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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (BIOLOGIA CELULAR E MOLECULAR)

Análise imunológica comparada da eficiência e especificidade da hialuronidase recombinante de *Polybia paulista* (Hymenoptera, Vespidae) expressa em bactéria e levedura

Débora Laís Justo Jacomini

Rio Claro
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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

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CERTIFICADO DE APROVAÇÃO

TÍTULO: ANÀLISE IMUNOLÓGICA COMPARADA DA EFICIÊNCIA E ESPECIFICIDADE DA HIALURONIDASE RECOMBINANTE DE Polybia paulista (HYMENOPTERA, VESPIDAE) EXPRESSA EM BACTÉRIA E LEVEDURA

AUTORA: DÉBORA LAÍS JUSTO JACOMINI

ORIENTADORA: Profa. Dra. MARCIA REGINA BROCHETTO BRAGA

Aprovada como parte das exigências para obtenção do Título de DOUTOR EM CIENCIAS BIOLÓGICAS (BIOLOGIA CELULAR E MOLECULAR) , pela Comissão Examinadora:

Profa. Dra. MARCIA REGINA BROCHETTO BRAGA
Departamento de Biologia / Instituto de Biociências / UNESP - Rio Claro

Prof. Dr. RICARDO DE LIMA ZOLLNER
Faculdade de Ciências Médicas - UNICAMP

Profa. Dra. SUSANA MARGARIDA GOMES MOREIRA
Universidade Federal do Rio Grande do Norte

Prof. Dr. MARIO SERGIO PALMA
Departamento de Biologia / Instituto de Biociências / UNESP - Rio Claro

Profa. Dra. KAREN CRISTIANE MARTINEZ DE MORAES
Universidade Federal de Ouro Preto (UFOP)

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Dedicatória

A minha mãe, que me deu a vida, que me deu seu carinho,
bondade e zelo que Norteiam os atos meus.

Ao meu esposo, pelo seu amor, companheirismo e compreensão,
obrigada por acreditar em mim.

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obrigada por encher minha vida de tanta alegria.

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"Os leitores vagarosos
são mais confiáveis do que aqueles
que tudo compreendem imediatamente".
Martin Heidegger

Resumo

RESUMO

Justo Jacomini, D.L. Análise imunológica comparada da eficiência e especificidade da hialuronidase recombinante de *Polybia paulista* (Hymenoptera, Vespidae) expressa em bactéria e levedura [tese]. Rio Claro: Instituto de Biociências da Universidade Estadual Paulista "Júlio de Mesquita Filho", 2013.

A vespa social *Polybia paulista* tem sido muito estudada nos campos da bioquímica, proteômica e imunologia pelas suas características de habitat, composição de seu veneno e pelas conseqüencias dos acidentes decorrentes de suas ferroadas. O cDNA de hialuronidase (GI: 302201582), um dos principais alérgenos desse veneno, foi clonado em vetores de expressão - pET-28a e pPICZαA - em bactéria *Escherichia coli* DE3 (BL21) e em levedura *Pichia pastoris*, respectivamente. A proteína Hyal recombinante (*Pp*-Hyal-rec) de bactéria foi expressa em corpúsculos de inclusão, enquanto que a de levedura na forma solúvel. Ambas foram purificadas por cromatografia de afinidade em resina Ni^{2+} (Ni-NTA-Agarose). A proteína nativa de Hyal (*Pp*-Hyal-nat) também foi obtida e purificada até a homogeneidade por meio de cromatografia de troca catiônica em coluna Hiprep FF CM, acoplado a um sistema de Akta FPLC, e as análises realizadas por espectrometria de massa em MALDI ToF/ToF-MS. Anticorpos policlonais foram produzidos em camundongos BALB/c contra a *Pp*-Hyal-nat e a *Pp*-Hyal-rec de bactéria, demonstrando alta especificidade nos testes de *imunoblotting* realizados. Estes alérgenos foram avaliados quanto ao reconhecimento de imunoglobulina E (IgE) *Pp*-Hyal-específico no soro de pacientes sabidamente alérgicos ao veneno desta vespa. Os soros imunes foram capazes de reconhecer especificamente, em maior intensidade as bandas correspondentes à proteína *Pp*-Hyal-rec de bactéria (43 kDa) e a *Pp*-Hyal-rec de levedura (37 kDa) em relação ao alérgeno *Pp*-Hyal-nat (39 kDa). No extrato de veneno bruto de *P. paulista*, os soros reconheceram outras proteínas provavelmente correspondentes aos demais alérgenos do veneno, tais como Fosfolipase (37 kDa), Antígeno 5 (25 kDa), e proteases. Os dados aqui obtidos com ambos alérgenos recombinantes indicam fortemente a possibilidade de sua utilização para o desenvolvimento de kit diagnóstico para alergia, bem como, em imunoterapia específica (SIT) contra as reações indesejáveis provocadas pelo veneno de *P. paulista*.

Palavras-chave: *Polybia paulista*, hialuronidase de veneno, alérgenos recombinantes, *E. coli*, *P. pastoris*, anticorpos.

Abstract

ABSTRACT

Justo Jacomini, D.L. Comparative analysis of immunological efficiency and specificity of recombinant hyaluronidase *Polybia paulista* (Hymenoptera, Vespidae), expressed in bacteria and yeast [thesis]. Rio Claro: Instituto de Biociências da Universidade Estadual Paulista "Júlio de Mesquita Filho", 2013.

The social wasp *Polybia paulista* has been well studied in fields of biochemistry, proteomics and immunology due to its habitat characteristics, venom composition and the consequences of accidents arising from their stings. The hyaluronidase cDNA (GI: 302201582), one of the major venom allergens, was cloned into expression vectors - pET-28a and pPICZαA - in *Escherichia coli* DE3 (BL21) and yeast *Pichia pastoris*, respectively. The recombinant protein Hyal (*Pp*-Hyal-rec) of bacteria was expressed in inclusion corpuscles, whereas the yeast in soluble form. Both were purified by affinity chromatography on Ni^{2+} (Ni-NTA-Agarose) resin. The native protein Hyal (*Pp*-Hyal-nat) was also obtained and purified to homogeneity by cation exchange chromatography on Hiprep CM FF column, to an Akta FPLC coupled system, and the analysis performed by mass spectrometry MALDI ToF / ToF-MS. Polyclonal antibodies were produced in BALB/c mice against *Pp*-Hyal-nat and *Pp*-Hyal-rec from bacteria, demonstrating an high specificity in the immunoblotting tests performed. These allergens were evaluated for recognition of immunoglobulin E (IgE) *Pp*-Hyal-specific in serum from patients known to be allergic to the venom of this wasp. The immune sera were able to recognize specifically in a higher intensity the bands corresponding to protein *Pp*-Hyal-rec of bacteria (43 kDa) and *Pp*-Hyal-rec of yeast (37 kDa) in relation to the allergen *Pp*-Hyal-nat (39 kDa). In the *P. paulista* crude venom extract, all sera recognized other proteins probably corresponding to other venom allergens, such as phospholipase (37 kD), antigen 5 (25 kDa), and protease. The data obtained here with both recombinant allergens strongly suggest the possibility of using these for development of diagnostic kit for allergy and, in specific immunotherapy (SIT) against undesirable reactions caused by *P. paulista* venom.

Keywords: *Polybia paulista*, venom hyaluronidase, recombinant allergens, *E. coli*, *P. pastoris* antibodies.

LISTA DE FIGURAS DO TEXTO

LISTA DE ABREVIATURAS E SIGLAS

AOX - álcool oxidase

Anti-*Pp*-Hyal-nat- Anticorpo policlonal da Proteína nativa Hyaluronidase de *P. paulista*

Anti-*Pp*-Hyal-rec- Anticorpo policlonal da Proteína recombinante Hyaluronidase

cDNA - Complementary DNA

DNA - Deoxyribonucleic Acid

His-Tag - cauda de histidina

IgE - Imunoglobulina E

Mut^S - Methanol utilization slow

Mut^+ - Methanol utilization plus

PCR - Reação em Cadeia da Polimerase

Pp-Hyal - Proteína Hyaluronidase de *Polybia paulista*

Pp-Hyal-rec - Proteína Hyaluronidase recombinante de *Polybia paulista*

SDS-PAGE - Polyacrylamide Gel Electrophoresis

SUMÁRIO

Introdução Geral

Introdução Geral

Dentre os insetos, a ordem Hymenoptera é um dos maiores grupos, compreendendo as vespas, abelhas e formigas. Possui aproximadamente 115.000 espécies já descritas na literatura (HANSON, GAULD, 1995), existindo somente no Brasil cerca de 500 espécies correspondente a um dos maiores números de espécies de vespas sociais de todo o planeta (RICHARDS, 1978). As vespas pertencentes ao grupo dos Hymenoptera Aculeata estão divididas em três grandes superfamílias: *Bethyloidea*, *Sphecoidea* e *Vespoidea* (BROTHERS, 1975). No Brasil a subfamília *Polistinae* é representada por três tribos a Polistini, Epiponini e Mischocyttarini. A tribo Epiponini é constituída por 23 gêneros e 405 espécies, dentre elas a espécie *Polybia paulista* (CHAUD-NETTO et al., 1994). As espécies brasileiras são tipicamente neotropicais, sendo seus venenos muito diferentes daqueles encontrados nas espécies de clima temperado (REISMAN et al., 1984, 1989), em termos de muitas das características individuais de seus componentes, mas não da composição geral.

Importantes dados mostram que os acidentes provocados por abelhas, vespas e formigas nativas das regiões sudeste e centro-sul dos Estados Unidos, representam um problema de saúde que vem crescendo rapidamente (MOFFIT, 2003). Há muitos anos atrás a vespa *Polybia paulista* já vinha sendo descrita na literatura por Machado (1984) como um importante inseto social de convivência estreita com os humanos, com ocorrência nos Estados de Goiás, Mato Grosso, Paraná e Mina Gerais, com registro no Paraguai e Argentina. No sudeste do Brasil, principalmente no Estado de São Paulo, essa vespa social é endêmica nesta região por possuir habitat caracteristicamente urbano, além de um perfil bastante agressivo, o qual tem levado à ocorrência de vários acidentes de importância médica, em razão da complexidade de composição de seu veneno, que inclui importantes alérgenos, entre eles a proteína alergênica hialuronidase (*Pp*-Hyal).

Em geral a convivência dos Hymenoptera sociais com os seres humanos, ocorre de maneira estreita fazendo com que os acidentes por ferroadas sejam bastante freqüentes, causando às vítimas, sensibilização ao veneno injetado e a possível ocorrência de reações alérgicas, desde as localizadas no sítio da ferroada até manifestações sistêmicas da anafilaxia, como o choque anafilático. A anafilaxia sistêmica ocasiona risco à vida do paciente alérgico, podendo promover, arritmia, bronco-espasmos, paradas cardíacas e respiratórias, choque e morte do paciente (REISMAN et al., 1989; FREEMAN, 2004). De acordo com Lorenzi

(2002) e Castro (2001) a maioria dos acidentes que resultam em anafilaxia está relacionada aos venenos de vespas, entretanto esta pode não ser uma regra geral.

A estimativa de sensibilização da população mundial aos venenos de Hymenoptera sociais varia entre 9.3% e 28.5% (ANTONICELLI ET AL., 2002). A incidência de reações sistêmicas na população geral, devido a esta ordem de insetos, está estimada entre 0.8-5.0%, e as de reações locais graves em 19%. Em um levantamento de acidentes com Hymenoptera, registrado no Hospital das Clínicas em São Paulo, foi observado que as vespas foram responsáveis por aproximadamente 32,6% dos acidentes, seguidas das abelhas com 28,2% e das formigas 26,8% (SOUZA, 2002).

Essas manifestações estão diretamente associadas à sensibilização mediada por IgE, para as proteínas presentes nos venenos das espécies de Hymenoptera sociais, como abelhas, vespas e formigas (MÜLLER et al., 1993; MÜLLER, 2010, 2011). Assim, o conhecimento sobre a estrutura e função dos componentes do veneno desses insetos, sobretudo no campo da alergia e imunologia clínica, é fundamental (ROSS et al., 2000; DRESCHLER et al., 2011). Os estudos relacionados aos acidentes provocados por ferroadas de Hymenoptera vêm sendo cada vez mais ampliados e aprofundados nas diferentes áreas das ciências, devido aos vários aspectos das reações apresentadas pelas vítimas, principalmente aqueles relacionados ao desencadeamento de manifestação sistêmica grave, que podem levar ao óbito (MÜLLER, 1990; BILÒ et al. 2005; BILÒ e BONIFAZI, 2009).

Os componentes dos venenos de vespídeos são, em geral, menos conhecidos do que aqueles que compõem o veneno de abelha. Em contraposição a isto, a vespa social *P. paulista*, tem sido muito estudada no campo da bioquímica, proteômica e imunologia pelas suas características de habitat, composição de seu veneno e pelas conseqüências causadas devido aos acidentes decorrentes de suas ferroadas. Seu veneno apresenta uma composição semelhante à maioria dos venenos dos Hymenoptera sociais, tais como, aminas biogênicas, peptídeos básicos e proteínas de elevadas massas moleculares, principalmente enzimas, entre elas a Hyal, as quais são responsáveis pelas reações alérgicas (CASTRO et al., 1994; MÜLLER, 2002; CASTRO, 2011). Os peptídeos biologicamente ativos, juntamente com as proteínas do veneno são os principais responsáveis pelas dores prolongadas, edema, eritema, reações alérgicas e sistêmicas. Além disso, a ocorrência de mortes devido às ferroadas de vespas estão diretamente relacionadas ao desencadeamento de reações imunológicas, bem como à toxicidade direta do veneno (MÜLLER, 1990; LORENZI, 2002; BILÒ et al. 2005; BILÒ e BONIFAZI, 2009).

A hipersensibilidade de tipo I, mediada por IgE, vem adquirindo grande importância no mundo, pois 25 % da população, em geral, sofrem de algum tipo de alergia, muitas destas reações são desencadeadas por acidentes com veneno de insetos (PRZYBILLA, RUËFF, 2012) gerando um alto custo para a saúde pública. Diante deste agravante, o diagnóstico e o tratamento para esse tipo de alergia dependem da disponibilidade de extratos alergênicos de qualidade, permitindo assim, sua utilização como ferramentas para o diagnóstico e o tratamento específico de alergias. As reações de hipersensibilidade a Hymenoptera são dirigidas contra as proteínas alergênicas presentes nos venenos dos insetos dessa ordem, sendo três as famílias causadoras de alergia - Apidae, Vespidae e Formicidae. As reações alérgicas causadas pelos venenos de insetos são o resultado da combinação dos alérgenos com mastócitos e anticorpos IgE, causando uma cascata de liberação de mediadores como histamina, leucotrienos, enzimas, peptídeos e ativador de plaquetas, os quais desencadeiam diversos efeitos (HOFFMAN, 1996). Estudos moleculares que identificam epítopos imunogênicos dos alérgenos são muito importantes, pois localizam as regiões da molécula do alérgeno, que interagem com os anticorpos da pessoa sensibilizada (BREITENEDER et al., 1999).

Além disso, podemos encontrar estudos que relatam um elevado grau de reatividade imunológica cruzada entre os alérgenos dos venenos de insetos Hymenoptera, os quais tornam difícil determinar qual o verdadeiro inseto responsável pelo desencadeamento das reações alérgicas. Pacientes previamente sensibilizados com veneno de um determinado inseto (p.ex. vespa) que, em uma segunda vez são ferroados com outro inseto diferente, podem apresentar em testes convencionais, a presença de anticorpos IgE inespecíficos. Disto, podem resultar respostas falso-positivas, devido à reatividade cruzada, com alérgenos de diferentes venenos, cujos epítopos possuem conformações similares, impossibilitando diferenciação pelas células B-1. Também podem ser encontrados resultados falso negativos, realizados por testes cutâneos, em função da baixa quantidade de IgE detectado, pelo baixo nível de sensibilidade do teste aplicado (p.ex., o teste de RAST) (HEMMER, 2008).

O diagnóstico e o tratamento dessas alergias dependem da disponibilidade de extratos alergênicos de qualidade, os quais podem ser usados como bioferramentas para o diagnóstico e promoção da dessensibilização específica. Comparativamente aos extratos alergênicos utilizados, o desenvolvimento de alérgenos recombinantes para medição de IgE específica representa um avanço extremamente importante (Karoui et al., 2013).

Com a tecnologia do DNA recombinante tem-se determinado seqüências de genes e a expressão dos principais constituintes do veneno de diversos organismos, os quais vêm

demonstrando aspectos bastante interessantes. Entre estes, podemos destacar a obtenção de dados sobre a origem e a organização dos genes, a possível estrutura tridimensional dos alérgenos recombinantes de veneno, a função comparativa destes alérgenos com os naturais, além da viabilidade de utilização das moléculas recombinantes no desenvolvimento de vacinas e kits diagnósticos que podem vir a ser aplicados em diversas áreas. Principalmente, no caso dos venenos de insetos, essas técnicas têm se constituído em ferramentas muito úteis no esclarecimento dos aspectos moleculares envolvidos nas reações imunológicas cruzadas entre os componentes alergênicos de venenos. Apesar dos avanços nos diferentes sistemas de expressão, incluindo bactérias, leveduras, células de mamíferos, e mesmo sistemas isentos de células, o sistema de expressão de proteínas recombinantes em bactérias ainda é o mais utilizado por ser de fácil manipulação, rápido crescimento e baixo custo de produção da proteína de interesse (DEMAIN, VAISHNAV, 2009; SONG et al. 2012). Adicionalmente, a *E.coli* é uma bactéria bem conhecida, cujo genoma já foi seqüenciado e apresenta elevados níveis de expressão protéica e rápido acúmulo de biomassa (BANEYX, MUJACIC, 2004; WALSH, 2004). A expressão de alérgenos recombinantes pelo sistema bacteriano também pode apresentar vantagem sobre os riscos oriundos dos cultivos com microorganismos patogênicos. Por outro lado, o enovelamento das proteínas recombinantes pode ocorrer de maneira incorreta pelo fato das modificações pós-traducionais estarem ausentes. Este processo não ocorre em células procarióticas, pois a condição conformacional e a maquinaria de ligação das proteínas são diferentes, levando a um acúmulo destas em agregados insolúveis (JEVSEVAR et al., 2005). Esses eventos podem ou não interferir na característica imunológica do alérgeno recombinante, conforme tem sido demonstrado em muitos trabalhos (FARRELL et al., 1995) uma vez que é conhecido que as proteínas recombinantes eucarióticas necessitam de modificações pós-traducionais para atingirem a conformação semelhante à nativa.

Além disso, a produção de proteínas em sistemas heterólogos vem sendo utilizada como uma estratégia para produzir proteínas que são requeridas para diversas aplicações em maiores quantidades, a exemplo do que ocorre na expressão em *Pichia pastoris*. Outras leveduras são empregadas industrialmente para a produção de proteínas heterólogas, entre elas, destacam-se *Saccharomyces cerevisiae*, *Hansenula polymorpha* e *Kluyveromyces lactis*. O uso dessa classe de organismos como sistema de expressão vem sendo realizado com sucesso crescente ao longo dos últimos 20 anos (DAMASCENO et al., 2012; DEMAIN, VAISHNAV, 2009). Neste estudo, além da expressão em bactéria, foi também realizada a expressão da proteína hialuronidase (*Pp*-Hyal) em levedura, por apresentar vantagens em

relação à alta produtividade, crescimento rápido com alta densidade celular, existência de linhagens recombinantes mais estáveis, secreção mais eficiente e processamento pós-traducional mais similar ao de mamíferos (DEMAIN, VAISHNAV, 2009). Todas estas condições, favorecem a obtenção, neste sistema, de proteínas recombinantes eucarióticas, em que as suas propriedades estruturais e funcionais resultantes sejam bastante semelhantes, ou mesmo idênticas às proteínas nativas, o que, conforme já mencionado, não ocorre no sistema de expressão procariótico de *E.coli* e portanto, pode comprometer ou mesmo impedir a sua utilização biotecnológica.

O presente estudo foi realizado primeiramente para proporcionar um entendimento mais preciso quanto às propriedades estruturais e imunogênicas de uma das isoformas do alérgeno hialuronidase nativo do veneno da vespa social *P. paulista* (Hymenoptera, Vespidae). Por meio de testes imunológicos convencionais, realizados com o soro de pacientes alérgicos a essa vespa e com o veneno de outros insetos sociais foram analisados respectivamente, a reatividade de IgE específica a este alérgeno, bem como, os níveis de reatividade cruzada. Todas as análises foram feitas de forma comparativa entre o alérgeno nativo e o alérgeno recombinante expresso nos dois diferentes sistemas - procariótico de *Escherichia coli* e eucariótico inferior de *Pichia pastoris*, com o objetivo de avaliar se as características imunogênicas das proteínas recombinantes seriam mantidas, implicando desta forma, em seu efetivo potencial de utilização em testes diagnósticos e imunoterapia específica.

Objetivos

Objetivo geral:

Obtenção e caracterização imunológica da proteína alergênica hialuronidase do veneno da vespa social *P. paulista* (*Pp*-Hyal) (Hymenoptera, Vespidae) expressa em dois diferentes sistemas, procariótico de *Escherichia coli* e eucariótico inferior de *Pichia pastoris*, buscando determinar possíveis e importantes diferenças antigênicas entre elas e, se as proteínas recombinantes mantêm as mesmas propriedades imunogênicas do alérgeno natural.

Objetivos específicos:

- Clonagem e expressão da enzima hialuronidase em bactéria *E. coli* e levedura *Pichia pastoris*;
- Produção de anticorpos policlonais em camundongos, contra a hialuronidase nas formas nativa (anti-*Pp*-Hyal-nat) e recombinantes (anti-*Pp*-Hyal-rec) purificadas*;*
- Testar a identidade imunológica dos anticorpos policlonais, anti-*Pp*-Hyal-nat e anti-*Pp*-Hyal-rec contra as proteínas purificadas e o veneno bruto da vespa *P. paulista;*
- Analisar a ocorrência de reação imunológica cruzada dos anticorpos policlonais, anti-*Pp*-Hyal-nat e anti-*Pp*-Hyal-rec, contra o veneno bruto de outros insetos sociais;
- Verificar e comparar os níveis de reconhecimento do soro de pacientes, sensibilizados pelo veneno de *P. paulista* pelas proteínas purificadas nas formas nativa e recombinantes.

Capítulo I

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

Débora Laís Justo Jacomini [a], Franco Dani Campos Pereira [a], José Roberto Aparecido dos Santos Pinto [b], Lucilene Delazari dos Santos [c], Antonio Joaquim da Silva Neto [d], Danielli Thieza Giratto [a], Mário Sérgio Palma [b], Ricardo de Lima Zollner [e], Márcia Regina Brochetto Braga [a,*]

[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] 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
[c] Rua José Barbosa de Barros, no. 1780, CEP 18610-307, Fazenda Experimental Lageado, Botucatu, SP, Brazil
[d] Faculdades Integradas Claretianas, Av. Sto. Antonio Maria Claret, nº 1724, CEP 13503-257, Cidade Claret, Rio Claro, SP, Brazil
[e] Laboratório de Imunologia E Alergia Experimental-LIAE, Faculdade de Ciências Médicas, FCM, Universidade Estadual de Campinas-UNICAMP, Rua Tessália Vieira de Camargo, nº 126, CEP 13083-887, Cidade Universitária "Zeferino Vaz", Campinas, SP, Brazil

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ABSTRACT

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

* Corresponding author. Fax: +55 19 3526 4135.
E-mail addresses: lucilene@cevap.org.br (L.D. dos Santos), yoakneto@gmail.com (A.J. da Silva Neto), mspalma@rc.unesp.br (M.S. Palma), zollner@unicamp.br (R. de Lima Zollner), mrbbraga@rc.unesp.br (M.R. Brochetto Braga).

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1. Introduction

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

Hyal have been described as "allergenic factors" in scorpion, bee, and wasp venoms, and are able to induce severe and fatal anaphylactic IgE-mediated reactions in humans (Lu et al., 1995; Kolarich et al., 2005). Hyal have already been characterized as glycoproteins (Kemeny et al., 1984; Jin et al., 2008) and analysis by high performance liquid chromatography and mass spectrometry revealed that the α-1,3-fucose-containing N-glycan is the fundamental structure responsible for their allergenicity (Kubelka et al., 1995; Kolarich and Altmann, 2000; Kolarich et al., 2005).

Since allergenic Hyal are phylogenetically more conserved among the other Hymenoptera allergens (e.g. Ag5 and PLA1), a significant degree of homology is observed among the sequences and 3D structures of these proteins, whether they are from different vespids or honeybee *Apis mellifera* venom (Api m 2) (Jin et al., 2010). In addition, a large percentage of patients allergic to Hymenoptera venom show reactivity to both bee and wasp venoms (known as cross-reactivity) in tests for the presence of IgE-specific antibodies (Hemmer, 2008). This makes selection of the most suitable venom for immunotherapy difficult. However, it is unclear whether this cross-reactivity is due to (a) sequence homology between these hyaluronidases; (b) sensitivity to the specific IgE antibodies; or (c) cross-reactive N-glycans (cross-reactive carbohydrate determinants [CCDs]), which have been investigated in allergens from different sources (Jin et al., 2010; Eberlein et al., 2012; Al-Ghouleh et al., 2012).

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

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

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

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

2. Material and methods

2.1. Insects and crude venom extracts

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

2.2. Protein determination

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

2.3. Preparation of total RNA and cDNA synthesis

RNA was extracted from 100 venom reservoirs with TRIzol® reagent (Life Technol, USA) and maintained at −85 °C for 7 days to increase the integrity of the total RNA. cDNA synthesis was performed by RT-PCR of 1 μg of RNA using a kit from Promega® (USA) and an oligo dT

primer. The complete cDNA sequence of *Pp*-Hyal was obtained with a degenerated forward primer designed based on the Hyal precursor sequence (GI: 5815250) of *Polistes annularis* venom (5′ TCC RAA AGA CCG AAA AGA GTG TTC ARC 3′) and a reverse primer (5′ CTA AAA GTT CAG GGA TGA TCT TCT 3′) designed based on the results of previous 3′ RACE experiments. Both primers were synthesized by Sigma–Aldrich (USA). PCR was performed using Platinum®Taq DNA Polymerase (Life Technol, USA) in a final volume of 25 μL containing 2 μg of cDNA, 1U of Taq DNA polymerase, 0.2 mM dNTPs, 2.0 mM $MgCl_2$ and 0.2 μM of the above primers under the following conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 47 °C for 1 min, and extension at 72 °C for 3 min, and a final extension at 72 °C for 15 min in an MJ Research PTC-100 Programmable Thermocycler.

2.4. Cloning

Pp-Hyal gene-specific PCR products were cloned into the pCR®8/GW/TOPO® vector (kit pCR®8/GW/TOPO® Cloning Kit, Invitrogen, USA) following the manufacturer's protocol. *Escherichia coli* One Shot®Mach1TMT1R cells chemically competent, were reared in SOC Medium (Tryptone 2.0%, yeast extract 0.5%, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) and used for transformation reactions. 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. Plasmid preparations were obtained using the QIAprep®Spin miniprep kit (Qiagen, Germany) and analyzed by restriction digestion with *Eco RI* enzyme (Fermentas UAB, Lithuania).

2.5. Sequencing

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

2.6. Structural modeling

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

2.7. Ion exchange liquid chromatography

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

2.8. Hyaluronidase activity

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

2.9. SDS-PAGE

Fractions showing hyaluronidase activity were collected, pooled, and lyophilized. The protein concentration was determined and 80 μg of total protein were separated by 15% (w/v) SDS-PAGE in a Mini-Protean II (BioRad) at 100 V. The gel was stained with *Coomassie Brilliant Blue R-250* (CBB) and scanned. For Western blotting experiments, 80 μg of total protein from venom extracts of different insects were separated by 15% SDS-PAGE. A pre-stained standard molecular weights ranging from 12,000 to 225,000 Da (High-Range Rainbow Molecular Weight Markers, Amersham Biosciences-GE Healthcare, USA) was run in parallel. Runs were carried out at 75 V in the stacking gel and 100–110 V on the resolving gel over a period of 2 h. Following separation, the proteins were transferred from the gels onto nitrocellulose membranes.

2.10. In-gel digestion

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

combined and vacuum dried. The concentrated digests were mixed with 0.5 μL of matrix containing 10 mg/mL α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile mixed with equal volume of 0.1% (v/v) trifluoracetic acid and spotted onto a MALDI plate.

2.11. Mass spectrometry

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

2.12. Protein identification

LaunchPad 2.8.4 (Shimadzu Biotech) was used to submit the MS data to MASCOT Protein Search Engine (version 2.2) using the National Center for Biotechnology Information (NCBI) Protein Database. The search parameters were as follows: no restrictions on protein molecular mass, one missed tryptic cleavage allowed, mass tolerance to peptide of 0.2 Da for MS spectra. Carbamide-methylation due to treatment of sulfhydryls with iodoacetamide and oxidation of methionine were specified in MASCOT as fixed and variable modifications, respectively.

2.13. Systemic sensitization and Pp-Hyal-specific antibodies preparation

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

2.14. Western blotting

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

3. Results

3.1. Analysis of Pp–Hyal: cDNA sequence, deduced protein and structural modeling

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

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

When the GenBank Hyal amino acid sequence for *Pp*-Hyal (ADL09135) from this study was aligned with the same allergen of *V. vulgaris* (PDB 2ATM), *P. annularis* (HUGA_POLAN), and *A. mellifera* (PDB 1FCQ_A), high levels of similarity were revealed (75%, 90%, and 54%, respectively). In Fig. 2, shaded blue areas indicate several regions of similarity mainly among the three first molecules. In addition, the amino acids DFE (highlighted by a red rectangle), which are present in the active site, are also highly conserved.

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

Based on its model (Fig. 3A,B), *Pp*-Hyal displays a structure comprised of a central barrel $(\beta/\alpha)_7$ containing seven α-helix and seven beta-sheets, in agreement with the expected structure for all hyaluronidases belonging to family 56 of glycoside hydrolases (Henrissat and Bairoch, 1996; Markovic-Housley et al., 2000; Skov et al., 2006). This model also reveals two important characteristics of the *Pp*-Hyal structure: the presence of two disulfide bonds between Cys 19–308 and Cys 185–197 (Fig. 3A) and putative glycosylation sites on residues Asn79, Asn187, and Asn325 (Fig. 3B). The sites Asn79 (5′ NITI 3′) and Asn325 (5′ NITI 3′)

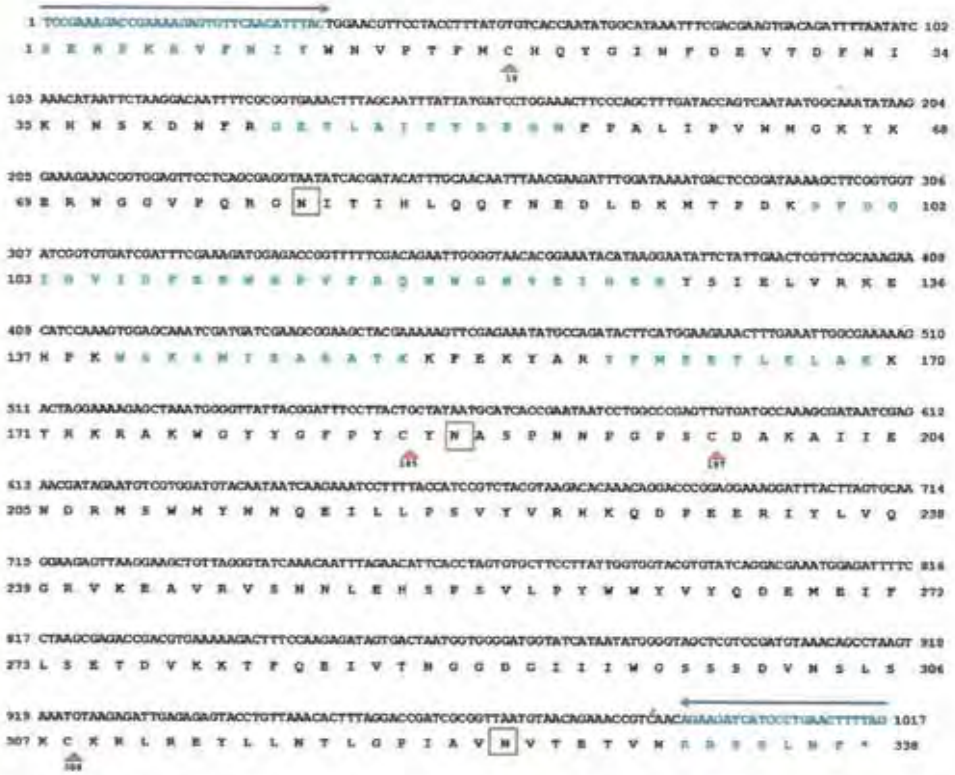

Fig. 1. cDNA and amino acid sequence of the mature *Pp*-Hyal transcript (338 amino acids, 1017 bp). Primers Forward (5′ TCC RAA AGA CCG AAA AGA GTG TTC ARC 3′) and a reverse (5′ CTA AAA GTT CAG GGA TGA TCT TCT 3′) used for PCR amplification are indicated by blue arrows at the beginning and end of the sequence, respectively. Brown boxes indicate the three immunogenic potential sites (N-glycosylated: residues of Asn79, Asn187, and Asn325). Disulfide bonds responsible for the *Pp*-Hyal protein structure stabilization are labeled with red arrows (▲): (Cys19/Cys308 and Cys185/Cys197). The four peptides generated by tryptic digestion, analyzed by MALDI ToF/ToF-MS and identified by MASCOT Software Protein Search Engine can also be visualized in light green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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

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

Sequences of potentially immunogenic regions were also identified (Fig. 5) by the Conformational Epitope Prediction Serve (CEP) (Kulkarni-Kale et al., 2005). According these authors for every antigen–antibody complex the total of antibody-binding sites corresponds to the sum of the residues that interact with the antibody plus those that are buried under the antibody. Using an implementation of Voronoi polyhedron (McConkey et al., 2002) to the calculation of percentage accessibility of residues and with base on the spatial distance cut-off among the involved atoms, Kulkarni-Kale et al. (2005) have stipulated a correction factor of ≤25% for identification of antigenic residues less accessible by the antibody binding.

So, in the *Pp*-Hyal 3D-structural model twelve antigenic sites were identified, located in regions of both the internal

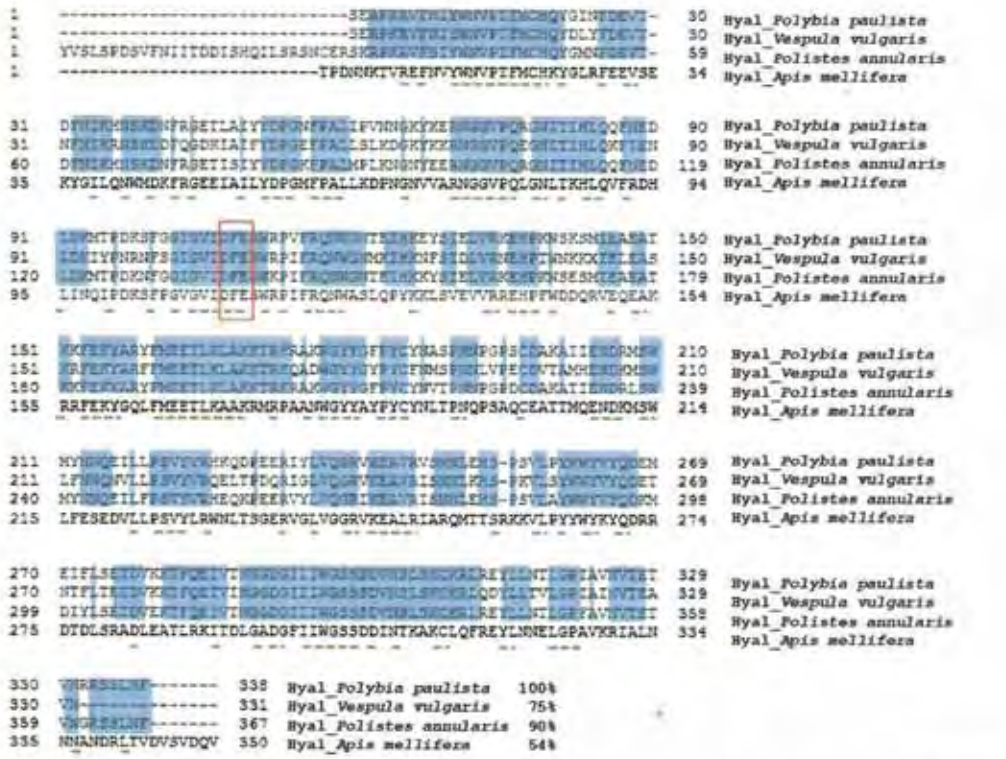

Fig. 2. Sequence alignment and percentage of similarity of the deduced *Pp*-Hyal protein (ADLO9135) with sequences of the same allergen from venoms of *Vespula vulgaris* (PDB 2ATM), *Polistes annularis* (HUGA_POLAN) and of *Apis mellifera* (PDB 1FCQ_A). In shaded blue are the regions of higher similarity among the three first molecules and dashed under the alignment corresponds to conserved residues in *Apis mellifera* enzyme, with respect to other three molecules from wasp venom. The three amino acid residues – DFE – extremely conserved and present in the active site of all of these molecules are highlighted by a red rectangle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and external loops revealing five conformational (displayed in green) and seven linear (presented in yellow) predicted epitopes. Thus, we can infer that in this allergen the presence of linear epitopes directly influence immune responses while the five conformational epitopes affect the humoral response mediated by B cells.

Through the model is also possible to note that even in the regions of linear epitopes some amino acid residues, as those shown in lowercase in L1 (Hys), L4 (Pro), L5 (Thr) and L7 (Phe and Ala), are more internally located in the tertiary structure of the molecule, both due to stereochemical arrangement of its radicals and also because of their localization within or very close to the grooves of α-helices, decreasing in consequence the accessibility to these residues by the antibodies.

3.2. *Purification of native* Pp-*Hyal*

The chromatographic profile of *P. paulista* crude venom (Fig. 6) produced eight peaks, designated A through H. Hyaluronidase activity was associated with peak F, with a total activity of 1.1 U/h. This corresponds to a recovery rate of 30%, taking into account that the total activity in crude venom was 3.6 U/h (100%). Thus, satisfactory recovery of specific hyaluronidase activity was obtained.

After collecting, pooling, and lyophilizing the samples with major *Pp*-Hyal activity (fractions 71–74 from peak F), 1.4 mg of total protein were obtained and 80 μg of which was subjected to SDS-PAGE to evaluate its level of purity, what was confirmed by the presence of only one band in the gel (Fig. 7). Fig. 8 shows the MALDI-ToF-ToF-MS spectra achieved after *in-gel* digestion of the *Pp*-Hyal protein band (from Fig. 7) with trypsin. Nine major tryptic peptide peaks were observed corresponding to ions with *m/z* 1060.51, *m/z* 1226.57, *m/z* 1342.63, *m/z* 1354.67, *m/z* 1372.72, *m/z* 1381.62, *m/z* 1913.84, *m/z* 2052.06, and *m/z* 2151.20. From these results, four peptides were identified by the Protein MASCOT Search Engine version 2.2, using the NCBI Protein Database, which revealed that they were similar to four regions of Hyal from *P. annularis* wasp venom (Q9U6V9), covering approximately 17% of this sequence (Score: 91, $p < 0.05$; see Supplementary Material). Through this

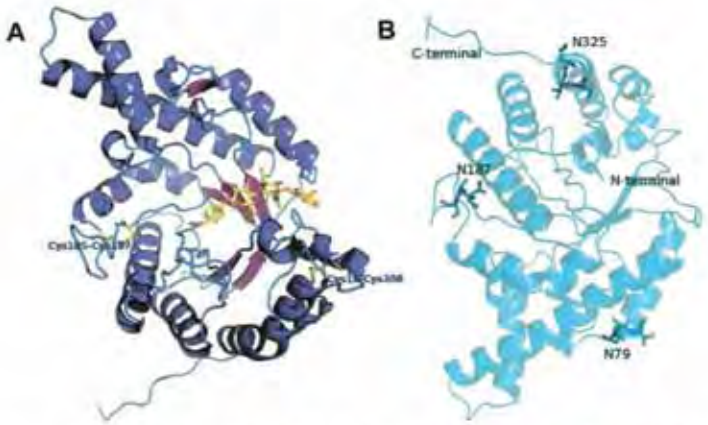

Fig. 3. (**A, B**) Two representations of the *Pp*-Hyal 3D-structural model (PMDB ID: PM0077230) are demonstrated in which it can be seen, respectively (**A**) two disulfide bonds formed between cysteine residues (Cys 185–197 and Cys 19–308) and (**B**) the putative sites of glycosylation (residues Asn79, Asn187, and Asn325), being the site Asn79 (5′ NITI 3′) coincident with that found in the rVes v 2 (PDB ID: 2ATM_A) (Skov et al., 2006) may be exerting direct influence on the molecule immunogenicity.

analysis it was also possible to determine a molecular mass of 43,277 Da and a calculated pI value of 8.13 for *Pp*-Hyal, while the values for the protein obtained by molecular cloning were a molecular weight of 39,648.8 Da and a pI of 8.77. These differences may result from the specificities of each technique and the degree to which the digested peptides retained their post-translational modifications, such as phosphorylation, acetylation, and glycosylation, which result in changes to the pI and molecular mass (Seo and Lee, 2004).

3.3. *Immunological analysis*

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

4. Discussion

Hyaluronidase of wasp venom is an allergen that has been extensively studied in several genders and species of

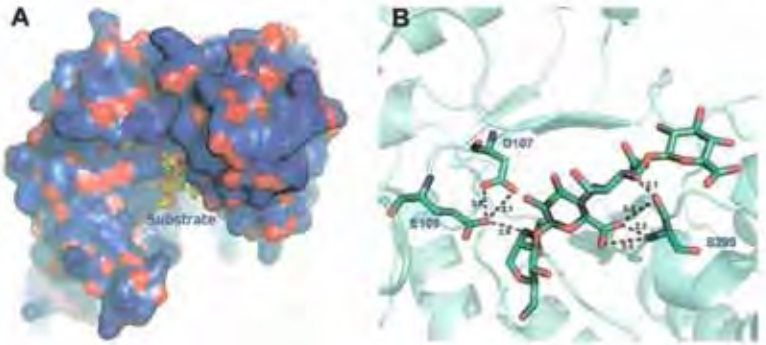

Fig. 4. (**A, B**) Show respectively (**A**) the topology of the *Pp*-Hyal molecule making evident the location of its active site when compared to the Hyal from *Apis mellifera* (PDB ID: 1FCQ) and (**B**) the predict amino acid residues in the model that are capable to establish interaction with the substrate Serine 299 (S299), Aspartate 107 (D107) and the Glutamate 109 (E109) what present hydrophilic character.

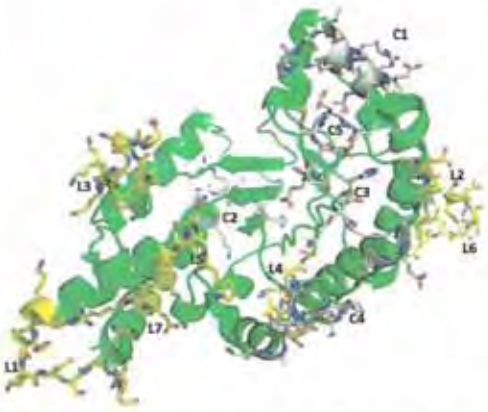

Fig. 5. Predicted antibody-binding sites (epitopes) in the *Pp*-Hyal 3D-structural model according analysis by ***Epitope Prediction Server*** (Kulkarni-Kale et al., 2005). **Five conformational (1C–5C) and seven linear (1L–7L)** epitopes can be observed, corresponding respectively to the positions 1C (295: NSLSKcKRIRE: 305); 2C (161: KKTrKRaK: 168); 3C (218: HKqDPEE: 224); 4C (181: SPNNPGPScDAK: 192); 5C (258: QDEMEI: 263). they are 1L (125: RKEhPKWSKSM: 135); 2L (266: SET: 268); 3L (51: IPVNNGKYKE: 60); 4L (241: NNIEHSpS: 248); 5L (110: WGNtE: 114); 6L (319: TETVNRRSSLNF: 330), and 7L (143: KKfE3KYaRY: 151). Amino acids in lowercase letters present percentage of accessibility by the antibody equal or less than 25% according to the CEP Server.

European and American wasps, but few studies have been conducted in Neotropical social wasps.

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

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

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

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

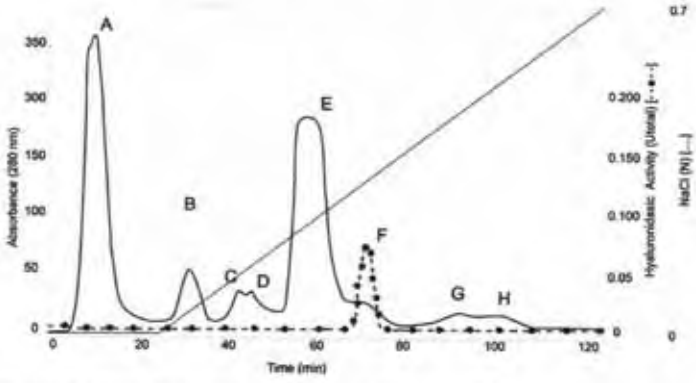

Fig. 6. Chromatographic profile of *P. paulista* crude venom extract (10 mg) obtained in a Hiprep FF CM column (160 mm × 10 mm, 20 mL – GE Healthcare) coupled to an AKTA-FPLC system. Column was washed with an isocratic buffer of 50 mM sodium acetate pH 5.2 and then the elution under a linear gradient from 0 to 1 M NaCl in the same buffer, at a flow rate of 2 mL/min collecting fractions of 2 mL. The protein was monitored by measuring the absorbance at 280 nm represented by continuous line (_____) while the dashed line (-·-·-·-) represents the *Pp*-Hyal activity.

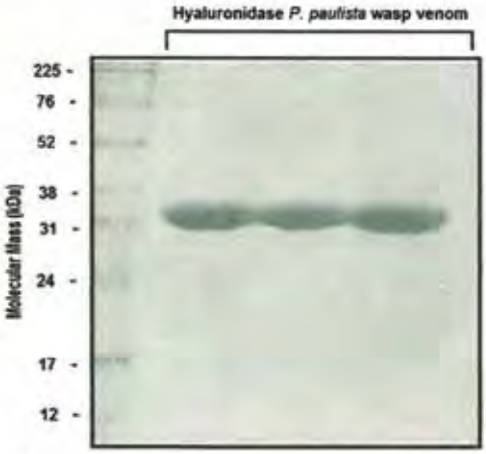

Fig. 7. Protein profile in SDS-PAGE (15%; w/v) of the purified fraction of native *Pp*-Hyal allergen (fractions F71 to 74 from peak F of cation exchange chromatography). About of 80 μg of total protein were applied on gel that was stained with *Coomassie Brilliant Blue R-250* (CBB).

identified it as a novel isoform of hialuronidase. All these differences can be ascribed to genetic variability as well as post-translational modifications.

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

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

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

Comparative analysis and superpositioning between the structures of Api m 2 co-crystallized with the substrate HA and that of *Pp*-Hyal revealed the presence of three amino acid residues that make contact with the polar hydroxyl nitrogen atoms of HA: Asp107, Glu109, and Ser299. In most glycosidases, two acidic residues play a central role in catalysis of the substrate, one of which acts as a proton donor while the other acts as a nucleophile (Markovic-Housley et al., 2000). In Api m 2, the only two residues that are highly conserved in the substrate binding site are Asp111 and Glu113, both of which appear to act as proton donors.

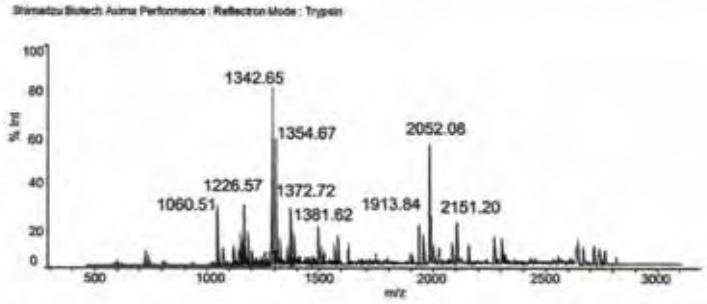

Fig. 8. MALDI-ToF/ToF-MS spectra of another form of *P. paulista* venom hyaluronidase after digestion with trypsin.

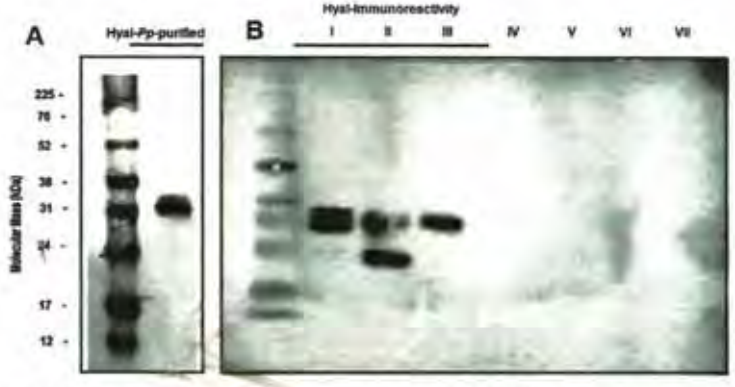

Fig. 9. (A, B) In **(A)** is shown the Western-blotting from a SDS-PAGE (15%; w/v) that contained 80 μg of *Pp*-Hyal protein FPLC purified run alongside a molecular standard mass **(M)**. Immunodetection was performed with *Pp*-Hyal-specific antibody diluted 1:1000 and anti-mouse IgG, alkaline phosphatase conjugate (Sigma–Aldrich, USA) diluted 1:5000 (2 μL/10 mL of blocking solution) as primary and secondary antibodies respectively, and revelation with colorimetric system of alkaline phosphatase. Notice that *Pp*-Hyal-specific antibody recognized only one band (38–39 kDa) in the pure fraction of *Pp*-Hyal native. In **(B)** is shown the Western blotting performed in the same condition, showing the *Pp*-Hyal-specific antibody high specificity in relation to *Pp*-venom crude extract (lane I) and different levels the cross-immunoreactivity with venom extracts of II – *Polybia sericea* and III – *Polybia ignobilis*, and no cross-reactivity with venoms of IV – *Agelaia pallipes pallipes*, V – *Polistes lanio lanio*, VI – *Apis mellifera* and VII – *Solenopsis invicta*. In each gel lane 80 μg of total protein of each extract were applied.

In the structure of *Pp*-Hyal characterized in this work, these two residues correspond to Asp107 and Glu109.

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

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

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

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

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

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2012.12.019.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Capítulo II

Title: Reactivity of IgE antibodies to the allergen hyaluronidase from *Polybia paulista* (Hymenoptera, Vespidae) venom in native and recombinant form

Débora Laís Justo Jacomini[a], Susana Margarida Gomes Moreira[b], Ricardo de Lima Zollner[c*] and Márcia Regina Brochetto Braga[a,d*].

[a] Laboratório de Biologia Molecular de Artrópodes-LBMA-IB-RC-UNESP (Univ Estadual Paulista), Av. 24-A, nº 1515, CEP 13506-900, Bela Vista, Rio Claro, SP, Brazil. mrbbraga@rc.unesp.br.

[b] Centro de Biociências. Universidade Federal do Rio Grande do Norte, Av. Sen. Salgado Filho, 3000, CEP 59072-970, Candelária, Natal, RN, Brazil. ssmargarida@gmail.com.

[c] Laboratório de Imunologia e Alergia Experimental-LIAE, Departamento de Clínica Médica, Faculdade de Ciências Médicas, FCM, Universidade Estadual de Campinas-UNICAMP, Rua Tessália Vieira de Camargo nº 126, Cidade Universitária "Zeferino Vaz", Campinas, SP, Brazil, CEP 13083-887. zollner@unicamp.br.

[d] Centro de Estudos de Venenos e Animais Peçonhentos-CEVAP (Univ Estadual Paulista), Rua José Barbosa de Barros, Fazenda Experimental Lageado-Botucatu, SP, Brazil, 1780, CEP 18610-307. mrbbraga@rc.unesp.br.

[*] Corresponding author. Phone/Fax: +55 19 3526 4135

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. mrbbraga@rc.unesp.br (Brochetto-Braga, MR)

Phone: 55+1932893709: Laboratório de Imunologia & Alergia Experimental, Rua Tessalia Vieira de Camargo, 126, Faculdade de Ciências Médicas, Unicamp, Campinas, SP, Brazil zollner@unicamp.br (Zollner, RL)

Abstract

Background: To date, there are no allergenic extracts or components available in Brazil to diagnosis and treatment of patients with venom allergy from social wasp (Vespidae Family; Polistinae Subfamily) despite of the great number of existing species.

Objetive: We evaluated the immunogenic potential of the Hyal recombinant protein (*Pp*-Hyal-rec) which was expressed in an insoluble form in comparison with the allergenic native protein (*Pp*-Hyal-nat) for recognition of immunoglobulin E (IgE) in the serum of allergic patients to venom of the endemic social wasp *Polybia paulista* from São Paulo State, Brazil.

Methods: Hyal cDNA from the venom of the social wasp *Polybia paulista* (*Pp*-Hyal) (GI: 302201582) was cloned into the expression vector pET-28a in *Escherichia coli* DE3 (BL21) cells. Solubilization and purification of *Pp*-Hyal-rec from inclusion bodies was performed using Ni^{2+} affinity chromatography (Ni-NTA-Agarose) under denaturing conditions. Both the native (*Pp*-Hyal-nat) and the recombinant (*Pp*-Hyal-rec) purified allergens were used for Western blotting to assess the levels of *Pp*-Hyal- IgE specific in the serum of 10 patients exclusively reactive to the venom of the social wasp *P. paulista*.

Results: The immune sera specifically recognized the band corresponding to the *Pp*-Hyal-rec protein (43 kDa) at a higher intensity than the native allergen (39 kDa). The sera recognized other proteins in *P. paulista* crude venom extract to a lesser extent, likely corresponding to other venom allergens such as phospholipase (37 kDa), Antigen 5 (25 kDa), and proteases.

Conclusion: The recognition pattern of the immune sera to the *Pp*-Hyal-rec allergen strongly suggests that this recombinant antigen could be used for developing a diagnostic allergy test as well as for specific immunotherapy (IT).

Keywords: *Polybia paulista*, venom, allergen, recombinant hyaluronidase, immunoglobulin E (IgE), cross-immunoreactivity.

Introduction

Brazil has more than 300 species of social wasp (Vespidae Family; Polistinae Subfamily) of which 104 are endemic.[1] Brazilian species are typically neotropical, with very different venoms from those found in temperate species. The Polistinae subfamily is represented by three tribes: Polistini, Epiponini and Mischocyttarini. The Epiponini tribe is constituted by 23 genera and 405 species, including the *Polybia paulista* species, which belongs to the Hymenoptera order. [2-3]

Because of the close coexistence of social Hymenoptera with humans, the incidence of stings has increased. When stings occur, victims who have been sensitized to the injected venom may experience severe allergic reactions to the venom at the sting site, potentially resulting in manifestations of systemic anaphylaxis such as anaphylactic shock. Systemic anaphylaxis is life threatening to the allergic patient, potentially causing arrhythmia, bronchospasm, respiratory and cardiac arrest, shock and death of the patient.[4,5]

An estimated 25% of the general population suffers from some type of allergy, including those triggered by incidents with insect venom.[6] Estimates of the proportion of individuals who are sensitive to social Hymenoptera venoms fall between 9.3% and 28.5%[7], indicating that these allergies are a significant public health concern. Systemic reactions to venom from this order of insects occur in approximately 0.8-5.0% of the overall population, and severe local reactions occur in 19%. These manifestations are directly associated with IgE-mediated sensitization to the proteins present in venoms of social Hymenoptera species, such as bees, wasps and ants.[8-10] Thus, knowledge about the structure and function of these insect venom components, especially in the fields of allergy and clinical immunology, is crucial.[11,12]

Although the components of vespid venom are generally less well characterized than those from bee venom, the habitat and venom composition of the social wasp *P. paulista* have been well studied in the fields of biochemistry, proteomics and immunology due to the accidents arising from its stings.[13-16] Its venom has a similar protein composition to most venoms of the social Hymenoptera, consisting of biogenic amines, basic peptides and proteins of high molecular masses, particularly enzymes, among them the hyaluronidase, one of the main proteins responsible for allergic reactions. The biologically active peptides, together with other venom proteins are the main responsible for the prolonged pain, edema, erythema and systemic reactions.[17,18]

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

allergens for measuring specific IgE represents an important advance in the diagnosis and treatment of IgE-mediated allergies.[19]

The use of recombinant DNA technology for gene sequencing and expression of the major venom constituents from various organisms has enabled the elucidation of the molecular mechanisms of immunological cross-reactions among venom allergenic components, as well as the development of diagnostic kits and allergen-specific vaccines. Although there have been great advances in various expression systems, including bacteria, yeast, insect, mammalian cells and even cell-free systems, the bacterial expression system is still the most used due to its easy handling, rapid growth and low cost of recombinant protein production.[20]

In this study, Hyal cDNA from the venom of the social wasp *P. paulista* (*Pp*-Hyal) (GI: 302201582) was cloned, expressed and analyzed immunologically in comparison to the natural allergen. The results demonstrate that the allergen *Pp*-Hyal-rec has great potential for use in immunotherapy, as well as in diagnostic kits for allergies caused by the venom from this and other closely related wasps.

Materials and Methods

Allergic Patients

The samples studied consisted of 10 sera from patients injured by stings from the social wasp *P. paulista,* with exclusive reactivity to this venom. All patient samples were obtained from the Ambulatório de Anafilaxia do Hospital das Clínicas, Faculdade de Ciências Médicas da Universidade Estadual de Campinas-UNICAMP regardless of sex or age. This institution offers treatment to patients from both the capital and the bordering municipalities of São Paulo state and southern Minas Gerais, Brazil. The study was approved by the Ethics Committee-FCM-UNICAMP under nº 187/2006. Informed consent was obtained from all study participants and study participation was voluntary. The samples were taken by collecting 5 ml of additional blood during the administration of routine tests ordered by the attending physician.

Insects

P. paulista nests captured around the campus of Universidade Estadual Paulista "Julio de Mesquita Filho"-UNESP, Rio Claro, SP, Brazil, were transported to the Laboratório de Biologia Molecular de Artrópodes (LBMA)- Departamento de Biologia - IB. After being subjected to analgesia at -20°C, wasp venom glands were dissected and used for RNA extraction.

Native Allergen (Pp-Hyal-nat)

Native hyaluronidase from the venom of the social wasp *P. paulista* (*Pp*-Hyal-nat) was isolated and purified by cation exchange chromatography on AKTA-FPLC and analyzed by mass spectrometry MALDI-ToF/ToF. All analyses were performed according to the protocol previously described.[21]

RNA extraction and cDNA synthesis

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

Cloning of Hyal coding sequence

The *Pp*-Hyal coding sequence was obtained by PCR from venom of the *P. paulista* social wasp using specific primers based on the Hyal cDNA complete sequence (GI: 302201582). Restriction sites for *Nde*I and *Eco*RI were, respectively included in the forward (5' TATA CATATG TCCGAAAGAAAGACCGAAAAGAGTGTTCAAC 3') and reverse (5'AGTG GAATTC CTAAAAGTTCAGGGATGATCT 3') primers (underlined) to allow the cloning of the coding sequence into the pET28a expression system. The *Pp*-Hyal sequence was also cloned in frame with a 6xHis tag at the N-terminal of the recombinant protein to allow purification of the recombinant protein by affinity chromatography. PCR was performed using Platinum Taq DNA Polymerase® (Life Technol, EUA) following the protocol previously described[21] and the *E. coli* XL1 Blue [recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F proAB lacIqZΔM15 Tn10 (Tetr)] (Stratagene, USA) was used as the cloning strain. The nucleotide sequence of the cloned region was verified by sequencing.

Expression and purification of recombinant Pp-Hyal

For the optimization of recombinant protein production, several clones of *E. coli* BL21 (DE3) cells [F- ompThsdSB (r-B mB-) gal demΔ(srl-recA) 306::Tn10(DE3)] (Novagen, USA) transformed with the expression vector pET28a_*Pp*-Hyal were tested under different expression conditions. Briefly, each selected clone of BL21-DE3 pET-28a_*Pp*-Hyal was inoculated into 5 mL of Luria Broth (LB) medium supplemented with kanamycin (50 μg/mL) and allowed to grow for 16 h at 37°C. These pre-inoculums were used to inoculate 50 mL of fresh medium. After cultures reached OD_{600nm} 0.3-0.6, they were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, Invitrogen, EUA) at different final concentrations (0.1, 0.5 and 1 mM) and different induction temperatures (37, 30 and 20ºC). Samples of 1.5 mL were collected at several times post-induction (0, 12, 24, 30, 48 and 72 h) and tested to determine the best induction conditions to produce recombinant protein *Pp*-Hyal.

Cells were collected by centrifugation (2,600 g, 10 min), resuspended in phosphate-buffered saline pH 8.0 and then lysed by sonication in an ice bath for 2 min (Chubby, GA450) using three pulses of 20 s, with 30 s between each pulse. The soluble and insoluble fractions were separated by centrifugation (9,200 g, 4°C, 30 min), analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 15%) and stained with *Coomassie Brilliant Blue R-250* (CBB).

After optimization of expression conditions, one *E. coli* BL21-DE3 clone carrying pET-28a_*Pp*-Hyal was selected for the production and purification of *Pp*-Hyal-rec protein. Ten milliliters of pre-inoculum, prepared as described, were used to inoculate 500 mL of LB/kanamycin (50 μg/mL) and the culture was incubated at 37ºC under agitation (200 rpm) until OD_{600nm} 0.6 was reached. To induce protein expression, IPTG was added at a final concentration of 1 mM and cells were incubated at 37ºC for 48 h. Cells were collected by centrifugation (10,000 g, 10 min, 4ºC) and lysed by sonication. Because recombinant protein was expressed in the insoluble fraction as inclusion bodies, its purification by affinity chromatography was performed under denaturing conditions, following the Ni-NTA-Agarose manufacturer's protocol (Qiagen, Germany). In brief, the cellular pellet was resuspended in 25 mL of lysis buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 5 mM imidazole and 0.2 mg/mL lysozyme) and incubated at 20ºC with gentle agitation for 25 min before the sonication procedure (5x30 s pulses, maintaining the samples in ice bath between the pulses). After centrifugation (15,000 g, 15 min, 4ºC), the insoluble fraction was resuspended in 10 mL of inclusion body solubilization buffer (8 M urea, 0.5 M NaCl, 50 mM Tris-HCl pH 8.0, 5 mM β-mercaptoethanol and 5 mM imidazole) and incubated overnight at 8ºC under gentle

agitation. The sample was then centrifuged (20,000g, 15 min, 4ºC) and the soluble fraction incubated (1 h at room temperature) with Ni^{2+}resin (Ni-NTA-Agarose, Qiagen, Germany) that had been previously equilibrated (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 8.0). This suspension was then applied to the column (5 mL) and washed with 10x column volume of washing buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 6.3). Recombinant protein was eluted using elution buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 4.5). Several samples were collected during the purification process for subsequent analysis by 15% SDS-PAGE.

IgE Analysis

Sera from 10 patients allergic to *P. paulista* venom were evaluated for specific IgE recognition using 80 µg of purified *Pp*-Hyal-nat and *Pp*-Hyal-rec proteins, as well as 100 µg of *Pp*-crude-venom extract that had been previously submitted to 12% (w/v) SDS-PAGE along with a pre-stained standard molecular weight protein (High-molecular Weight Range Rainbow Markers, Amersham Biosciences, GE Healthcare, USA). The proteins were transferred to a nitrocellulose membrane using a semi-dry system (Nova Blot Unit, Multiphor II, Pharmacia Biotech). Transfer efficiency was assessed by staining the gel with Coomassie Blue G-250. The sera were previously diluted (1:50) in blocking solution (TBS-20% Tween containing 1% non-fat milk) and incubated overnight at 4°C. Immunodetection was performed using monoclonal antibody Anti-human IgE alkaline phosphatase conjugated (Sigma-Aldrich, USA) diluted 1:5000. Bands were visualized with alkaline phosphatase/BCIP®/NBT (Sigma-Aldrich, USA).

IgE Cross-reactivity

To analyze the IgE immune cross-reactivity of the allergic patients' sera, 5 sera among the 10 previously tested were randomly chosen. Venom extracts were prepared from other wasp species (*Polybia sericea*, *Polybia ignobilis*, *Polybia occidentalis*, *Polybia scutellaris*, *Polistes lanio lanio* and *Agelaia palipes palipes*) as well as bee (*Apis mellifera*) and ant (*Solenopsis invicta*) and quantified for total protein, as described previously.[21] From each one of these extracts, 80 µg of total protein was subjected to gel electrophoresis for immunoblotting analysis.

Results

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

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

Pp-Hyal-rec expression in E. coli

The allergenic protein *Pp*-Hyal-rec was heterologously expressed with a 6xHis tail at the N-terminal for its purification by affinity chromatography with Ni^{2+}-agarose (Ni-NTA-Agarose) resin. Different expression conditions were tested, including induction with varying concentrations of IPTG as well as different times and temperatures of induction. The best results were obtained with clone 8 under conditions of 1 mM IPTG and growth at 37°C for 48 hours, which resulted in the highest quantity of protein expressed (**Figure 1B**). In times less than 24 hours, low levels of protein expression were observed.

Analysis of *Pp*-Hyal-rec protein in the soluble and insoluble fractions of bacterial lysate showed that expression occurred in inclusion bodies, as seen in **Figure 1** (**A, B**), independent of the conditions used for induction of expression.

Inclusion Body Solubilization and Pp-Hyal-rec Purification

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

Specific IgE immunodetection

The IgE immunoreactivity of the *Pp*-Hyal-rec protein was assessed by Western blotting with sera from patients exclusively reactive to the venom of the social wasp *P. paulista*. Specific reactivity was tested against *P. paulista* crude venom extract as well as the purified native and recombinant forms of the *Pp*-Hyal allergenic protein. Serum from a non-sensitive individual was used as negative control. **Figure 3** (**A, B**) shows that sera from the 10 patients tested recognized the crude venom extract as well as both native and recombinant *Pp*-Hyal proteins,

with the band corresponding to recombinant protein *Pp*-Hyal (43 kDa) recognized at a higher intensity than the native allergen (39 kDa). In *P. paulista* crude venom extract, sera also recognized additional proteins besides Hyal with less intensity; these proteins are likely other allergenic components of the venom.

Analysis of IgE Immune Cross-reactivity

Immune cross-reactivity of 5 randomly chosen sera was tested against venom extracts from several related species. As shown in **Figure 4** (**A-E**), we can verify the existence of immune cross-reactivity due to IgE recognition of a protein with molecular weight between 39-43 kDa, likely corresponding to the allergenic protein Hyal, in venom extracts of *Polybia sericia*, *ignobilis* and *scutellaris*, as well as in *Polistes lanio lanio.* No IgE binding was detected with crude venom extracts of *Apis mellifera* and *Solenopsis invicta*. The different profiles and intensities of reactions observed in the patients tested demonstrate the differences in IgE specificity and production levels. Additionally, we can observe in both **Figure 4** (**A-E**) and **Figure 3** the recognition of other proteins in addition to the Hyal isoforms, which likely corresponds to other allergenic proteins from the venom of these wasps.

Discussion

Scientific studies of Hymenoptera stings have expanded into different areas of the biological sciences due to the diverse aspects of the reactions presented by victims after injury, especially those that trigger severe systemic manifestations that may lead to death.[22] The social wasp *P. paulista*, endemic to southeastern Brazil, has a characteristically urban habitat and a very aggressive profile. Its stings result in many clinically significant incidents due to the complexity of its venom composition, which includes important allergens such as hyaluronidase (*Pp*-Hyal).

Dotimas and Hider[23] reported that the allergens hyaluronidase and phospholipase are capable of provoking severe immunological reactions; these allergens are the major proteins responsible for the onset of allergic manifestations, triggering the production of specific IgE and causing hypersensitivity reactions in individuals stung by Hymenoptera. In turn, this class of antibody can bind to the high affinity Fc receptor (FceRI) on the surface of mast cells and basophils, which consequently become sensitized, causing degranulation and the release of other factors.[24,25] In this study, the *Pp*-Hyal-rec, obtained by recombinant DNA techniques, was retained in bacterial inclusion bodies. Alternatives to minimize the formation of these aggregates, such as decreasing temperature and changing the concentration of the expression

inducer, were tested, but did not successfully increase the expression of the soluble form of the *Pp*-Hyal-rec protein.

Proteins expressed in bacterial inclusion bodies are usually expressed at high levels, in some cases reaching 30% of the total protein from the host cell. These proteins thus exhibit lower levels of degradation, resistance to proteolytic attack by cellular proteases and greater homogeneity of the protein of interest, all of which help to reduce the number of purification steps for recovery of the pure protein.[26,27] Furthermore, the purified proteins can be used with increased reliability for the production of specific polyclonal antibodies, as well as for the production of diagnostic tests and possibly gene-specific immunotherapy.

The immunogenic potential of *Pp*-Hyal-rec expressed in *E. coli* was compared to the native allergen (*Pp*-Hyal-nat) by measuring the IgE reactivity of sera from 10 patients known to be allergic to *P. paulista* venom, as well as by determining the cross reactivity of these IgEs with venoms from different species of Hymenoptera (wasp, bee and ant).

Despite being in denatured form, after solubilization and purification the recombinant protein was able to bind to serum IgE of all allergic patients tested, and to a greater extent than the binding observed with *Pp* Hyal-nat. These results strongly indicate that the linear epitopes, already described by structural modeling[21] remained available in *Pp*-Hyal-rec and could be recognized by all sera IgEs from the allergic patients previously exposed to *P. paulista* venom. This also implies that the *Pp*-Hyal-rec protein is immunologically reactive even in its denatured form and thus has great potential to be used in specific immunotherapy.

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

Regarding the IgE cross-reactivity of the sera studied here, with venoms of other species of Hymenoptera, we found that except for the reactions observed only with wasp venoms, IgE-mediated cross-reactivity with venoms from *Apis mellifera* and *Solenopsis invicta* was not detected under the conditions tested. These results confirm those previously obtained with *Pp*-Hyal-nat[21] but differ from those obtained by Aalberse et al.[28], Hoffman et al.[29], Hoffman[30] and Egner et al.[31] showing the occurrence of cross-reactivity with bee and wasp venoms from temperate climates in diagnostic tests applied to Hymenoptera allergic patients before the beginning of IT. In these studies, the authors indicate that, similar to our results, hyaluronidase is the main protein responsible for this response. According to King et al.[32],

patients allergic to wasp venoms exhibit responses to more than one venom due to cross-reactivity among homologous allergens present in the venoms of different species. This phenomenon is due to the presence of common epitopes reactive to specific IgE[33] because the antibodies are raised against highly conserved regions in these proteins.[34]

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

Recombinant protein technology has allowed for the production of a large number of individual allergens and pools of allergens to produce new therapeutic products, as well as tools for diagnosis and for allergen-specific immunotherapy. Considering that robust reactivity of allergic patient sera against *Pp*-Hyal-rec was verified by our immunoblotting results, this recombinant allergen has potential for use in the development of more sensitive and standardized specific immunodiagnostic tests to assist in the monitoring and immunotherapy (IT) of patients allergic to this social insect that is quite abundant in southeastern Brazil.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

Legends

Figure 1. SDS-PAGE (12%) showing the expression profile of *Pp*-Hyal-rec in *E. coli* BL21-DE3 cells as a function of the cultures' growing time at 37°C and after induction with 1 mM IPTG. **(A)** represents the soluble fraction of the bacterial lysate, indicating the absence of *Pp*-Hyal-rec in all cultivation periods tested (24 h, 30 h, 48 h and 72 h), **(B)** represents the insoluble phase (inclusion bodies) showing that expression of *Pp*-Hyal-rec (MW 39 kDa) reached the highest levels after 48 h of cultivation.

Figure 2. Protein profile in 12% SDS-PAGE representing steps of solubilization and purification of the *Pp*-Hyal-rec attached to 6xHis, in an Agarose-Ni^{2+} (Ni-NTA-Agarose) affinity column previously equilibrated with buffer (50 mM Tris-HCl pH 8.0, 0.5 M NaCl, 5 mM β-mercaptoethanol, 5 mM imidazole and 8 M urea). **(A)** depicts in channel **(1)** proteins that did not bind to the resin and in **(2)** the fraction obtained after washing the column with washing buffer (100 mM NaH2PO4, 10 mM Tris-HCl, 8 M urea, pH 6.3). **(B)** shows the purified protein with a molecular mass of 39 kDa that was eluted with 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 4.5. **(M)** Represents a standard molecular mass marker. Gels were stained with Coomassie Brilliant Blue R-250 (CBB).

Figure 3. Western blot from a 12% SDS-PAGE demonstrating the reactivity of IgE immune sera from 10 allergic patients to 80μg of purified native and recombinant forms of *Pp*-Hyal as well as to 100μg of total protein from *P. paulista* crude venom extract. In **(A)**, the channel **(-)** represents a negative control containing sera from a insensitive patient, and the channel (***Pp*_crude venom**) represents the IgE-mediated reactivity of serum of 1 of the 10 samples (randomly selected) against the allergenic protein *Pp*-Hyal (39 kDa, higher intensity band), and the lesser intensity of IgE recognition to other allergenic proteins present in the venom crude extract of this wasp (e.g., AG5, PLA1 and proteases). **(B)** and **(C)** show allergen-specific IgE binding to the native protein (*Pp*-Hyal-nat, 39 kDa) and to the recombinant protein (*Pp*-Hyal rec, 43 kDa), respectively, for 10 immune sera. Note that all sera recognized the recombinant allergen at a higher intensity than the native one. **(MW)** represents the standard molecular mass marker. Immunodetection was performed using Anti-human IgE alkaline phosphatase conjugated monoclonal antibody (Sigma-Aldrich, USA) diluted 1:5000, followed by visualization with the alkaline phosphatase/BCIP®/NBT colorimetric system (Sigma-Aldrich, USA).

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

Figures

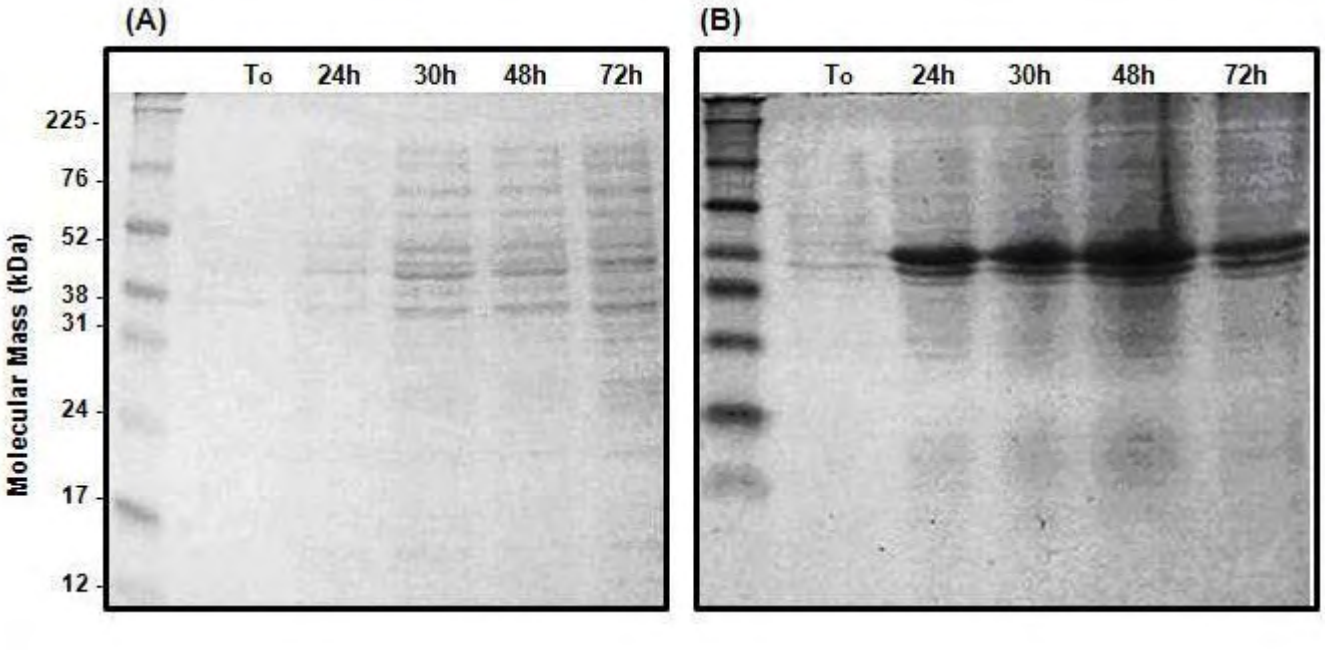
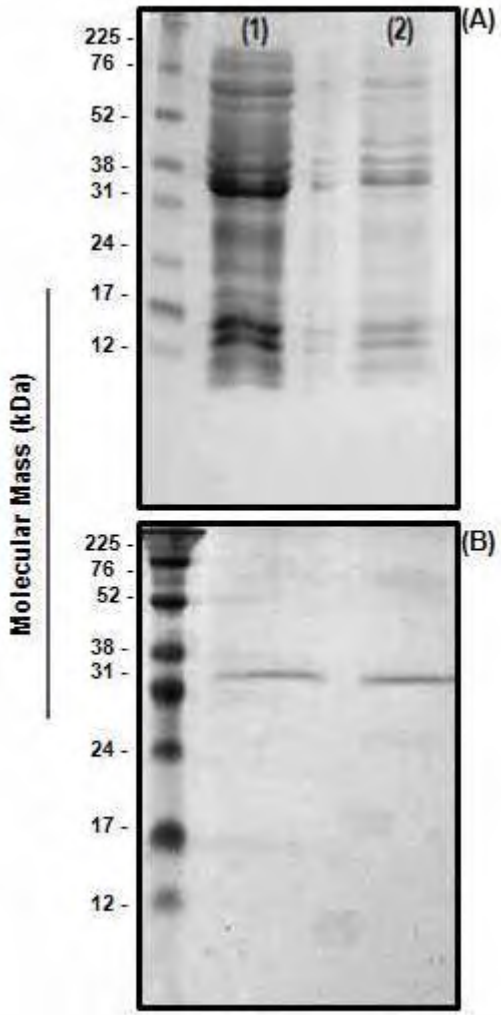


Fig.1


(A)
(1)
(2)
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Molecular Mass (kDa)
(B)
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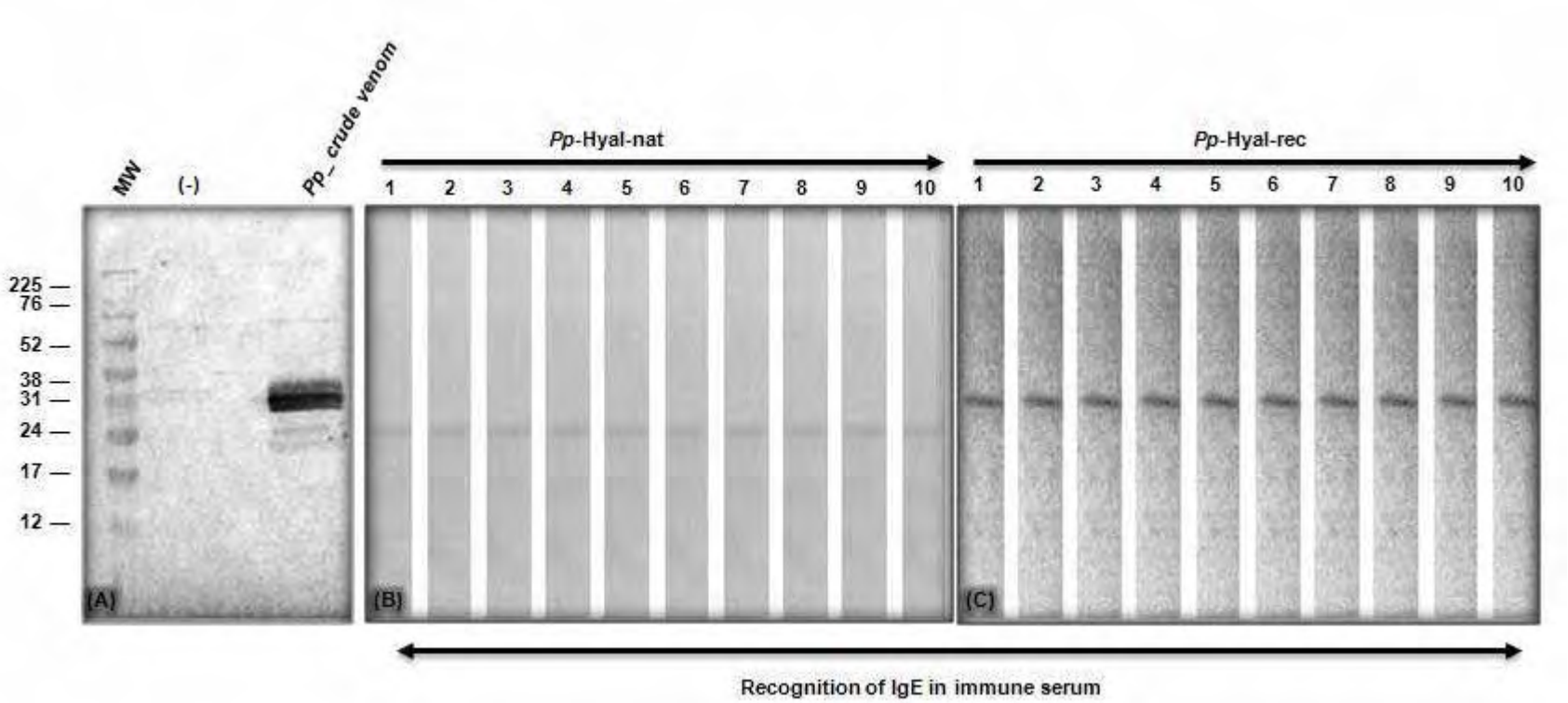
Fig 2


MW
(-)
Pp_ crude venom
Pp-Hyal-nat
Pp-Hyal-rec
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1 2 3 4 5 6 7 8 9 10
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31
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(A)
(B)
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Recognition of IgE in immune serum

Fig 3

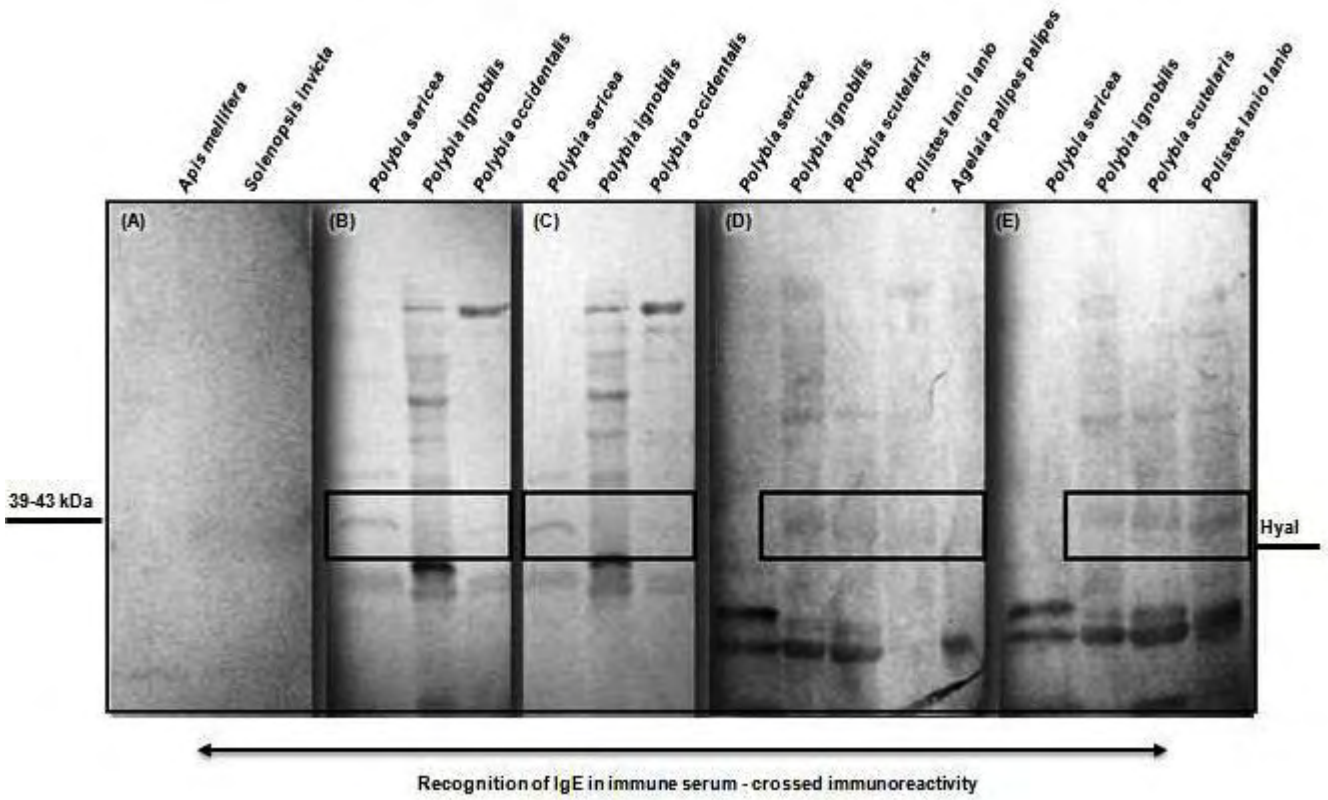
Apis mellifera
Solenopsis invicta
Polybia sericea
Polybia ignobilis
Polybia occidentalis
Polybia sericea
Polybia ignobilis
Polybia occidentalis
Polybia sericea
Polybia ignobilis
Polybia scutellaris
Polistes lanio lanio
Agelaia palipes palipes
Polybia sericea
Polybia ignobilis
Polybia scutellaris
Polistes lanio lanio
(A)
(B)
(C)
(D)
(E)
39-43 kDa
Hyal
Recognition of IgE in immune serum - crossed immunoreactivity

Fig 4

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Capítulo III

III.1 Introdução

Conforme mencionado anteriormente, os sistemas de expressão mais utilizados para a produção de proteínas recombinantes atualmente são baseados em culturas de bactérias, leveduras, fungos filamentosos, células de insetos (via baculovírus) e células de mamíferos ou em organismos transgênicos (plantas e mamíferos) (DEMAIN, VAISHNAV, 2009).

As vantagens no uso de leveduras são a alta produtividade, crescimento rápido e em alta densidade celular, linhagens recombinantes mais estáveis, secreção mais eficiente e processamento pós-traducional mais similar ao de mamíferos (DEMAIN, VAISHNAV, 2009). A principal desvantagem é a presença de um padrão de glicosilação diferente do que ocorre nos humanos (HINNEN et al., 1994; JUNG, WILLIAMS, 1997; TATE, GRISSHAMMER, 1996).

A levedura *P. pastoris* tem revelado excelente potencial para a expressão heteróloga, pois possibilita vantagens tais como, a capacidade deste organismo de realizar modificações pós-traducionais, conforme normalmente ocorre em eucariotos superiores e o fácil aumento de escala de produção da proteína de interesse, permitindo desta forma, que altos níveis de expressão de genes heterólogos e a secreção de diferentes proteínas com importância comercial e medicinal sejam obtidos (CEREGHINO, CREGG, 2000). Além disso, este sistema de expressão tem se mostrado como um eficiente hospedeiro para a síntese e secreção de proteínas heterólogas para aplicações acadêmicas ou industriais (COS et al., 2005).

P. pastoris é uma levedura pertencente ao reino Fungi, divisão Eumycota, subdivisão Ascomycotina, classe Hemoascomycetes, ordem Endomycetales, família Saccharomycetaceae e subfamília Saccharomycetoideae. Kurtzman (2009) reclassificou esta levedura no novo gênero *Komagataella* e a dividiu em três espécies: *K. pastoris*, *K. phaffii* e *K. pseudopastoris*.

A mais notável característica fisiológica da levedura *P. pastoris* é o fato dela ser metilotrófica, ou seja, capaz de crescer em meio contendo metanol como única fonte de carbono. Isto ocorre graças à hiperexpressão de uma enzima peroxissomal chamada álcool oxidase, que pode atingir até 30% das proteínas intracelulares na presença de metanol (COUDERC, BARATTI, 1980).

O sistema de expressão da *P. pastoris* foi desenvolvido exatamente com base na sua habilidade de crescer em metanol como única fonte de carbono e energia. Neste organismo, dois genes cromossômicos *AOX* (*AOX*1 e *AOX*2) que codificam álcool oxidase foram identificados. O promotor do gene AOX1 (álcool oxidase 1) é fortemente induzido durante o crescimento das células em presença de metanol, sendo responsável pela maior parte da

atividade da álcool oxidase na célula. Esta enzima, que constitui aproximadamente 20-30% da proteína celular total, catalisa a primeira etapa do metabolismo de metanol, isto é, sua oxidação em formaldeído, utilizando oxigênio molecular (ROMANOS et al., 1992; CREGG et al., 1993; FABER et al., 1995; LAL et al., 1997; KATAKURA et al., 1998). *P. pastoris* também abriga um segundo gene funcional de álcool oxidase, *AOX*2, apresentando homologia de 97% e aproximadamente a mesma atividade específica que *AOX*1. A interrupção do gene estrutural *AOX*1 produz uma linhagem que cresce lentamente em metanol (MutS, *AOX*1*AOX*2), podendo resultar em uma proporção acentuada de acúmulo de proteínas heterólogas quando comparada com a linhagem Mut+ (*AOX*1, *AOX*2) (FABER et al., 1995). Uma vez que o fenótipo Mut (utilização de metanol) afeta a taxa de crescimento, durante a indução, as linhagens MutS podem produzir altos níveis de proteínas heterólogas corretamente enoveladas e a velocidade de enovelamento é um fator limitante.

A secreção de proteínas heterólogas, por meio do sistema de expressão de *P. pastoris*, é complexa e depende não apenas de fatores como dosagem do gene e fenótipo Mut, mas também de fatores como seqüência sinal, processamento, proteólise e glicosilação, os quais afetam diretamente no rendimento e na qualidade do produto (ROMANOS, 1995).

Devido aos requisitos de estabilidade e dobramento correto da proteína, a opção de secreção para o meio de cultura é a reservada para proteínas heterólogas que são normalmente secretadas nos organismos de origem (CEREGHINO, CREGG, 2000). Entretanto, para se obter uma linhagem produtiva há necessidade de um grande esforço de investigação a fim de que os fatores que limitam a produção da proteína heteróloga possam ser controlados.

Adicionalmente, este tipo de sistema não apresenta problemas como produção de endotoxinas como ocorre com bactérias, nem tampouco, problema de contaminação viral como costuma ser encontrado na expressão de proteínas em cultura de células animais (LIN-CEREGHINO et al., 2002).

Outro fator importante é que geralmente, a utilização dos códons em *P. pastoris* é semelhante à usada para expressão em *Saccharomyces cerevisiae* (DE SCHUTTER et al, 2009) e totalmente diferente da utilizada em bactérias ou insetos. Assim, a análise do "*codon usage*" e sua otimização são etapas essenciais para melhorias substanciais na expressão da proteína recombinante em *P. pastoris*.

Resumidamente, a relativa facilidade na manipulação genética aliada aos baixos custos de produção faz desta levedura um versátil sistema de expressão sem precedentes na Biotecnologia moderna.

Portanto, para a execução da etapa de expressão da proteína alergênica hialuronidase (*Pp*-Hyal) em *P. pastoris*, fizemos primeiramente análises cuidadosas para otimização do "*codon usage*", com conseqüentes modificações na seqüencia de cDNA de *P. paulista* (NCBI-GenBank-GI: 302201583) de forma que esta proteína pudesse ser clonada corretamente e expressa por este sistema.

III.2 Material e Métodos

III.2.1 Otimização do "*codon usage*" de cDNA (NCBI-GenBank-GI: 302201583)

Para obtenção da proteína recombinante na forma secretada, a otimização cuidadosa do "*codon usage*" foi a primeira etapa a ser realizada. Para isto, a seqüencia de cDNA de *Pp*-Hyal especificada acima, foi analisada pelo *Software Graphical Codon Usage Analyser*, o qual revelou a necessidade de muitas modificações nos códons desta seqüencia de cDNA para obtenção da proteína em estudo. Estas modificações implicavam na necessidade de um longo processo experimental de ***mutagênese dirigida***, tendo-se ainda muitas incertezas quanto à obtenção final da seqüencia correta a ser expressa, devido aos subprodutos que seriam gerados pelas várias etapas de PCRs requeridas.

Por esta razão, optamos pelo processo de síntese direta do gene em estudo por uma empresa especializada (*GenScript* - USA). Com isso, os experimentos relacionados à expressão da molécula madura do alérgeno *Pp*-Hyal na levedura *P. pastoris* foram realizados a partir do DNA sintetizado por esta empresa.

III.2.2 Descrição do vetor pPICZαA

Para expressão da *Pp*-Hyal de forma a ser secretada no meio de cultura da levedura *P. pastoris* foi utilizado o vetor pPICZαA (Invitrogen®, USA) e a cepa de *Pichia* X-33, este vetor permite que altos níveis de proteínas recombinantes sejam expressos. O vetor pPICZαA (**Figuras 1** e **2**) possui 3,6 kb, apresenta a sequência sinal "fator α" de *Saccharomices* que permite com que a proteína expressa seja endereçada para o meio extracelular, exibe a possibilidade de ser digerido com enzimas de restrição específicas na região *polylinker* [*Xho* I (sítios 1185, 1247), *Not* I (sítio 1259), entre outras enzimas] e possui também, uma seqüencia na região N-terminal que codifica seis histidinas denominadas "His-Tag". Dessa forma, o gene de interesse é clonado em fusão com esta seqüencia, facilitado a purificação da proteína expressa, por cromatografia de afinidade. Se necessário, a cauda de histidina pode ser removida, após a clivagem com a protease trombina. A seleção dos clones de leveduras recombinantes (contendo vetor + inserto) deve ser feita com o antibiótico Zeocina, uma vez que o vetor usado possui um gene que confere resistência a este antibiótico.

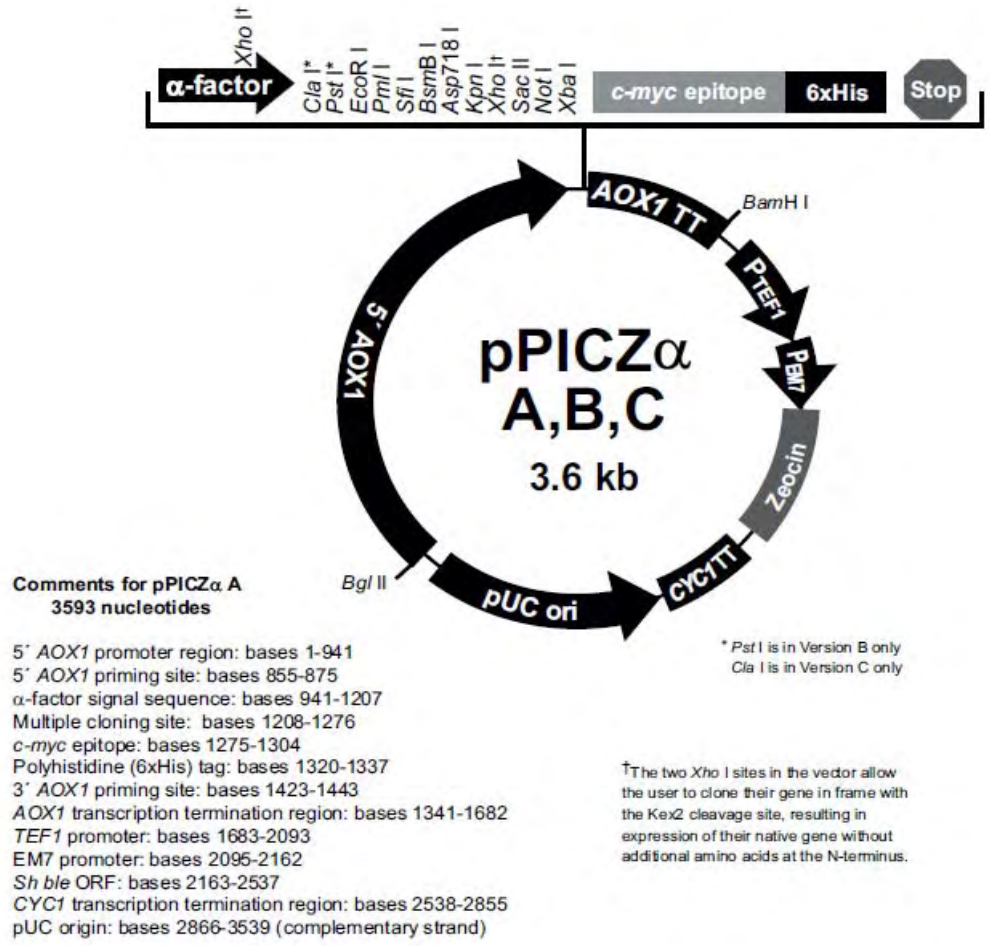

Figura 1. Mapa dos Vetores de expressão pPICZαA, B e C. Notar que as especificações do vetor pPICZαA estão à esquerda. Fonte: pPICZα A, B, and C. *Pichia* Expression Vectors for Selection on Zeocin and Purification of Secreted, Recombinant Proteins (Invitrogen -Catalog no. V195-20).

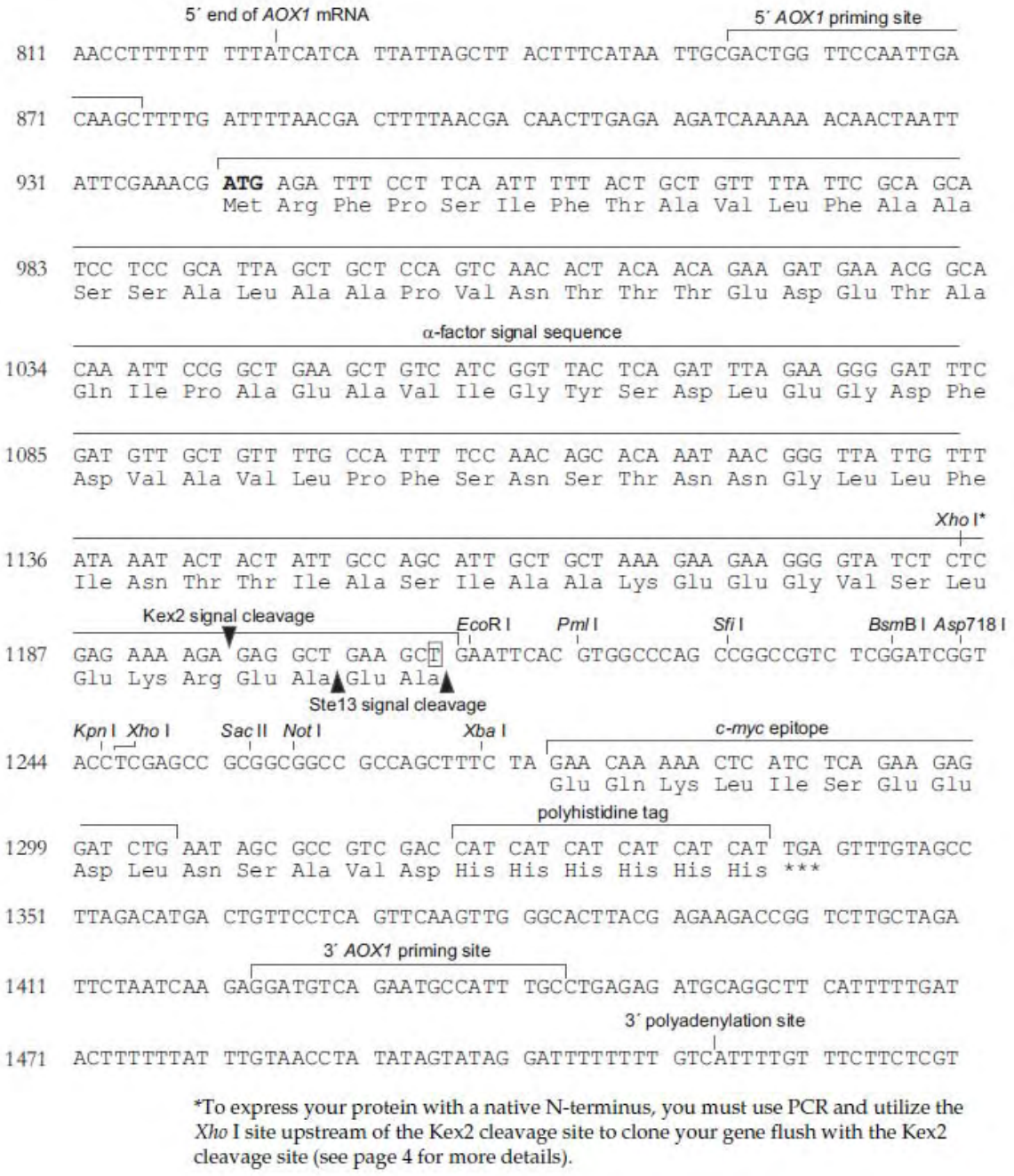

Figura 2. Sítio de múltipla clonagem no vetor pPICZ α A. Os sítios de restrição estão marcados para indicar o sítio de clivagem. Os nucleotídeos nos retângulos indicam a região variável. Fonte: pPICZα A, B, and C. *Pichia* Expression Vectors for Selection on Zeocin and Purification of Secreted, Recombinant Proteins (Invitrogen - Catalog no. V195-20).

III.2.3 Síntese do gene de hialuronidase de *P. paulista,* otimizado para expressão em *Pichia pastoris,* clonagem em vetor de entrada e sub-clonagem em vetor de expressão, para amplificação, em células de *E. coli*

Após otimização e síntese da seqüência de DNA de hialuronidase de *P. paulista*, para expressão em *Pichia* e sintetizada, foi realizado o processo de clonagem inicial em vetor pUC57 e em cepa de *E. coli* DH5α [F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1] para amplificação, e após isolamento destes plamídeos, digestão e purificação da sequência, foi feita a sua ligação no vetor de expressão pPICZαA, o qual, após confirmação da presença do inserto, foi transformado em bactérias *E. coli*. A seguir, foram preparadas minipreps, de acordo com o protocolo descrito previamente (QIAprep, Qiagen, Germany), a partir das colônias de bactéria *E.coli* contendo o vetor recombinante *Pp*-Hyal-pPICZαA. O DNA plasmidial (correspondente ao vetor recombinante) foi então cortado com a enzima de restrição *Pme* I, uma das três enzimas usadas que permitem a linearização do vetor para correta integração do mesmo no genoma de *Pichia* e então este DNA foi usado para a transformação de leveduras *P. pastoris* por eletroporação. Estes primeiros passos foram realizados pela empresa *GenScript*-USA.

III.2.4 Transformação em *Pichia pastoris*

A partir da obtenção do plasmídeo recombinante de *Pp*-Hyal-pPICZαA (*GenScript*) a transformação foi realizada em células competentes de *P. pastoris* linhagem X-33, em meio YPDS [Yeast Extract Peptone Dextrose/Glucose Medium with Sorbitol: (1% de extrato de levedura, 2% de peptona, 2% de glicose, 18% de sorbitol e 2% de ágar)] por eletroporação, conforme consta no manual *pPICZα A, B, and C- Pichia Expression Vectors for Selection on Zeocin™ and Purification of Secreted Recombinant Proteins* (Catalog no. V195-20, Invitrogen®). Para a reação de transformação das células competentes de *P. pastoris* X-33 foram utilizados 10 µg do vetor construído (*Pp*-Hyal-pPICZαA) e previamente linearizado conforme mencionado no item acima.

III.2.5 Seleção dos clones transformantes de *Pichia pastoris* para expressão

Para a análise dos transformantes foi realizado o crescimento dos mesmos em meio líquido seletivo, YPDS (1% de extrato de levedura, 2% de peptona, 2% de glicose e zeocina a 100 µg/mL) e a confirmação das colônias por PCR com os primers direto *5'AOX1 sequencing*

(5'-GACTGGTTCCAATTGACAAGC-3') e reverso *3'AOX1 sequencing* (3'-GCAAATGGCATTCTGACATCC-3'). As preparações plasmidiais do vetor de expressão pPICZαA "vazio" bem como do vetor + inserto *Pp*-Hyal, obtidas dos transformantes, foram submetidas a reações de dupla digestão com as endonucleases *Xho* I e *Not* I respectivamente, nas extremidades 5' e 3' do gene.

III.2.6 Expressão da *Pp-Hyal-pPICZαA* em *Pichia pastoris*

A inserção da sequência de DNA de hialuronidase de *P. paulista* no genoma da *P. pastoris* foi confirmada pela obtenção de quatro clones recombinantes, que foram avaliados para o efeito da presença deste gene no crescimento da estirpe hospedeira. Dos quatro clones, somente um foi usado para determinação da curva de crescimento e otimização da expressão.

Para a expressão da proteína, as células foram crescidas em meio sólido *Yeast Extract peptone Dextrose Medium*-YPDS [1% yeast extract, 2% peptone, 1M sorbitol, 2% ágar, 100µg/mL Zeocin], a 30 ºC, 250 rpm, durante a noite. No dia seguinte, a biomassa foi transferida para 100 mL de meio líquido *Yeast Extract peptone Dextrose Medium*-YPD [1% yeast extract, 2% peptone, 100µg/mL Zeocin] contendo 2% de dextrose. Estas células de *P. pastoris* X-33 transformadas com o vetor pPICZαA foram incubadas durante 16-18h, a 30 ºC e 250 rpm.

Para a preparação do primeiro inóculo, antes da indução da proteína foram realizadas as seguintes etapas: (a) 50 mL da cultura foram transferidos para frascos *Erlenmeyer* de 500 mL nas seguintes condições: temperatura de 30 ºC e agitação de 250 rpm, sendo os frascos tampados com apenas 3 camadas de gaze, pois esta cepa de levedura requer muito oxigênio para crescer. O crescimento das culturas foi acompanhado pelos valores de densidade óptica a 600 nm; (b) em seguida, foi feita a centrifugação das células a 5.300g, 4 ºC de temperatura, sendo o sobrenadante desprezado e o precipitado usado para a fermentação em meio *Buffered Minimal Methanol-complex Medium*-BMMY [1% yeast extract, 2% peptone, 100 mM potassium phosphatase, pH 6.0, 1,34% YNB, $4x10^{-5}$ biotin, 1% methanol].

A otimização da expressão foi testada por este meio BMMY, porque é específico para expressar proteínas secretadas, o qual, confere características tamponadas importantes garantindo maior estabilidade e atividade da proteína secretada. Para isso, as células foram colhidas em OD_{600} = 4,0-6,0 e ressuspensas para OD_{600} em 50 mL de BMMY. Para a indução da expressão da *Pp*-Hyal uma amostra inicial, correspondente ao tempo de "zero horas" (T_0) foi retirada, e para o restante da suspensão foi adicionado metanol passo a passo, até atingir

uma concentração final de 1%, ou seja, a cada 24 horas adicionou-se 0,5% de metanol, 2x/dia durante 6 dias, incubando a cultura a 18 ºC, com agitação de 250 rpm durante 5 a 7 dias. Para o controle negativo, foram seguidos os mesmos procedimentos de indução, entretanto, a fermentação foi induzida por glicerol nas mesmas concentrações acima descritas. Amostras das culturas foram recolhidas de tempos em tempos, para acompanhamento das densidades ópticas e do valor de pH do meio, até atingir a fase estacionária do crescimento. Após todo esse período, foi realizada uma centrifugação em baixa velocidade (3.000g), o sobrenadante e as células foram recolhidas, lisadas e também analisadas por SDS-PAGE 12 %, conforme descrito a seguir.

III.2.7 Preparação de lisado celular de *Pichia pastoris*

Embora o sistema de expressão utilizado possua o fator α para secreção das proteínas heterólogas produzidas, existem relatos de que o processamento da seqüencia deste peptídeo sinal não ocorre de forma correta, levando ao acúmulo da proteína no interior das células (BOYSEN et al., 2004; KJELDSEN et al., 1999). Portanto, é conveniente proceder a lise celular e investigar o conteúdo protéico de cada fase do extrato celular - a solúvel e a insolúvel. Desta forma, as células obtidas das fermentações dos clones positivo e negativo, respectivamente transformadas com o vetor recombinante *Pp*-Hyal-pPICZα A e com o mesmo vetor vazio, foram recolhidas e lisadas. Estas células foram primeiramente lavadas em tampão de lise *Breaking Buffer* [50 mM de tampão fosfato de sódio, pH 7,4; 1 mM de PMSF; 1 mM de EDTA; 5% de glicerol (v/v)], efetuando-se a ressuspensão das mesmas no tampão e centrifugando-se por 5 a 10 minutos a 3.000 g, a 4 ºC. Em seguida, o *pellet* foi ressuspenso num volume de tampão de lise (BB) o suficiente para se obter uma suspensão celular com densidade óptica OD_{600} de 50-100 em BB, sendo a seguir adicionado a ele igual volume de esferas de vidro (de 0,5 mm de diâmetro) com posterior agitação em vortex por 30 segundos, colocando-se a mistura em gelo por mais 30 segundos. Este ciclo foi efetuado oito vezes. Em seguida, a amostra foi centrifugada nas condições acima descritas e o sobrenadante, que se apresentava translúcido e correspondia à fração de proteína solúvel, foi transferido para um novo tubo. A fração insolúvel foi ressuspensa numa solução de Triton X-100 (1%, v/v) (Sigma) e a proteína presente em cada uma das frações foi quantificada pelo método de Bradford.

III.2.8 Análise de proteínas por eletroforese sob condição denaturante

A análise das proteínas de cada uma das frações celulares foi efetuada por SDS-PAGE a 12% segundo o sistema de Laemmli (1970). Após a corrida, os géis foram corados [metanol 50%, (v/v), ácido acético 10%, (v/v), *Coomassie Blue R* 0,05% durante 30 minutos, seguidos da aplicação do descorante [metanol 5%, (v/v), ácido acético 7% (v/v)] para que as bandas protéicas fossem visualizadas.

III.2.9 Purificação da *Pp*-Hyal-rec de *Pichia pastoris*

A purificação da hialuronidase recombinante expressa em *P. pastoris* (**Figura 5**) foi obtida por cromatografia de afinidade em resina de Ni^{2+} (Ni-NTA-Agarose) seguindo o protocolo do fabricante (Protocol 14, The QIAexpressionist, Qiagen, Germany) em que foram utilizados os seguintes tampões, todos a pH 8.0 (ajustado com NaOH): (i) Tampão de lise (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM de Imidazol); (ii) Tampão de lavagem (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM de Imidazol); (iii) Tampão de eluição (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM de Imidazol). O único parâmetro de diferenciação dos tampões do Protocolo original consistiu na concentração final de imidazol desde a calibração da resina até a eluição da proteína.

III.2.10 *Western blotting* para avaliação da especificidade da *Pp*-Hyal-rec de levedura

Para análise da especificidade e pureza da hialuronidase recombinante de *P. paulista* expressa em *P. pastoris*, 80 µg desta proteína foi aplicada em SDS-PAGE 12 % e testada contra o anticorpo anti-*Pp*-Hyal-nat. Para a produção deste anticorpo policlonal em camundongos BALB/c, a proteína Hyal nativa do veneno de *P. paulista* (*Pp*-Hyal-nat) foi obtida e purificada até a homogeneidade, por meio de cromatografia de troca catiônica em coluna Hiprep FF CM, acoplado a um sistema de Akta FPLC, sendo as análises realizadas por espectrometria de massa em MALDI ToF/ToF-MS (Justo Jacomini et al. 2013).

III.2.11 Análise imunológica comparativa da *Pp*-Hyal nas formas nativa e recombinantes, expressas pelos dois diferentes sistemas (bactéria e levedura)

Para esta análise foram aplicadas 80 µg de cada uma destas proteínas em gel a 12 % (SDS-PAGE), as quais, foram testadas contra o anticorpo anti-*Pp*-Hyal-nat, para avaliação comparativa quanto à identidade das proteínas recombinantes em função da especificidade deste anticorpo, já previamente determinada (Justo Jacomini et al. 2013). Para isso, foram

utilizados amostras protéicas da hialuronidase nativa (*Pp*-Hyal-nat), recombinante (*Pp*-Hyal-rec) expressa em levedura *P. pastoris*, recombinante (*Pp*-Hyal-rec) expressa em bactéria DE3-BL21, junto a um padrão pré-corado de proteínas de massa molecular (High-Range Rainbow Molecular Weight Markers, Amersham Biosciences-GE Healthcare, USA). Após a corrida do gel, foi realizada a transferência dessas amostras para uma membrana de nitrocelulose, utilizando o sistema semi-seco (Nova Unidade de Blot Multiphor II, Pharmacia Biotech). A eficiência de transferência foi analisada pela coloração do gel eletrotransferido com Coomassie Blue G-250 e, a imunodetecção com o anticorpo anti-*Pp*-Hyal-nat, seguido da revelação pelo sistema colorimétrico de fosfatase alcalina/BCIP®/NBT (Sigma-Aldrich, USA).

III.2.12 Análise da ligação de IgE específica à proteína *Pp*-Hyal-rec expressa em levedura, por *Western blotting*

Os soros de 2 pacientes alérgicos ao veneno de *P. paulista* foram selecionados ao acaso, para serem avaliados quanto à presença e o reconhecimento de IgE específica à proteína *Pp*-Hyal-rec de levedura. Para esta análise, 100 µg dessa proteína, bem como a mesma quantidade da proteína *Pp*-Hyal-rec expressa em bactéria e 100 µg des proteínas totais do extrato de veneno bruto da vespa em estudo foram submetidos à corrida em SDS-PAGE 12 %, ao lado de um padrão de massa molecular de proteínas pré-corado (High-Range Rainbow Molecular Weight Markers, Amersham Biosciences-GE Healthcare, USA). O soro de um indivíduo não alérgico foi utilizado neste experimento, como controle negativo. Após a corrida, as proteínas foram transferidas para uma membrana de nitrocelulose, conforme descrito acima, sendo também a eficiência de transferência analisada pela coloração do gel eletrotransferido com Coomassie Blue G-250. Os 2 soros dos pacientes alérgicos foram diluídos (1:50) em solução de bloqueio (TBS-Tween 20% contendo 1% de leite desnatado) e incubados overnight a 4º C. A imunodetecção foi realizada com o anticorpo humano monoclonal Anti-IgE conjugado com fosfatase alcalina (Sigma-Aldrich, USA) diluído a 1:5000, seguido da revelação pelo sistema colorimétrico de fosfatase alcalina/BCIP®/NBT (Sigma-Aldrich, USA).

III.3 Resultados e Discussão

De acordo com a análise de *codon usage* em *Pichia pastoris*, para otimização da expressão da *Pp*-Hyal neste sistema, a seqüência de DNA codificadora da enzima

hialuronidase (EC 3.2.1.35), nativa do veneno de *P. paulista* e determinada previamente por clonagem em bactéria e sequenciamento, teve que ser modificada pela inserção de mais 12 nucleotídeos (realizado pela empresa *GenScript*- USA). Este passo foi essencial para a clonagem e obtenção do inserto a ser expresso por este sistema. Na **Figura 3** estão descritas, de forma suscinta, as duas etapas de clonagem do DNA de hialuronidase sintetizado.

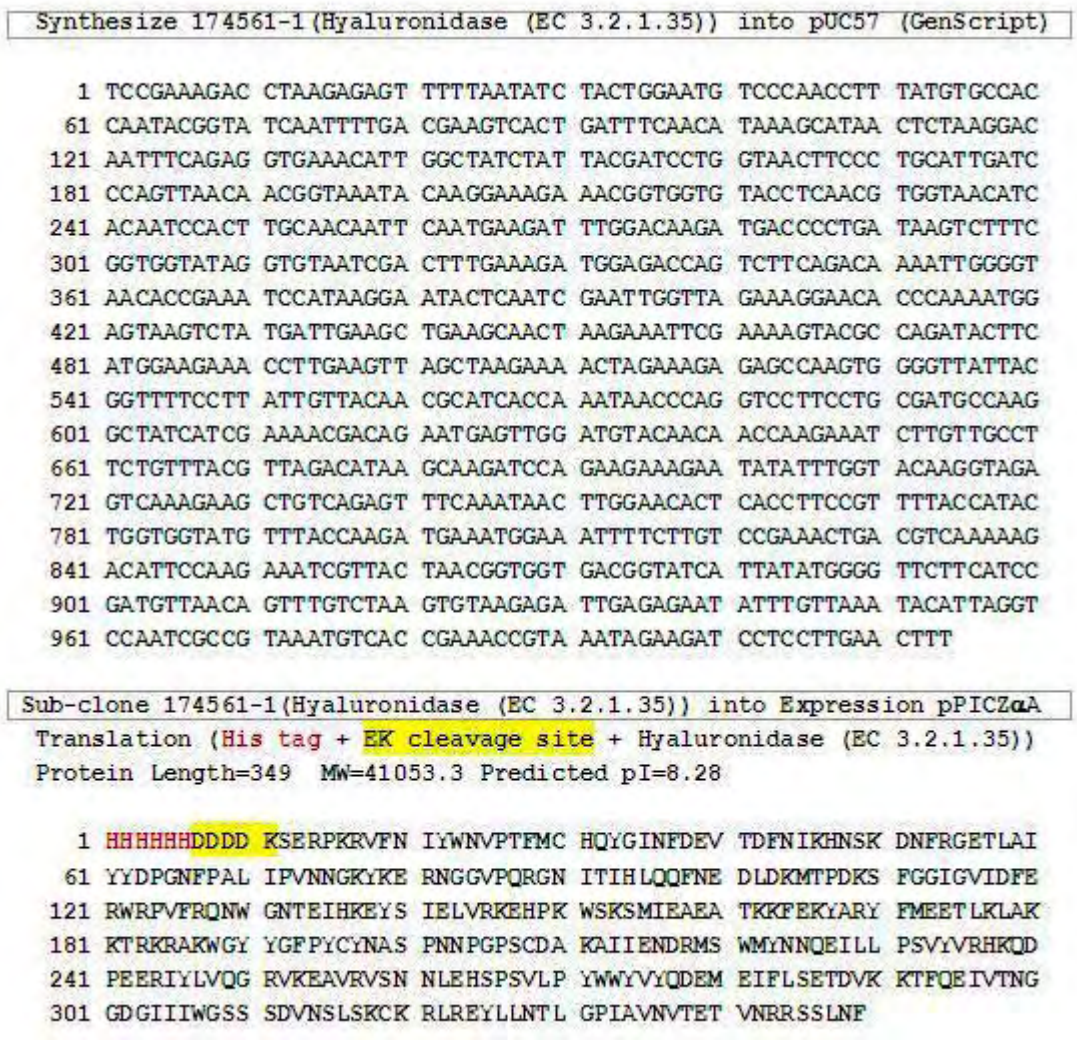

Figura 3. Seqüencia de DNA correspondente à proteína hialuronidase no veneno da vespa social *P. paulista*, a qual foi sintetizada de acordo com a análise de *codon usage* da levedura *Pichia pastoris* (**primeira parte da Figura**) e a proteína transcrita (**segunda parte da Figura**). Nesta imagem também podemos verificar como foi a estratégia de clonagem da sequência com o 6X(His tag) - EK (sítio de clivagem) - além do inserto de hialuronidase (EC 3.2.1.35) num total de 1026 nucleotídeos, resultando numa proteína com 349 aminoácidos, massa molecular de 41 kDa e ponto isoelétrico (pI) teórico de 8.28.

Após os passos de síntese desta seqüência de DNA, clonagem em vetor pUC57, sub-clonagem em vetor de expressão pPICZα, o qual foi inicialmente utilizado para transformação de bactérias *E.coli* DH5α-competentes, para propagação e análise dos transformantes por restrição para confirmação de ocorrência de fusão do gene *in frame* com peptídeo sinal de

secreção (fator α) e o resíduo C-terminal, os plasmídeos foram isolados da cultura bacteriana, purificados e linearizados para poderem ser utilizados na transformação de células competentes da levedura *P. pastoris*. Por seleção em meio contendo o antibiótico zeocina e PCR de colônias, puderam ser confirmados nove transformantes positivos de *E. Coli*-DH5α para a presença do inserto (DNA de *Pp*-Hyal sintetizada) no vetor de expressão pPICZαA conforme pode ser observado na **Figura 4**.

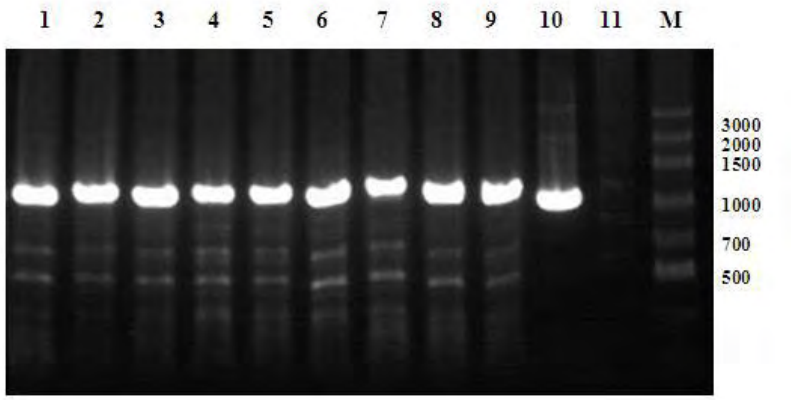

Figura 4. Perfil eletroforético, em gel de agarose a 1,5%, da análise da presença do inserto de DNA sintetizado, realizado por PCR de colônias. Os fragmentos foram amplificados com os iniciadores *5'AOX1 sequencing* direto e *3'AOX1 sequencing* reverso**.** As bandas correspondentes à hialuronidase apresentam 1026 pb. Nas colunas de **1-9**: transformantes contendo o DNA sintetizado de hialuronidase (EC 3.2.1.35); coluna **10**: vetor *Pp*-Hyal-pPICZα A vazio; coluna **11**: cepa não transformada como controle negativo; **M**: DNA marker.

Destes transformantes apenas um único clone foi selecionado para otimização da expressão pela avaliação em pequena escala. No final do 6º dia de fermentação, após centrifugação, para separação das células do meio de cultura, verificamos que o volume da biomassa chegou a cerca de metade do volume total do meio de cultura, permitindo que purificação da *Pp*-Hyal-rec expressa em *P. pastoris*, por cromatografia de afinidade, ocorresse com êxito, conforme pode ser visualizado na **Figura** 5. Por esta análise, a proteína obtida apresentou massa molecular de 37 kDa, diferindo do valor teórico de 41 kDa fornecido pela empresa *GenScript*, o que é perfeitamente aceitável. Além disto, estes valores de massas moleculares são coerentes com aqueles descritos na literatura para as hialuronidases. Nesta mesma figura verifica-se ainda que a proteína pode ser purificada com um excelente grau de pureza, uma vez que apenas 01 banda protéica pôde ser detectada na fração da coluna de afinidade eluída com 500 mM de imidazol (canaletas **5** e **6** do gel). Essa proteína purificada foi usada para a sensibilização em camundongos BALB/c para a produção de anticorpos policlonais específicos para posteriores análises imunológicas.

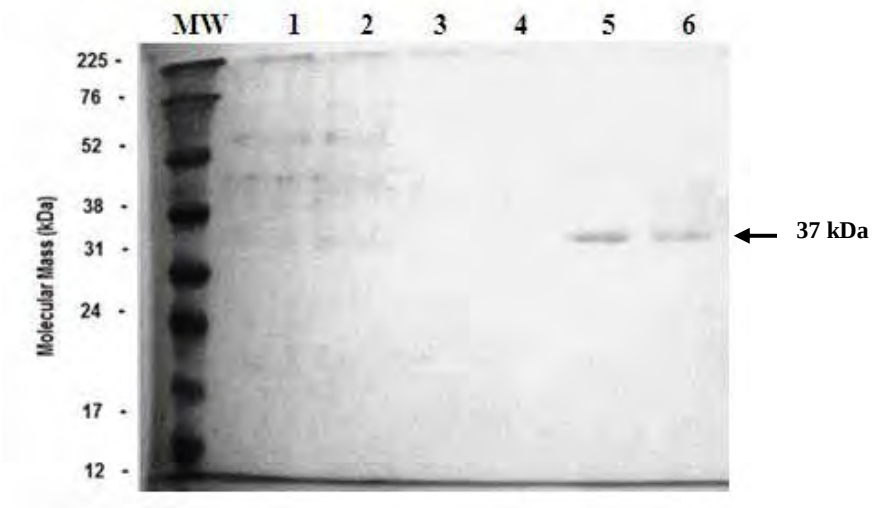

Figura 5. Análise do perfil protéico, em SDS-PAGE 12%, obtido durante as fases de purificação da *Pp*-Hyal-rec expressa em *P. pastoris.* **MW** - padrão de massas moleculares (GE- Healthcare); **(1)** volume "vazio" da coluna (proteínas não aderidas); **(2)** eluído da lavagem com 20 mM de Imidazol; **(3)** eluído da lavagem final com 40 mM de Imidazol; **(4)** eluído da lavagem final com 250 mM de Imidazol; **(5,6)** eluição com 500 mM de Imidazol, recolhendo um total de 1,5 mL de amostra, onde pode ser observada a proteína hialuronidase correspondente a massa molecular de 39 kDa. O gel foi corado com *Coomassie Blue.*

Uma vez purificada, a *Pp*-Hyal-rec expressa em *P. pastoris* foi testada quanto à sua identidade como hialuronidase, o que pode ser confirmado pelos resultados da análise de *Western blotting*, utilizando o anticorpo policlonal anti-*Pp*-Hyal-nat, demonstrados na **Figura 6.**

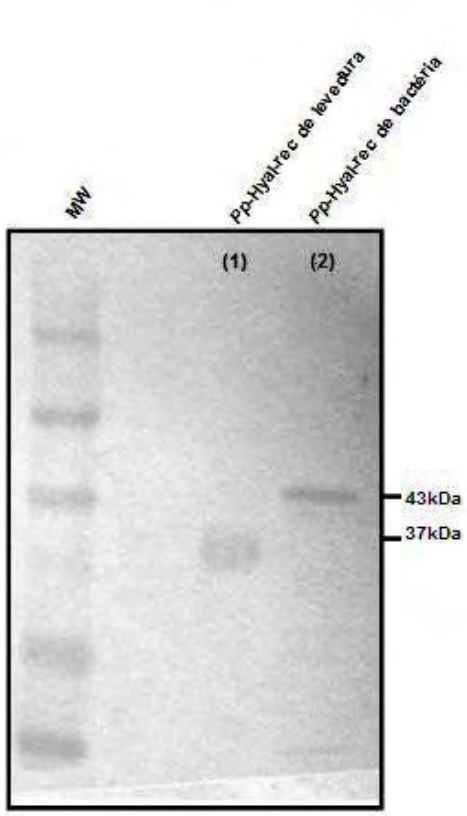

Figura 6. *Western blotting*, mostrando o reconhecimento do anticorpo policlonal anti-*Pp*-Hyal-nat pela **(1)** proteína hialuronidase recombinante expressa em levedura *P. pastoris* (37 kDa); **(2)** proteína recombinante expressa em bactéria DE3-BL21 (43 kDa). (**MW**) representa o padrão de massa molecular de proteínas de (Sigma-Aldrich, USA).

Nesta figura verifica-se que o anticorpo policlonal anti-*Pp*-Hyal-nat, produzido em camundongos BALB/c, além de demonstrar a identidade da proteína expressa em levedura como sendo realmente hialuronidade, foi capaz de reconhecer a *Pp*-Hyal obtida neste estudo nas 2 formas recombinantes expressas por dois diferentes sistemas, as quais apresentaram massas moleculares aparentes ligeiramente diferentes, o que pode ser devido às características de corrida de cada uma destas proteínas no gel, tendo em conta, que ambas proteínas recombinantes foram expressas com 6 resíduos de Histidina no N-terminal. Um fato importante a ser abordado é também que a proteína recombinante expressa em bactéria foi transferida para a membrana de nitrocelulose, após ser submetida à solubilização dos corpúsculos de inclusão com 8 M de uréia, que pode fazer com que a proteína sofra completo ou parcial desdobramento na sua estrutura (CLARK, 2001), mesmo assim, o anticorpo anti-*Pp*-Hyal-nat foi capaz de reconhecer e se ligar a epítopos específicos da *Pp*-Hyal-rec expressa em bactéria. O reconhecimento específico, dessas proteínas denaturadas (pela fervura com β-mercaptoetanol antes de serem aplicadas no gel), pelo anticorpo utilizado ocorreu muito

provavelmente devido a identidade dos resíduos de aminoácidos dos epítopos lineares que se mantiveram íntegros e foram expostos pela desnaturação, já que poderiam estar interiorizados nas estruturas protéicas expressas (JAMES et al., 2003). O fato da ocorrência de reconhecimento pelo anticorpo anti-*Pp*-Hyal-nat é muito importante também, porque indica uma enorme potencialidade de utilização deste e dos dois outros anticorpos policlonais produzidos neste trabalho (contra as proteínas recombinantes, produzidas em bactéria e levedura) em estudos imunológicos e bioquímicos relacionados, respectivamente, tanto à verificação de ocorrência de reatividade cruzada em venenos de insetos como também à purificação da enzima hialuronidase em venenos das espécies de vespa mais proximamente relacionadas à *P. paulista*.

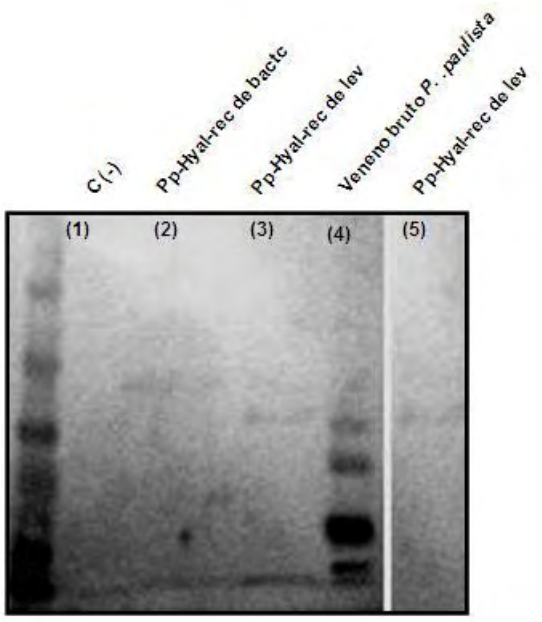

Figura7. *Western blotting* mostrando o reconhecimento de IgE dos soros imunes de 2 pacientes alérgicos à *P. paulista* selecionados ao acaso, entre os demais testados. Em (**2**) e (**3**) tem-se respectivamente, o reconhecimento de IgE de 1 dos soros à *Pp*-Hyal-rec expressa em bactéria (43 kDa), *Pp*-Hyal-rec expressa em levedura (37 kDa) e ao extrato de veneno bruto de *P. paulista*, respectivamente. No extrato de veneno bruto (**4**) verificamos o reconhecimento de outras bandas que provavelmente devam corresponder a outros alérgenos presentes neste veneno (ex. Ag5, PLA1 e proteases). Nas canaletas **1 c(-)**, (**5**) tem-se respecivamente, o controle negativo relativo ao soro de um paciente não alérgico e a ligação de IgE com o outro soro testado ao acaso, ao *Pp*-Hyal-rec expressa em levedura. (**MW**) representa o padrão de massa molecular de proteínas de (Sigma-Aldrich, USA).

A análise imunológica comparativa da eficiência e especificidade da hialuronidase recombinante de *Polybia paulista* expressa pelos dois diferentes sistemas - bactéria e levedura- em relação ao mesmo alérgeno presente no extrato bruto deste veneno, foi avaliada por meio de *Western blotting*, pela reatividade de IgE específica de soros de 2 pacientes exclusivamente reativos ao veneno desta vespa. Os resultados estão apresentados na **Figura 7**, a qual mostra que tanto o extrato de veneno bruto, como ambas proteínas recombinantes,

foram reconhecidas pelo soro de 1 dos 2 pacientes testados, em intensidade ligeiramente maior com a banda correspondente à proteína *Pp*-Hyal-rec de bactéria (43 kDa) em relação a proteína *Pp*-Hyal-rec de levedura (37 kDa). No extrato de veneno bruto de *P. paulista*, além do reconhecimento da Hyal, este soro também reconheceu outras proteínas que muito provavelmente devem corresponder aos outros componentes alergênicos do veneno. O segundo soro foi testado apenas com a *Pp*-Hyal-rec expressa em levedura e demonstrou o mesmo perfil de reconhecimento do soro 1. Estes resultados são extremamente importantes do ponto de vista imunológico, pois nos permite dizer que as proteínas obtidas por expressão em bactéria e levedura mantiveram suas características imunogênicas relativamente à proteína alergênica hialuronidase na forma nativa, e este dado abre perspectivas de utilização destes alérgenos recombinantes na imunoterapia de pacientes alérgicos a esta vespa bem como no desenvolvimento de kits diagnósticos de alergia mais específicos e sensíveis ao veneno deste inseto.

III.4 Conclusão

Nesta etapa do estudo, de acordo com o que se tem descrito até o momento na literatura, pela primeira vez foi expresso o alérgeno hialuronidase de veneno de Hymenoptera sociais em levedura *Pichia pastoris* - no caso, a proteína *Pp*-Hyal-rec, cujo alérgeno nativo é proveniente do veneno da vespa social *P. paulista.* A proteína recombinante foi expressa na forma solúvel, e após ter sido purificada, teve sua identidade confirmada por *immunoblotting* quando testada contra o anticorpo policlonal *Pp*-Hyal-nat, produzido previamente contra a proteína nativa, em camundongos BALB/c. Além disso, pela metodologia de *Western blotting*, foi possível também verificar que o soro de pacientes exclusivamente reativos ao veneno de *P. paulista* apresentaram níveis praticamente semelhantes de reatividade de IgE tanto a esta proteína como a expressa em bactéria. Desta forma, por estes dados, concluímos que ambas têm potencial de utilização na imunoterapia de pacientes alérgicos a esta vespa bem como no desenvolvimento de kits diagnósticos de alergia mais específicos e sensíveis ao veneno deste inseto.

Outros experimentos poderão ser ainda posteriormente realizados para obtenção da proteína *Pp*-Hyal-rec expressa em levedura, em maior quantidade e/ou concentração, principalmente com o intuito de testá-la em análises, *in vitro* e *in vivo*, viabilizando assim, efetivamente a sua utilização nos processos e protocolos imunológicos relacionados ao tratamento e diagnóstico de alergia ao veneno da vespa social *P. paulista.*

Conclusão Final

Conclusão

- A sequência completa de cDNA (GI: 302201582) da molécula madura de Hyal, do veneno de *P. paulista*, contendo parte da sequência precursora, apresentou 1315pb. A proteína deduzida por tradução, apresentou 338 aminoácidos, massa molecular de 39.648,8 kDa e pI de 8,77, valores que diferem daqueles encontrados por Espectrometria de Massas, para a proteína nativa de Hyal (*Pp*-Hyal-nat), a qual apresentou massa molecular de 43,277 e pI de 8.13. Por outro lado, a proteína traduzida mostrou-se muito similar às outras hialuronidases de veneno de Hymenoptera sociais, também pertencentes à família das glicosil-hidrolases.

- Os anticorpos policlonais produzidos em camundongos, contra a hialuronidase obtida nas formas nativa e recombinante (proteína Hyal expressa em bactéria) mostraram-se altamente específicos porque ambos foram capazes de reconhecer, com altos níveis de sensibilidade, as proteínas contra as quais foram produzidos, bem como a banda protéica correspondente ao mesmo alérgeno presente no extrato bruto do veneno de *P. paulista*. Além disso, ambos também foram capazes de revelar, de forma coerente, a existência de reatividade cruzada de *Pp*-Hyal com o mesmo alérgeno nos extratos de veneno bruto de outras espécies de *Polybia (sericea*, *ignobilis, occidentalis*, *scutelaris*) bem como no extrato de *Polistes lanio lanio*. Entretanto, ao contrário dos resultados encontrados para este alérgeno no veneno das vespas de clima temperado, ambos anticorpos não revelaram a ocorrência de reatividade cruzada entre a *Pp*-Hyal e qualquer dos componentes homólogos dos extratos de veneno de abelha (*Apis mellifera*) e formiga (*Solenopsis invicta*). Estes resultados são extremamente importantes para a seleção de extratos de veneno, caso venham a ser utilizados em imunoterapia.

- Tanto o alérgeno nativo (*Pp*-Hyal-nat) como os recombinantes (*Pp*-Hyal-rec) expresso em bactéria e levedura, quando utilizados para avaliação dos níveis de reconhecimento pela imunoglobulina E (IgE) *Pp*-Hyal-específica, presente no soro de pacientes sabidamente alérgicos ao veneno dessa vespa, puderam ser especificamente reconhecidos, o que foi demonstrado pela visualização da reação das IgEs destes soros às bandas correspondentes à proteína nativa (39 kDa), a

proteína recombinante de bactéria (43 kDa) e a proteína recombinante de levedura (37 kDa), nas análises de *Western blotting*.

- Todos os dados aqui obtidos indicam que as proteínas recombinantes mantiveram suas propriedades imunológicas, portanto dessa forma, tem grande potencial para serem posteriormente utilizadas no desenvolvimento de kits diagnóstico para alergia, bem como, em imunoterapia específica contra alergia ao veneno de *Polybia paulista*

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Débora Laís Justo Jacomini

Profa. Dra. Márcia Regina Brochetto Braga