Toxicon 124 (2016) 44–52

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

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

Amilcar Perez-Riverol [a], Franco Dani Campos Pereira [a, b], Alexis Musacchio Lasa [c], Luis Gustavo Romani Fernandes [d], José Roberto Aparecido dos Santos-Pinto [e], Débora Lais Justo-Jacomini [a], Gabriel Oliveira de Azevedo [f], Murilo Luiz Bazon [a], Mario Sergio Palma [e], Ricardo de Lima Zollner [d], Márcia Regina Brochetto-Braga [a, g, *]

[a] *Laboratório de Biologia Molecular de Artrópodes-LBMA-IBRC-UNESP (Univ Estadual Paulista), Av. 24-A, nº 1515, CEP 13506-900, Bela Vista, Rio Claro, SP, Brazil*
[b] *Laboratório de Mutagênese Ambiental, Avenida 24-A, nº 1515, Bela Vista, Rio Claro, São Paulo, CEP 13506-900, Brazil*
[c] *Center for Genetic Engineering and Biotechnology, Biomedical Research Division, System Biology Department, Ave. 31, e/ 158 and 190, P.O. Box 6162, Cubanacan, Playa, Havana, 10600, Cuba*
[d] *Laboratório de Imunologia Translacional, Faculdade de Ciências Médicas, FCM, Universidade Estadual de Campinas-UNICAMP, Rua Vital Brasil, nº 300, CEP 13083-887, Cidade Universitária "Zeferino Vaz", Campinas, SP, Brazil*
[e] *Centro de Estudos de Insetos Sociais-CEIS-IBRC-UNESP (Univ Estadual Paulista), Av. 24-A, nº 1515, CEP 13506-900, Bela Vista, Rio Claro, SP, Brazil*
[f] *Instituto de Pesquisa em Bioenergia (IPBEN) (Univ Estadual Paulista), Av. 24-A, nº1515, CEP 13506-900, Bela Vista, Rio Claro, SP, Brazil*
[g] *Centro de Estudos de Venenos e Animais Peçonhentos-CEVAP (Univ Estadual Paulista), Rua José Barbosa de Barros, 1780, Fazenda Experimental Lageado, Botucatu 18610-307, SP, Brazil*

ARTICLE INFO

Article history:
Received 20 June 2016
Received in revised form
5 September 2016
Accepted 3 November 2016
Available online 5 November 2016

Keywords:
Polybia paulista
Venom
Allergy
Diagnosis
Recombinant phospholipase A1
Immunoglobulin E (IgE)

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 *P. 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.

* Corresponding author. Laboratório de Biologia Molecular de Artrópodes-LBMA-IBRC-UNESP (Univ Estadual Paulista), Av. 24-A, nº 1515, CEP 13506-900, Bela Vista, Rio Claro, SP, Brazil.

E-mail addresses: aperezriverol@gmail.com (A. Perez-Riverol), franko_mg@hotmail.com (F.D. Campos Pereira), alexis.musacchio@cigb.edu.cu (A. Musacchio Lasa), luisgrf1982@gmail.com (L.G. Romani Fernandes), jrbio04@rc.unesp.br (J.R.A. Santos-Pinto), dmjacomini@gmail.com (D.L. Justo-Jacomini), gabriel.azevedo4@outlook.com (G. Oliveira de Azevedo), bazonmurilo@gmail.com (M.L. Bazon), mspalma@rc.unesp.br (M.S. Palma), zollner@unicamp.br (R.L. Zollner), mrbbraga@rc.unesp.br (M.R. Brochetto-Braga).

http://dx.doi.org/10.1016/j.toxicon.2016.11.006
0041-0101/© 2016 Elsevier Ltd. All rights reserved.

(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.

© 2016 Elsevier Ltd. All rights reserved.

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%–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 dominulus* 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 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. 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 (*nPoly 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 n° 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 °C 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 °C), 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 °C. 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 μL of Trizol® (Life Technologies, USA), macerated, and then stored for 7 days at −80 °C. After RNA extraction, the first-strand cDNA synthesis was performed using 1 μg 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'CTT A 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 μg/mL spectinomycin 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)]} (Stratagene, 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)] (Novagen, 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 μg/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 μL 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 (16 × 10 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 μm 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 dominulus* (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

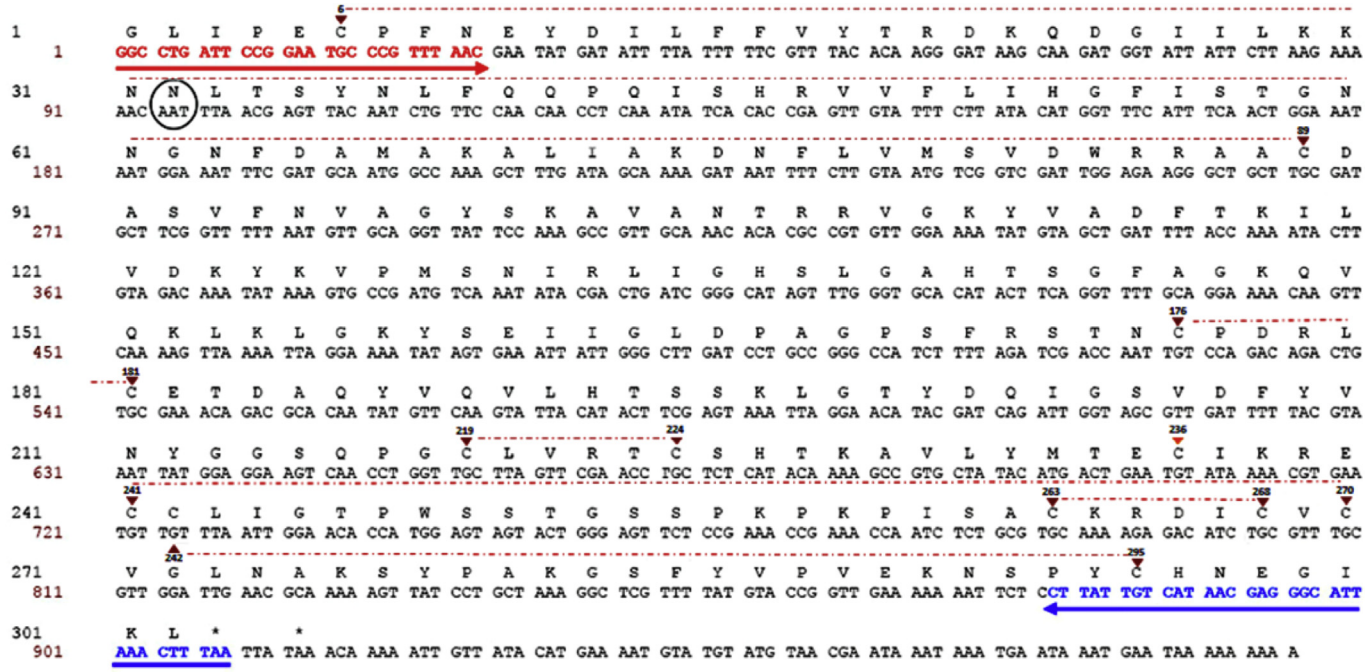

Fig. 1. cDNA and predicted amino acid sequence of the mature r*Poly p* 1 (Gen Bank: ADT89774.1). Forward (red arrow) and reverse (blue arrow) primers used for gene specific amplification are indicated. Black oval mark shows putative N-glycosylation site. Disulfide bridges and the cysteine residues involved 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 °C 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 (8–20%), 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 °C, 30 °C or 25 °C, 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 18–21) 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

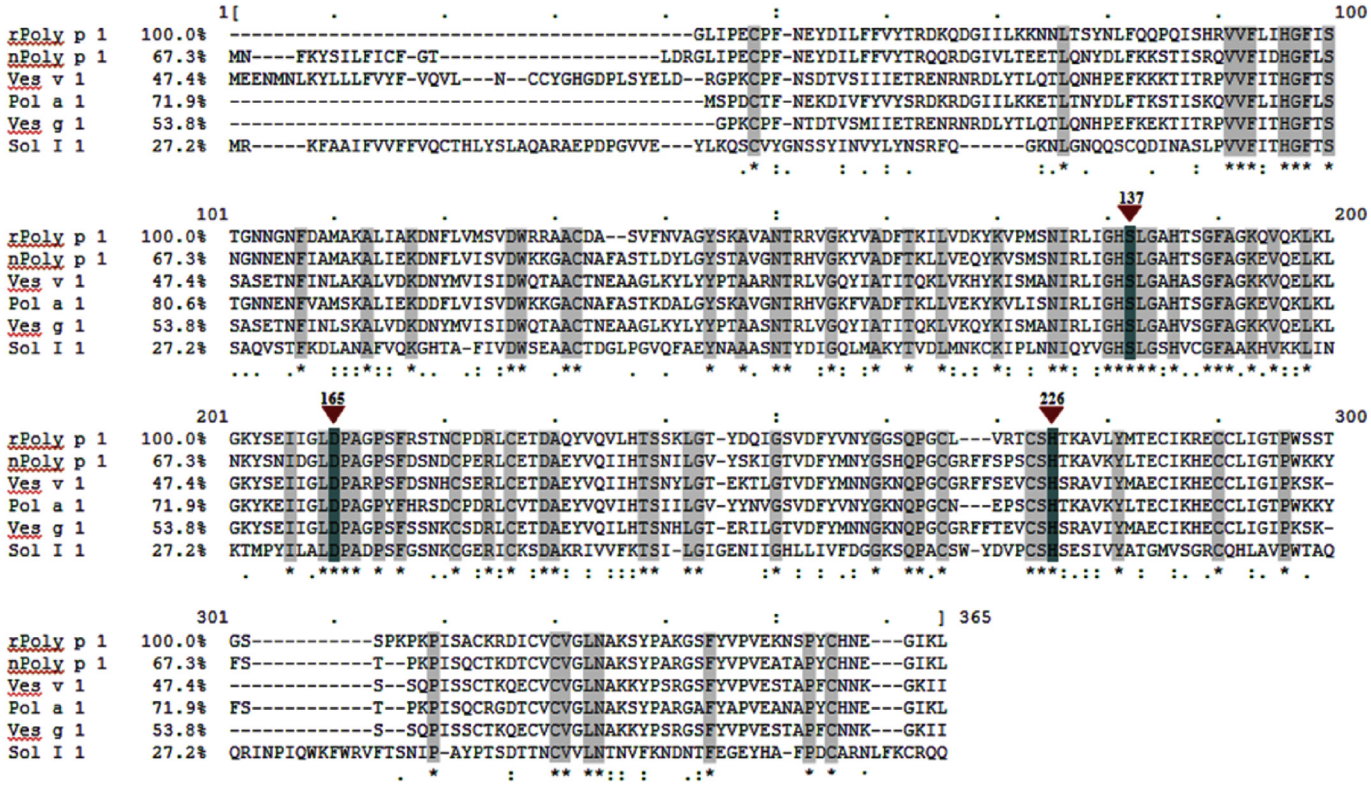

Fig. 2. Sequence alignment and identity of the predicted r*Poly p* 1 protein (GenBank: ADT89774.1) with the primary sequences of n*Poly p* 1, other vPLA1s (*Pol a* 1, *Ves v* 1, *Ves g* 1) and the ant PLA1 from *Solenopsis invicta* venom (*Sol i* 1). In green are the amino acid residues of the catalytic 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.)

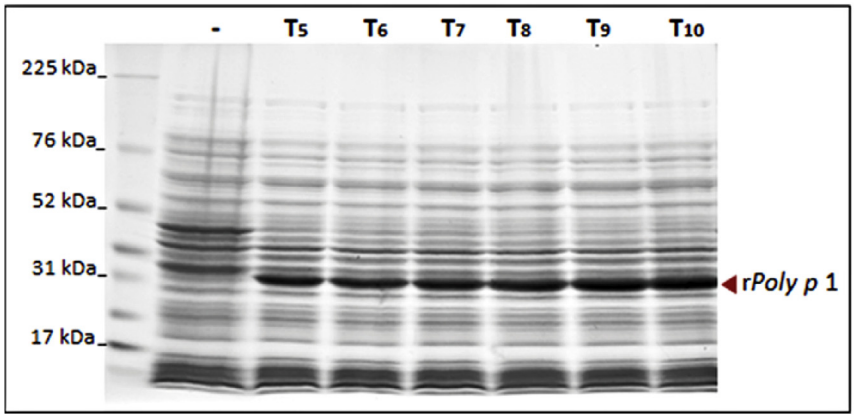

Fig. 3. SDS-PAGE (8–20%) analysis showing the expression profile of r*Poly p* 1 in *E. coli* BL21 (DE3) cells at different times after induction (20 °C, 1 mM IPTG). T_5–T_{10} represent hours after induction. (–) corresponds to a negative control (non-induced clone at T_{10}).

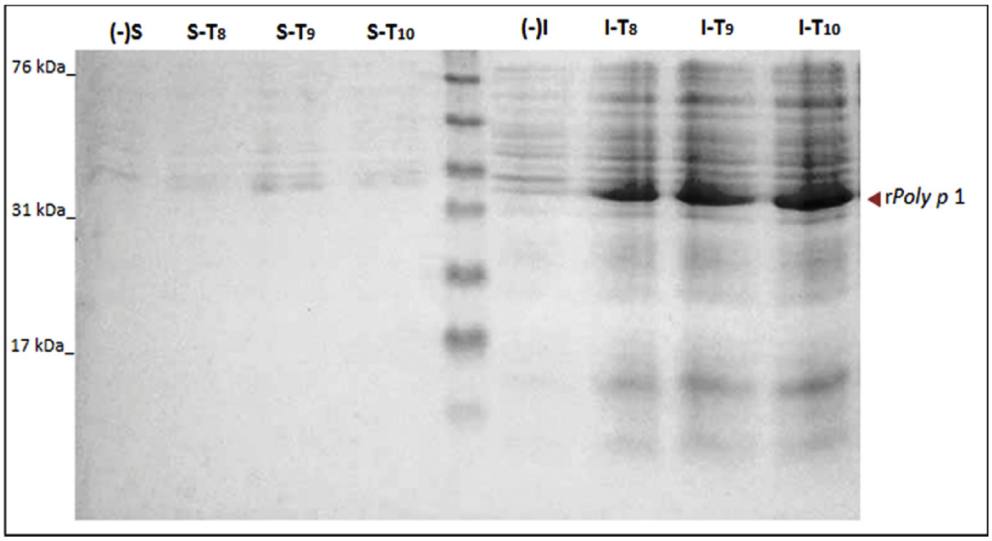

Fig. 4. SDS-PAGE (12%) analysis of r*Poly p* 1 *E. coli* BL21 (DE3) cell lysate. (S) soluble and (I) insoluble fraction after cell disruption and centrifugation. T_8–T_{10} represent hours after induction. (–) corresponds to a non-induced r*Poly* p 1 clone at T_{10}, used as a negative control.

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. 8**B**) and r*Poly p* 1 (Fig. 8**C**) 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

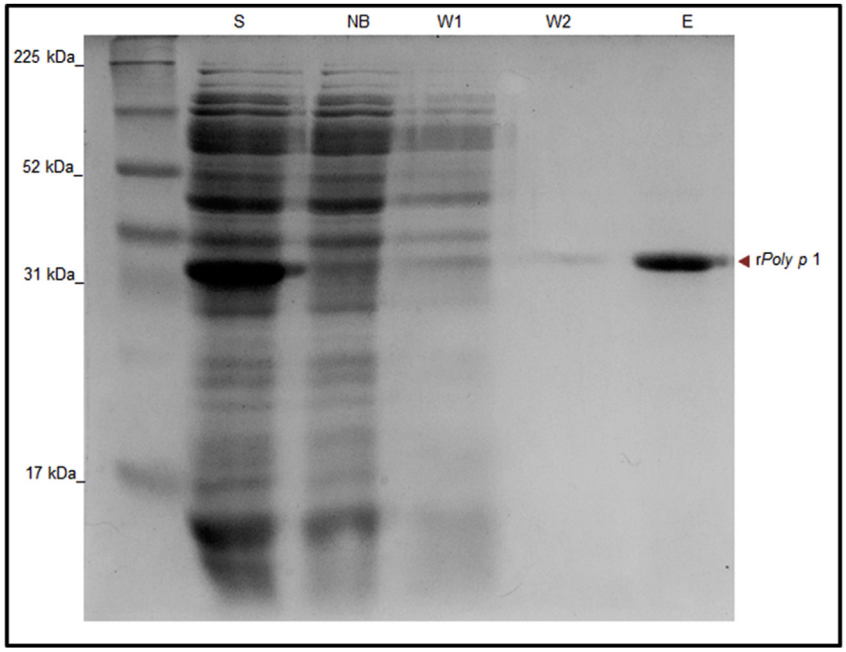

Fig. 5. SDS-PAGE (12%) analysis of fractions obtained during r*Poly p* 1 purification process. Samples: S = Solubilized proteins (8M urea); NB = Protein not bound to the column; W1–W2 = fractions corresponding to column-washing steps with 75 and 100 mM imidazole; E = fraction obtained after elution with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

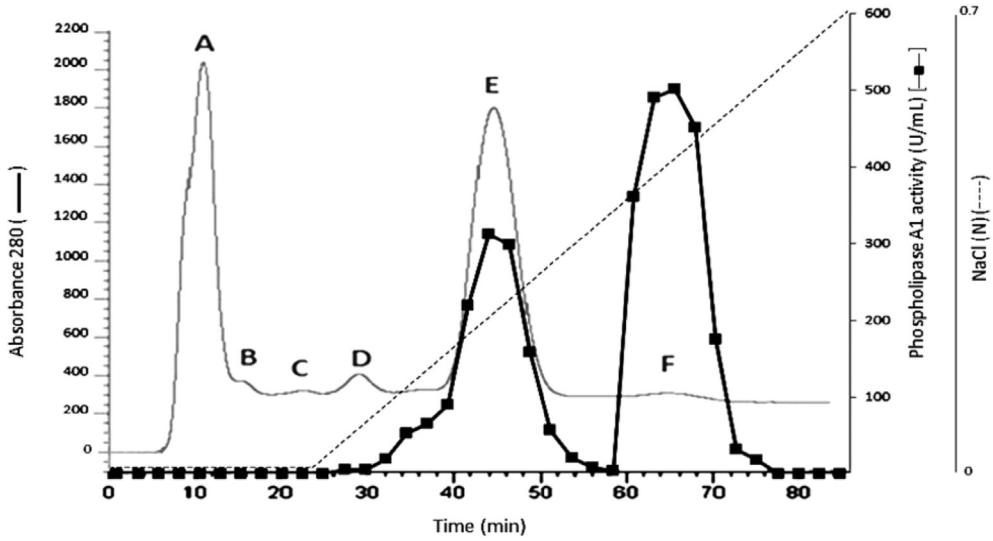

Fig. 6. Cation-exchange chromatography profile of *P. paulista* crude venom extract (23 mg), obtained in a Hiprep FF CM column (16 × 10 mm, 20 mL; GE Healthcare) coupled to an AKTA-FPLC system. The column was washed with an isocratic buffer of 50 mM sodium acetate pH 5.2. The elution was performed under 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 (—■—).

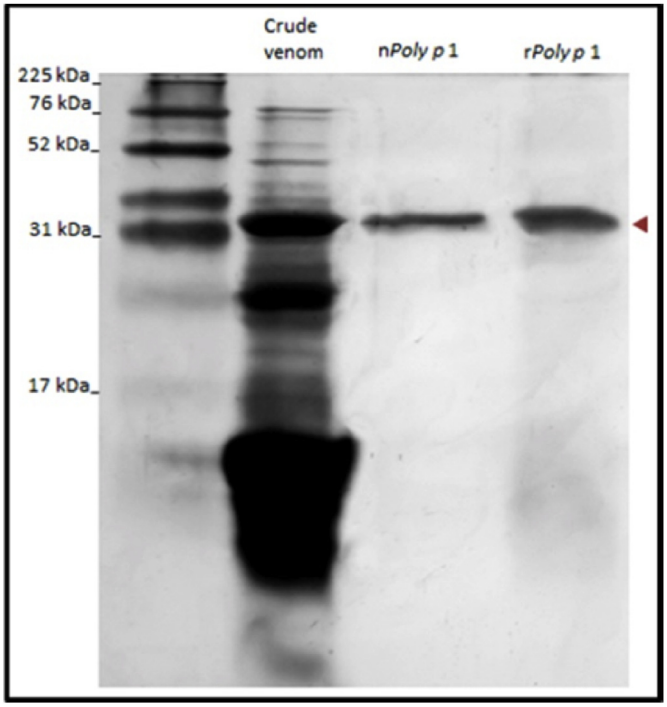

Fig. 7. SDS-PAGE (12%) analysis of crude venom extract, n*Poly p* 1 and r*Poly p* 1. Gel was staining with silver-nitrate.

temperature (6–10 °C) (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. 8**B**). 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 material—obtained from individuals of different nests—used 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 18–21 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

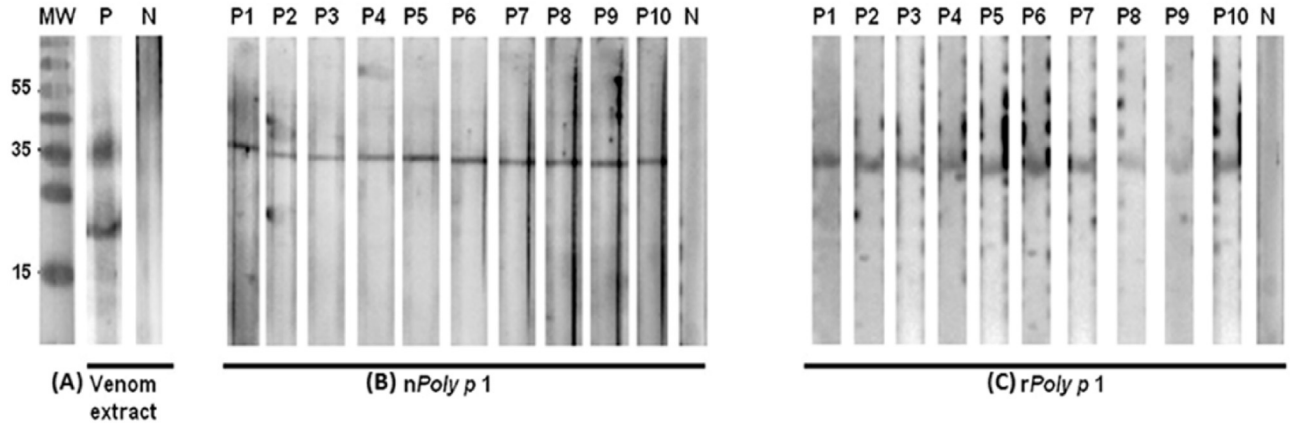

Fig. 8. (A, B, C). Antigenicity of *Poly p* 1 protein evaluated using a Western blot. Recognition of **(A)** *Poly p* 1 in crude venom, **(B)** native-purified (n*Poly p* 1) and **(C)** recombinant (r*Poly p* 1) by serum IgE in *P. paulista* venom-sensitized patients. Serum samples: P1–P10 = individual 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.

Acknowledgments

We acknowledge the financial support from FAPESP (São Paulo Research Foundation, Grant numbers: 2006/54799-6, 2014/13936-7) and from FUNDUNESP-UNESP (Fundação para o Desenvolvimento da Univ. Estadual Paulista, SP, Brazil; Grant number: 01197/10-DFP). The authors also thank to CAPES-DS (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), Postgraduate Program of Biological Sciences (Cellular and Molecular Biology) at UNESP, Rio Claro for providing Master and Doctoral scholarships to F.D.C.P and A.P.R. respectively, and to FAPESP (São Paulo Research Foundation, Proc.2009/51539-1) for Doctoral schoolarship to D.L.J.J.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2016.11.006

References

Bilò, M.B., 2011. Anaphylaxis caused by Hymenoptera stings: from epidemiology to treatment. Allergy 66, 35–37.
Bilò, M.B., Bonifazi, F., 2009. The natural history and epidemiology of insect venom allergy: clinical implications. Clin. Exp. Allergy 39, 1467–1476.
Borodina, I., Jensen, B.M., Wagner, T., Hachem, M.A., Søndergaard, I., Poulsen, L.K., 2011. Expression of enzymatically inactive wasp venom phospholipase A1 in *Pichia pastoris*. PLoS One 6, e21267.
Correa, A., Oppezzo, P., 2015. Overcoming the solubility problem in *E. coli*: available approaches for recombinant protein production. In: García-Fruitós, E. (Ed.), Insoluble Proteins: Methods and Protocols. Humana Press, Springer Inc., New York, pp. 27–45.
Floch, B.L., Bussières, L., Airouche, S., Lautrette, A., Bouley, J., Berjont, N., Horiot, S., Huet, A., Jain, K., Lemoine, P., Batard, T., Mascarell, L., Baron-Bodo, V., Tourdot, S., Nony, E., Moingeon, P., Chabre, H., 2012. Expression and characterization of natural-like recombinant Der p 2 for sublingual immunotherapy. Int. Arch. Allergy Immunol. 158 (2), 157–167.
Frick, M., Müller, S., Bantleon, F., Huss-Marp, J., Lidholm, J., Spillner, E., Jakob, T., 2015. rApi m 3 and rApi m 10 improve detection of honey bee sensitization in Hymenoptera venom-allergic patients with double sensitization to honey bee and yellow jacket venom. Allergy 70, 1665–1668.
Hou, M.H., Chuang, C.Y., Ko, T.P., Hu, N.J., Chou, C.C., Shih, Y.P., Ho, C.L., Wang, A.H.J., 2016. Crystal structure of vespid phospholipase A 1 reveals insights into the mechanism for cause of membrane dysfunction. Insect. biochem. Mol. Biol. 68, 79–88.
Jennings, A., Duggan, E., Perry, I.J., Hourihane, J.O., 2010. Epidemiology of allergic reactions to hymenoptera stings in Irish school children. Pediatr. Allergy Immunol. 21, 1166–1170.
Justo-Jacomini, D.L., Gomes-Moreira, S.M., Campos-Pereira, F.D., Zollner, R.L., Brochetto-Braga, M.R., 2014. Reactivity of IgE to the allergen hyaluronidase from *Polybia paulista* (hymenoptera, Vespidae) venom. Toxicon 82, 104–111.
Justo-Jacomini, D.L., Campos-Pereira, F.D., dos Santos-Pinto, J.R., dos Santos, L.D., Silva-Neto, A.J., Giratto, D.T., Palma, M.S., Zollner, R.L., Brochetto-Braga, M.R., 2013. Hyaluronidase from the venom of the social wasp *Polybia paulista* (Hymenoptera, Vespidae): cloning, structural modeling, purification, and immunological analysis. Toxicon 64, 70–80.
King, T.P., Lu, G., Gonzalez, M., Qian, N., Soldatova, L., 1997. Yellow jacket venom allergens, hyaluronidase and phospholipase: sequence similarity and antigenic cross-reactivity with their hornet and wasp homologs and possible implications for clinical allergy. J. Allergy Clin. Immunol. 98, 588–600.
Korošec, P., Valenta, R., Mittermann, I., Celesnik, N., Silar, M., Zidarn, M., Košnik, M., 2012. High sensitivity of CAP-FEIA r*Ves v* 5 and r*Ves v* 1 for diagnosis of *Vespula* venom allergy. J. Allergy Clin. Immunol. 129, 1406–1408.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Lockwood, S.A., HaghiPour-Peasley, J., Hoffman, D.R., Deslippe, R.J., 2012. Identification, expression, and immuno-reactivity of *Sol i* 2 & *Sol i* 4 venom proteins of queen red imported fire ants, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). Toxicon 60 (5), 752–759.
Monsalve, R.I., Veja, A., Marques, L., Miranda, A., Fernandez, J., Soriano, V., Cruz, S., Domínguez-Noche, C., Sánchez-Morillas, L., Armisen-Gil, M., Guspi, R., Barber, D., 2012. Component-resolved diagnosis of vespid venom-allergic individuals: phospholipases and antigen 5 are necessary to identify *Vespula* or *Polistes* sensitization. Allergy 67, 528–536.
Müller, U.R., Schmid-Grendelmeier, P., Hausmann, O., Helbling, A., 2012. IgE to recombinant allergens *Api m 1*, Ves v. 1, and *Ves v* 5 distinguish double sensitization from cross reaction in venom allergy. Allergy 67, 1069–1073.
Ollert, M., Blank, S., 2015. Anaphylaxis to insect venom allergens: role of molecular diagnostics. Curr. Allergy Asthma Rep. 15 (26), 1–11.
Perez-Riverol, A., Justo-Jacomini, D.L., Zollner, R.D.L., Brochetto-Braga, M.R., 2015. Facing hymenoptera venom allergy: from natural to recombinant allergens. Toxins 7, 2551–2570.
Rosano, G.L., Ceccarelli, E.A., 2014. Recombinant protein expression in *Escherichia coli*: advances and challenges. Front. Microbiol. 5 (172), 1–17.
San-Miguel, T., Pérez-Bermúdez, P., Gavidia, I., 2013. Production of soluble eukaryotic recombinant proteins in *E. coli* is favoured in early log-phase cultures induced at low temperature. SpringerPlus 2 (1), 1–4.
Santos, L.D., Menegasso, A.R.S., Santos-Pinto, J.R., Santos, K.S., Castro, F.M., Kalil, J.E., Palma, M.S., 2011. Proteomic characterization of the multiple forms of the PLAs from the venom of the social wasp *Polybia paulista*. Proteomics 11, 1403–1412.
Santos, L.D., Santos, K.S., de Souza, B.M., Arcuri, H.A., Cunha-Neto, E., Castro, F.M., Kalil, J.E., Palma, M.S., 2007. Purification, sequencing and structural characterization of the phospholipase A1 from the venom of the social wasp *Polybia paulista* (Hymenoptera, Vespidae). Toxicon 50, 923–937.
Santos, L.D., Santos, K.S., Pinto, J.R., Dias, N.B., de Souza, B.M., dos Santos, M.F., Perales, J., Domont, G.B., Castro, F.M., Kalil, J.E., Palma, M.S., 2010. Profiling the proteome of the venom from the social wasp *Polybia paulista*: a clue to understand the envenoming mechanism. J. Proteome Res. 9, 3867–3877.
Sedmak, J.J., Grossberg, S.E., 1977. A rapid, sensitive and versatile assay for protein using Coomassie brilliant blue G250. Anal. Biochem. 79, 544–552.
Seismann, H., Blank, S., Cifuentes, L., Braren, I., Grunwald, T., Ollert, M., Spillner, E., 2010. Recombinant phospholipase A1 (*Ves v* 1) from yellow jacket venom for improved diagnosis of hymenoptera venom hypersensitivity. Clin. Mol. Allergy 8, 1–8.
Sina, M., Farajzadeh, D., Dastmalchi, S., 2015. Effects of environmental factors on soluble expression of a humanized anti-TNF-α scFv antibody in *Escherichia coli*. Adv. Pharm. Bull. 5 (4), 455–461.
Skov, L.K., Seppala, U., Coen, J.J.F., Crickmore, N., King, T.P., Monsalve, R., Kastrup, J.S., Spangfort, M.D., Gajhede, M., 2006. Structure of recombinant Ves v 2 at 2.0 angstrom resolution: structural analysis of an allergenic hyaluronidase from wasp venom. Acta Crystallogr. D. Biol. Crystallogr. 62, 595–604.
Song, J.M., An, Y.J., Kang, M.H., Lee, Y.H., Cha, S.S., 2012. Cultivation at 6–10°C is an effective strategy to overcome the insolubility of recombinant proteins in *Escherichia coli*. Protein Expr. Purif. 82, 297–301.
Spillner, E., Blank, S., Jakob, T., 2014. Hymenoptera allergens: from venom to venome. Front. Immunol. 5 (77), 1–7.
Tanyaratsrisakul, S., Malainual, N., Jirapongsananuruk, O., Smith, W.A., Thomas, W.R., Piboonpocanun, S., 2009. Structural and IgE binding analyses of recombinant *Der p* 2 expressed from the hosts *Escherichia coli and Pichia pastoris*. Int. Arch. Allergy Immunol. 151 (3), 190–198.
Vinzón, S.E., Pirpignani, M.L., Nowicki, C., Biscoglio de Jimenez-Bonino, M., 2010. Molecular cloning and expression in *Pichia pastoris* of a hypoallergenic antigen 5. Protein Expr. Purif. 73, 23–30.
Vu, T.T.T., Jeong, B., Yu, J., Koo, B.K., Jo, S.H., Robinson, R.C., Choe, H., 2014. Soluble prokaryotic expression and purification of crotamine using an N-terminal maltose-binding protein tag. Toxicon 92, 157–165.