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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).

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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

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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.

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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**)

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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.

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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