insect venom allergy. The molecular basis for the wasp venom PLA1-based cross-reactivity was elucidated. Furthermore, for the first time the absence of CCDs in venoms from Neotropical wasps was informed suggesting that crude venoms from these clinically relevant insects could be used in diagnosis without the interference of CCD-specific IgE. This result suggests that CCD-devoid allergens are typical features for members of the Polistinae Sub-family of wasps. Overall, this Thesis provides important data that will improve specific diagnosis of wasp venom allergy, specially, but not only, in Neotropical regions and showed that the rPoly p 1 could be used in routine diagnosis of *P. paulista* venom allergy.

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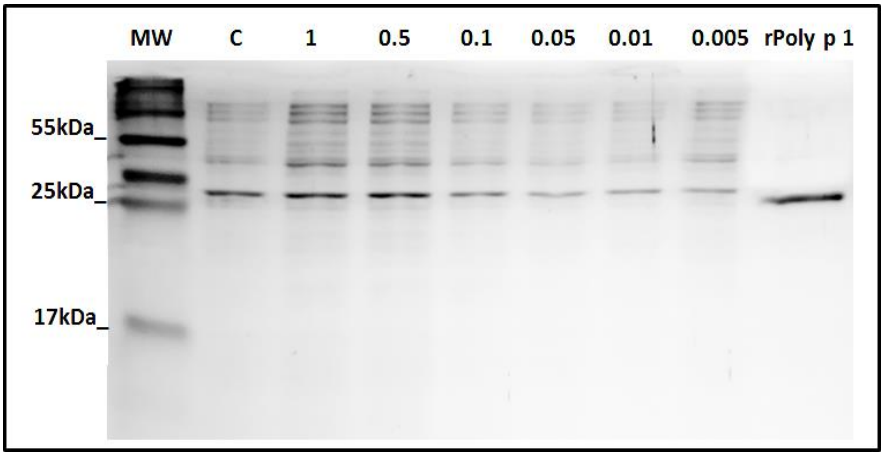
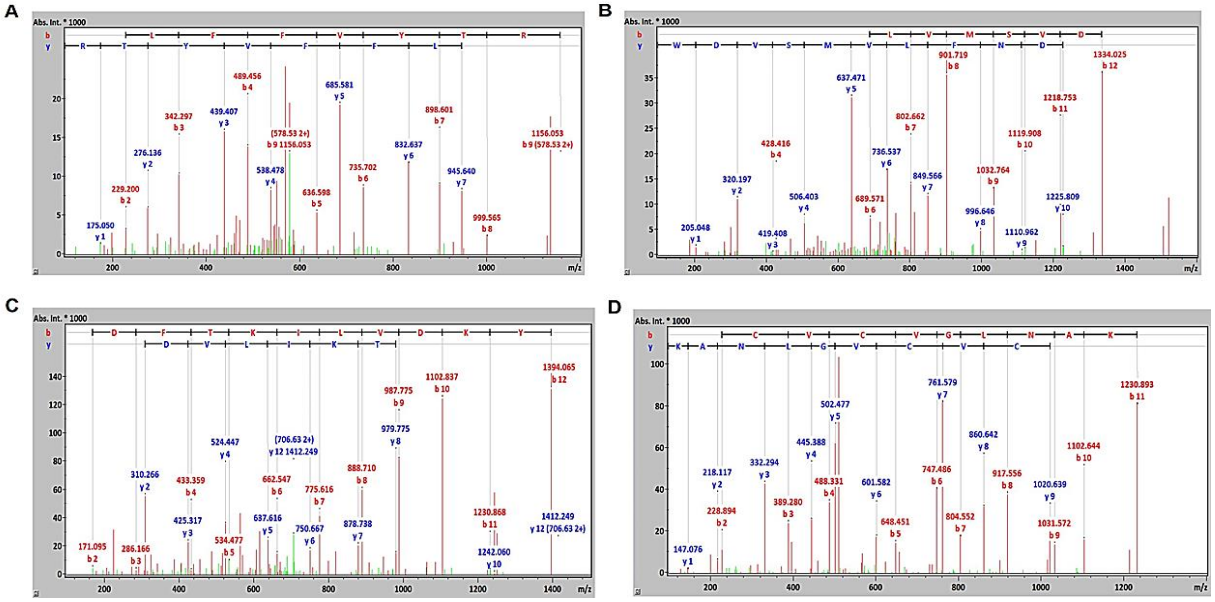
9. APPENDIX

9.1 Supplementary data from CHAPTER 3.

Manuscript 4- Improved production of the recombinant phospholipase A1 from *P. paulista* wasp venom expressed in *E.coli*



Data S1 Figure 1 (12%) SDS-PAGE analyses of the cell lysate soluble fractions from *E. coli* BL21 (DE3) obtained after rPoly p 1 expression (16°C, 10 h post-induction). Samples: N= negative control (non-transformed *E. coli* BL21 (DE3) induced with 1 mM IPTG, C= positive control (rPoly p 1 expression under previously condition tested (PEREZ-RIVEROL et al., 2016)) and 1-0.005= transformed *E. coli* BL21 (DE3) cells after induction with 1, 0.5, 0.1, 0.05, 0.01 and 0.005 mM IPTG.



Data S1 Figure 2. Representative mass spectra of recombinant phospholipase A1 from *P. paulista* venom. (A) CID spectrum of Asp-N protease peptide Y.DILFFVYTR.D (12-20) selecting the m/z 587.397 $[M + 2H]^{2+}$ as precursor ion; (B) CID spectrum of chymotryptic

peptide L.IAKDNFLVMSVDW.R (72-84) selecting the m/z 769.465 $[M + 2H]^{2+}$ as precursor ion; (C) CID spectrum of chymotryptic peptide Y.VADFTKILVDKY.K (113-124) selecting

Table S1 Table 1: Amino acid sequence of recombinant phospholipase A1 from the venom of the social wasp *P.paulista* obtained after *in-gel* protein digestion with different proteolytic enzymes. Enzyme used, amino acid position, m/z value observed (Z), number of missed cleavage sites, peptide sequences and MASCOT ion score were listed for all identified peptide.

Enzymes	Amino acid Position	m/z value observed (Z)	Missed cleavage	Peptide Sequences	Ions Score
Trypsin	31-47	1030.517 (+2)	0	K.NNLTSYNLFQQPQISHR.V	65
Trypsin	48-69	784.803 (+3)	0	R.VVFLIHGFISTGNNGNFDAMAK.A	62
Trypsin	75-85	691.379 (+2)	0	K.DNFLVMSVDWR.R	79
Trypsin	87-101	780.400 (+2)	0	R.AACDASVFNVAGYSK.A	91
Trypsin	102-111	536.625 (+2)	2	K.AVANTRRVGK.Y	85
Trypsin	112-118	422.314 (+2)	0	K.YVADFTK.I	24
Trypsin	124-132	554.382 (+2)	1	K.YKVPMSNIR.L	22
Trypsin	133-148	518.358 (+3)	0	R.LIGHSLGAHTSGFAGK.Q	41
Trypsin	158-172	811.445 (+2)	0	K.YSEIIGLDPAGPSFR.S	91
Trypsin	173-196	937.127 (+3)	1	R.STNCPDRLCETDAQYVQVLHTSSK.L	29
Trypsin	180-196	989.987 (+2)	0	R.LCETDAQYVQVLHTSSK.L	73
Trypsin	197-222	955.826 (+3)	0	K.LGTYDQIGSVDFYVNYGGSQPGCLVR.T	80
Trypsin	223-228	675.760 (+1)	0	R.TCSHTK.A	62
Trypsin	229-238	614.386 (+2)	0	K.AVLYMTECIK.R	70
Trypsin	229-239	692.402 (+2)	1	K.AVLYMTECIKR.E	35
Trypsin	266-276	624.900 (+2)	0	R.DICVCVGLNAK.S	81
Trypsin	277-281	564.640 (+1)	0	K.SYPAK.G	58
Trypsin	282-290	513.390 (+2)	0	K.GSFYVPVEK.N	50
Trypsin	291-302	716.426 (+2)	1	K.NSPYCHNEGIKL.-	27
Chymotrypsin	19-28	579.860 (+2)	0	Y.TRDKQDGIIL.K	31
Chymotrypsin	51-64	745.965 (+2)	2	F.LIHGFISTGNNGNF.D	33
Chymotrypsin	51-71	731.430 (+3)	3	F.LIHGFISTGNNGNFDAMAKAL.I	22
Chymotrypsin	56-71	812.939 (+2)	1	F.ISTGNNGNFDAMAKAL.I	23
Chymotrypsin	72-84	769.465 (+2)	2	L.IAKDNFLVMSVDW.R	58
Chymotrypsin	78-84	849.400 (+1)	1	F.LVMSVDW.R	21
Chymotrypsin	113-120	453.894 (+2)	1	Y.VADFTKIL.V	58
Chymotrypsin	113-124	706.479 (+2)	2	Y.VADFTKILVDKY.K	53
Chymotrypsin	154-171	631.423 (+3)	3	L.KLGKYSEIIGLDPAGPSF.R	31
Chymotrypsin	159-171	1302.606 (+1)	1	Y.SEIIGLDPAGPSF.R	62
Chymotrypsin	159-187	1090.799 (+3)	3	Y.SEIIGLDPAGPSFRSTNCPDRLCETDAQY.V	17
Chymotrypsin	192-209	673.455 (+3)	3	L.HTSSKLGTYDQIGSVDFY.V	54
Chymotrypsin	198-209	682.885 (+2)	2	L.GTYDQIGSVDFY.V	60
Chymotrypsin	201-209	1043.472 (+1)	1	Y.DQIGSVDFY.V	30
Chymotrypsin	201-212	710.384 (+2)	2	Y.DQIGSVDFYVNY.G	52
Chymotrypsin	233-248	685.364 (+3)	1	Y.MTECIKRECCLIGTPW.S	19
Glu-C/V8 protese	66-75	524.473 (+2)	0	D.AMAKALIAKD.N	19
Glu-C/V8 protese	161-182	825.768 (+3)	1	E.IIGLDPAGPSFRSTNCPDRLCE.T	55
Glu-C/V8 protese	183-207	909.490 (+3)	2	E.TDAQYVQVLHTSSKLGTYDQIGSVD.F	20
Asp-N protease	12-20	587.397 (+2)	0	Y.DILFFVYTR.D	66
Asp-N protease	75-82	924.489 (+1)	0	K.DNFLVMSV.D	39
Asp-N protease	115-121	418.335 (+2)	0	A.DFTKILV.D	44
Asp-N protease	178-200	895.476 (+3)	2	P.DRLCETDAQYV	33
Asp-N protease	184-200	637.435 (+3)	0	T.DAQYVQVLHTSSKLGTY.D	69
Asp-N protease	240-265	968.793 (+3)	0	R.ECCLIGTPWSSTGSSPKPKPISACKR.D	22

9.2 Supplementary data from CHAPTER 4.

Manuscript 5- Venoms from several clinically relevant Neotropical wasps are devoid of cross-reacted carbohydrate determinants

S1

S1.1 Immunoblotting analyses

For the analyses of the general glycosylation pattern and CCDs presence by Western blot, the venoms were submitted to (12%) SDS-PAGE and then transferred to a 0.22 mm nitrocellulose membrane using a semi-dry system (Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell, Bio-Rad, US). For HRP detection the membranes were blocked 30 min at room temperature with 4% nonfat dry milk powder (AppliChem, Darmstadt, Germany) in TBS buffer (20 mM Tris-HCl 150 mM NaCl; pH 7.4). The membrane was then incubated overnight (4 °C, 150 rpm) with rabbit anti-HRP serum (1:5000) diluted in TBS buffer with 2% non-fat dried milk. After washing (3 times with TBS-T: TBS buffer containing 0.5 % Tween) the membrane was incubated (1 hr, room temperature and gently shaking) with a goat anti-rabbit IgG (Sigma, UK) (1:20 000) conjugated with alkaline phosphatase. The bands were visualized using BCIP/NBT Color Development Substrate (Sigma, Denmark).

For the detection of general glycosylation, the membranes containing the venoms were blocked using a Blocking Solution (Vector Labs, US) (1 hr, at room temperature). After washing with TBS-T, Concanavalin A (Vector Labs, US) (2µg/mL) was added and followed by incubation for 30 minutes at room temperature. The membranes were washed and then incubated with a Streptavidin (1:70 000) (Sigma, Uk) conjugated to alkaline phosphatase (30 min, room temperature, gently shaking). Similarly, the bands were visualized using BCIP/NBT Color Development Substrate (Sigma, Denmark).

S1.2 ELISA

For detection of CCDs by the rabbit anti-HRP serum and sIgE in patient sera, 384-well microtiter plates (Nunc, Thermo Fisher Scientific, Ulm, Germany) were coated with 20 ng of the venoms (4 °C, overnight). Coated plates were then blocked with 4% nonfat dry milk powder (AppliChem, Darmstadt, Germany) in TBS at room temperature for 1h. After washing with TBS, rabbit anti-HRP serum (1:5000) and patient sera (1:2) (diluted in TBS and 2% nonfat dry milk powder) were added to the plates and incubated overnight at 4 °C. For HRP detection, a goat anti-rabbit IgG (1:20 000) (Sigma aldrich, UK) conjugated to alkaline phosphatase was added. For detection of CCDs by patients sera a goat anti-human IgE (1:20 000) (Biozol, Germany) was added, followed by incubation with a rat anti-goat IgG (1:20 000) (Sigma, UK) also conjugated to alkaline phosphatase (1 hour, gently shacking at room temperature). Detection was performed by adding 50 µL per well of the substrate solution (5 mg/mL 4-nitrophenylphosphate; AppliChem, Darmstadt, Germany). The absorbance was read at 405 nm. A

signal that duplicates the means of the negative control was interpreted as possitive results. Three independent expreriments for each type of antibodies were conducted.

S1.3 Polyacrilamide gel electrophoresis and protein quantification

SDS-PAGE was performed according to Laemmli (1970) (LAEMMLI, 1970), using a Mini-Protean® Tetra Cell System (BioRad). After running, the gels were stained with Coomassie Brilliant Blue R-250 (CBB). Protein fractions and purified natural or recombinant allergens were quantified using the modified Bradford method and bovine serum albumin (Sigma, US) as a standard (SEDMAK; GROSSBERG, 1977).

9.3 Supplementary data from CHAPTER 4.

Manuscript 6- Cross-reactivity analyses of the recombinant phospholipase A1 from *Polybia paulista* wasp venom

S1 Materials and Methdos

S1.1 Immunoblotting analyses

For the analyses of the PLA1s-mediated cross-reactivity among Neotropical species, proteins were submitted to (12%) SDS-PAGE and then transferred to a 0.22 mm nitrocellulose membrane using a semi-dry system (Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell, Bio-Rad, US). Prior to the addition of sera from patients' or from rPoly p 1-sensitized mice, membranes were blocked 2 hour at room temperature with 4% non-fat dried milk in TBS buffer (20 mM Tris-HCl 150 mM NaCl; pH 7.4). After washing (3 times with TBS-T: TBS buffer containing 0.5 % Tween), the membrane with the different allergenic materials were incubate overnight (4 °C, 150 rpm) with patients' (1:50) or a pool of mice (1:200) sera diluted in TBS buffer with 2% non-fat dried milk. For the analyses with patient sera the immunodetection was performed using an anti-human IgE (Ɛ-chain specific) peroxidase conjugate antibody (Sigma Aldrich, US) diluted at 1:5000 (TBS and 2% non-fat dried milk). For mice sera, the membrane was washed and then incubated with a rat anti-mouse IgE (BD Biosciences, Brazil) (1:500) for 2 hours at room temperature followed by incubation with a goat anti rat IgG (Sigma Aldrich, US) peroxidase conjugated. The bands were visualized in Image Quant 400 (GE Healthcare, Sweden) using the chemiluminescent substrate Luminata ™ Forte Western HRP substrate (Millipore, USA). In the case of immunoblott analyses with venoms and allergens from European species, after the overnight incubation with mice sera a goat anti-mouse IgE (icllab, US) (1:250) was added followed by incubation with a rabbit anti-goat IgG alkaline phosphatase conjugated (Sigma, Denmark) (1:20 000). The detection was performed using BCIP/NBT Color Development Substrate (Sigma, Denmark).

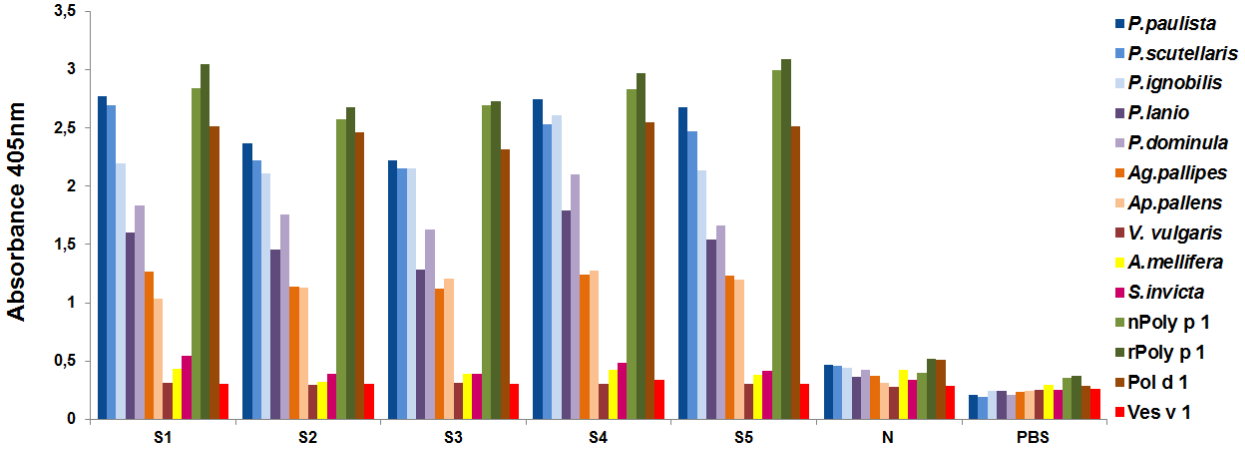
S1.2 ELISA

For ELISA analyses, 384-well microtiter plates (Nunc, Thermo Fisher Scientific, Ulm, Germany) were coated with 20 ng of venoms and purified allergens (4 °C, overnight). Coated plates were then blocked with 4% nonfat dry milk powder (AppliChem, Darmstadt, Germany) in TBS at room temperature for 1h. After washing with TBS, individual sera diluted 1:100 (TBS and 2% nonfat dry milk powder) were added to the plates and incubated overnight at 4 °C. After washing with TBS-T, either a goat anti-mouse IgG conjugated to alkaline phosphatase (1:30 000) or a goat anti-mouse IgE (icllab, US) (1:250) diluted in TBS and 2% nonfat dry milk powder were added and incubated by gently shaking, 1 or 2 hour at room temperature, respectively. For sIgE, a secondary rabbit anti-goat IgG (Sigma Aldrich, US) (1:20 000) conjugated to alkaline phosphatase was added (1 hour, gently shacking at room temperature). Detection was performed by adding 50 µL per well of the substrate solution (5 mg/mL 4-nitrophenylphosphate; AppliChem, Darmstadt, Germany). The absorbance was read at 405 nm. A signal that duplicates the means of the negative control was interpreted as possitive results. Three independent expreriments for each type of antibodies were conducted.

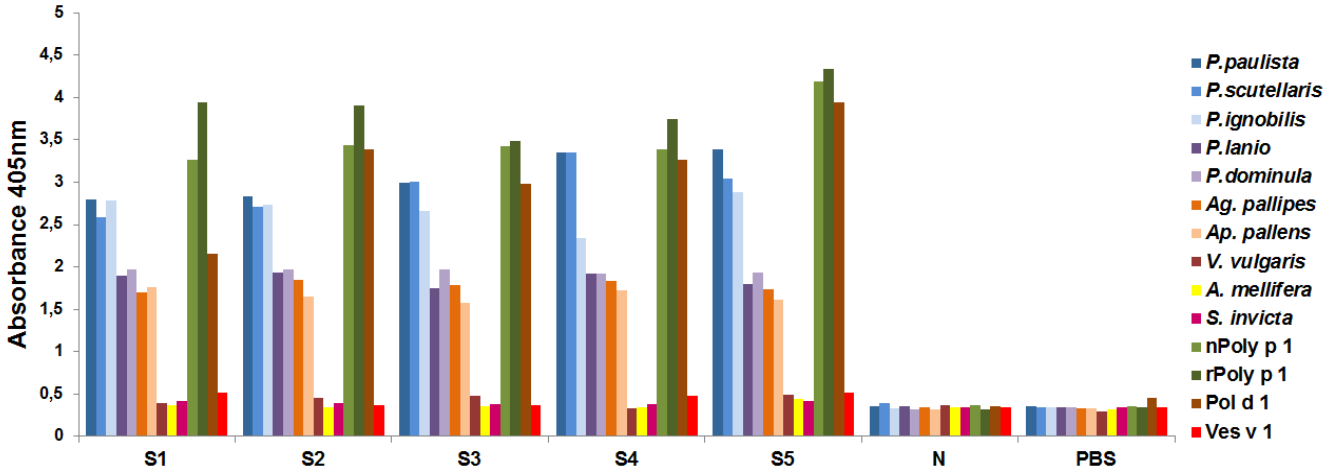
S1.3 Polyacrilamide gel electrophoresis and protein quantification

SDS-PAGE was performed according to Laemmli (1970) (LAEMMLI, 1970), using a Mini-Protean® Tetra Cell System (BioRad). After running, the gels were stained with Coomassie Brilliant Blue R-250 (CBB). Protein fractions and purified natural or recombinant allergens were quantified using the modified Bradford method and bovine serum albumin (Sigma, US) as a standard (SEDMAK; GROSSBERG, 1977).

S2 Results



Data S2 Figure 1. Detection of cross-reactive sIgE in the sera from rPoly p 1-sensitized mice measured by ELISA. S: individual mouse serum. N= negative control (serum from mouse immunized with a cell lysate of *E.coli* BL21 (DE3) transformed with an empty pET-28a plasmid). PBS: reagents control. The results represent the media of three replicates of the experiment.



Data S2 Figure 2. Detection of cross-reactive sIgG in the sera from rPoly p 1-sensitized mice measured by ELISA. S: individual mouse serum. N= negative control (serum from mouse immunized with a cell lysate of *E.coli* BL21 (DE3) transformed with an empty pET-28a plasmid). PBS: reagents control. The results represent the media of three replicates of the experiment.