

Proteomic Characterization of the Hyaluronidase (E.C. 3.2.1.35) from the Venom of the Social Wasp *Polybia paulista*

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Abstract: *Polybia paulista* wasp venom possesses three major allergens: phospholipase A₁, hyaluronidase and antigen-5. To the best of our knowledge, no hyaluronidase from the venom of Neotropical social wasps was structurally characterized up to this moment, mainly due to its reduced amount in the venom of the tropical wasp species (about 0.5% of crude venom). Four different glycoproteic forms of this enzyme were detected in the venom of the wasp *Polybia paulista*. In the present investigation, an innovative experimental approach was developed combining 2-D SDS-PAGE with *in-gel* protein digestion by different proteolytic enzymes, followed by mass spectrometry analysis under collision-induced dissociation (CID) conditions for the complete assignment of the protein sequencing. Thus, the most abundant form of this enzyme in *P. paulista* venom, the hyaluronidase-III, was sequenced, revealing that the first 47 amino acid residues from the N-terminal region, common to other Hymenoptera venom hyaluronidases, are missing. The molecular modeling revealed that hyaluronidase-III has a single polypeptide chain, folded into a tertiary structure, presenting a central (β/α)₅ core with alternation of β-strands and α-helices; the tertiary structure stabilized by a single disulfide bridge between the residues Cys¹⁸⁹ and Cys²⁰¹. The structural pattern reported for *P. paulista* venom hyaluronidase-III is compatible with the classification of the enzyme as member of the family 56 of glycosidase hydrolases. Moreover, its structural characterization will encourage the use of this protein as a model for future development of “component-resolved diagnosis”.



Keywords: Allergen, hyaluronidase, mass spectrometry, molecular modeling, peptide sequencing, wasp venom.

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INTRODUCTION

Stinging of the Hymenoptera insects, such as bees, wasps and ants may induce allergic reactions and, occasionally, fatal anaphylaxis. The allergens found in wasp venoms stimulate the immune system to produce highly specific antibodies, and to trigger allergic reaction symptoms in sensitized patients [1]. Immunochemical studies have shown that the major allergenic proteins are phospholipases, hyaluronidases, antigens-5 [2, 3, 4] and serine-proteases [5].

Hyaluronidase is naturally found in several human organs and body fluids, and may also be found in snakes, scorpions, bees, wasps, spiders, lizards, and leeches [6]. This enzyme breaks down the hyaluronic acid, a glycosaminoglycan of high molecular weight, which is one of the major components of the extracellular matrix of vertebrates. The hyaluronic acid is found in almost all tissues and body fluids, and it is particularly abundant in the connective tissue, synovial fluid and the eye vitreous humor [7, 8].

In animal venoms, hyaluronidases are known as “spreading factors”, because they facilitate the spread of toxins through the tissues by promoting the hydrolysis of glycosaminoglycans, thereby contributing to the local and systemic effects of envenomation due to the increase in absorption rates and diffusion of the venom through tissues [9].

Several studies have reported the characterization of hyaluronidase from the venoms of *Lycosa* [10], *Hyppasa* [11] and *Loxosceles* [12, 13] spiders, as well as from the venoms of the scorpions *Tityus serrulatus* [14] and *Buthus martensi* [15] and from the venoms of the snakes *Naja naja* and *Vipera russellii* [16]. In addition, hyaluronidases have been reported in the venoms of social Hymenoptera insects including the social wasps *Vespula maculifrons*, *Vespula maculata*, *Vespula arenaria* [17], *Dolichovespula maculata*, *Vespula squamosa*, *Polistes exclamans* [18], *Vespula vulgaris* [3] *Polybia paulista* [4]; as well as in *Apis mellifera* bee venom [19] and *Solenopsis invicta* ant venom [20].

Hyaluronidase has been isolated from the venoms of social Hymenoptera by using several chromatographic steps, including gel filtration and cation exchange [21]. Studies of Markovic-Housley *et al.* (2000) [22] have elucidated the three-dimensional structure of hyaluronidase from *Apis mel-*

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lifera venom, characterizing it as presenting 350 amino acid residues with two disulfide bridges and four sites of glycosylation. The hyaluronidase from the venoms of social wasps are relatively conserved in relation to that found in honeybee venom [19], its molecular weight ranges from 33 to 45 kDa, and its different molecular forms bear significant amounts of carbohydrates attached to polypeptide chains [23-25]. It has been speculated that carbohydrate moieties may be responsible for the cross-reactivity of this protein with its congener from honeybee venom in sera from allergic patients [3, 4].

Polybia paulista is an extremely aggressive social wasp endemic in Southeast Brazil, where it provokes hundreds of stinging accidents of medical importance for humans every year. Santos *et al.* (2010) [4] identified four different molecular forms of hyaluronidase in the venom of *P. paulista*. This protein is one of the major allergens of this wasp venom, and is responsible for triggering inflammatory reactions in stinging victims [26]. Despite its importance, this enzyme has not been characterized yet. It is well known that hyaluronidase represents less than 1% of the crude venom of Neotropical social wasps, making difficult to obtain reasonable amounts of this enzyme for purification and structural characterization [4]. Thus, in order to achieve a better understanding of the molecular structure of this enzyme, herein, we used a proteomic approach to identify and to sequence the most abundant form of this protein in the venom of the social wasp *P. paulista*. An innovative experimental approach was developed combining 2-D SDS-PAGE with *in-gel* protein digestion by different proteolytic enzymes, followed by mass spectrometry analysis under collision-induced decomposition (CID) conditions for complete assignment of protein sequencing.

MATERIAL AND METHODS

Polybia paulista Venom

Workers of *Polybia paulista* were captured in the University Campus, at Rio Claro, SP, Southeast Brazil. The freshly collected wasps were immediately frozen and dissected. The venom reservoirs were removed from the sting apparatus by pulling them out of bodies with forceps and microscissors. The venom reservoirs were then carefully washed and suspended in small volumes of a solution containing a cocktail of protease inhibitors (2 mM AEBSF, 0.3 μ M aprotinin, 130 μ M bestatin, 1 mM EDTA, 14 μ M E-64 and 1 μ M leupeptin, Sigma-Aldrich). Subsequently, they were thawed, punctured, washed three times with the protease inhibitor solution to extract the venom, and centrifuged at $10.000 \times g$ for 10 min at 4°C. The supernatants were collected, lyophilized and maintained at -80°C until use.

Ion Exchange Liquid Chromatography

The freeze-dried venom (23.4 mg) was solubilized in 50 mM sodium acetate buffer, pH 5.2, and submitted to cation exchange chromatography in a HiPrep FF CM column (160 mm x 10 mm, 20 mL, GE Healthcare) coupled to an Akta-FPLC system. The column was previously equilibrated with the same solubilization buffer and the elution was performed under isocratic conditions with 50 mM sodium acetate buffer, pH 5.2, during the first 20 min, and then under a lin-

ear gradient from 0 to 1 M NaCl in the same buffer from 20.1 to 180 min at a flow rate of 2 mL/min; then, fractions of 5 mL were collected. The elution was monitored by measuring the absorbance at 280 nm and hyaluronidase activity was assayed. The acquisition and processing of data from chromatography were performed using the UNICORN software.

Hyaluronidase Activity

The hyaluronidase activity was measured by the turbidimetric method [27], employing chondroitin sulfate as substrate. The reaction medium contained 150 mM calcium chloride, 10 mM sodium acetate buffer pH 5.2, chondroitin sulfate 0.05 mg/mL in a final volume of 2 mL. After protein addition, the fractions/crude venom were incubated at 37°C for 1 hour. The absorbance was measured at 400 nm. One unit of hyaluronidase activity was defined as the amount of enzyme that hydrolyses the release of 2.566 μ mol chondroitin sulfate.h⁻¹ in 1 mL of the reaction medium.

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2-D SDS-PAGE

Samples (200 μ g protein) were applied by rehydration to 7 cm IPG strips, pH 3-10. Isoelectric focusing (IEF) was performed using an IPGphor system (GE Healthcare) at 3500 V for 7.100 Vh. IPG strips were incubated in an equilibration buffer [50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS] containing 0.5% (w/v) DTT for 15 min, followed by an equilibration buffer containing 4% (w/v) iodoacetamide for 15 min. The second dimension was run on home casted SDS-PAGE gels [15% (w/v) polyacrylamide and 0.8% (w/v) bis (*N,N'*-methylenebisacrylamide)] at 10 mA/gel for 15 min and 20 mA/gel for 1 hour, at 10°C in a MiniVe system (GE Healthcare). Gels were stained overnight with Coomassie Brilliant Blue R-250 (CBB) and after were scanned and digitized (BioImage, GE Healthcare) for documentation. Images were analyzed using Image Master Platinum software v.7 (GE Healthcare).

In-gel Digestion

Gel pieces were destained twice for 30 min at 25°C with 50 mM ammonium bicarbonate/50% (v/v) acetonitrile, dehydrated in acetonitrile, dried, and treated with three different proteolytic enzymes: 20 μ g/mL of trypsin (Promega, USA) in 50 mM ammonium bicarbonate, pH 7.9, at 37°C, during 18 hours; 20 μ g/mL of chymotrypsin (Sigma, USA) in 50 mM ammonium bicarbonate, pH 7.9, at 3°C, during 3 hours; and 20 μ g/mL of endoproteinase Glu-C/V8 protease (Sigma, USA) in 50 mM ammonium bicarbonate, pH 7.9, at 25°C, during 18 hours. Digests were extracted from gel pieces with 60% (v/v) acetonitrile/water and 0.1% (v/v) formic acid, combined, desalted and cleaned with PerfectPure C18 pipet tips (Eppendorf, Germany) according to the manufacturer's instructions and vacuum dried. The concentrated digests were mixed with 0.5 μ L of matrix [10 mg/mL α -cyano-4-hydroxycinnamic acid in methanol/acetonitrile (1:1, v/v) mixed with an equal volume of 0.1% (v/v) aqueous

trifluoroacetic acid (TFA)] and spotted onto a MALDI plate target and allowed to dry at room temperature.

Glycosylation Assay

Gels were incubated in 50% (v/v) methanol for 30 to 45 min, washed twice in 3% (v/v) acetic acid for 5 to 10 min each, and glycans were then oxidized by incubation in 1% periodic acid, 3% (v/v) acetic acid for 20 to 30 min. The gel was then washed four times for 5 to 10 min each time in 3% (v/v) acetic acid to remove the residual periodate. Then, the gel was incubated in Pro-Q Emerald 300 dye solution (GE Healthcare) for 30 to 120 min. This solution is prepared by diluting the stock Pro-Q Emerald 300 dye solution 1:50 in Pro-Q Emerald 300 dilution buffer just prior to staining. Afterwards, the gel was incubated in 3% (v/v) acetic acid, two times for 5 to 10 min each time. Detection of glycoproteins was visualized by measurement of absorption at 300 nm by a photo documentation system for electrophoresis gels through VDS Image Master (GE Healthcare).

MALDI-ToF/ToF Mass Spectrometry Data

Mass spectrometric analysis was performed using a MALDI ToF/ToF-MS (matrix-assisted laser desorption ionization time of flight/time of flight-mass spectrometry) instrument (Shimadzu, mod. Axima Performance). Both MS and MS/MS spectra were acquired in the positive ion reflectron mode using a N₂ laser. Typically, 250 to 500 laser shots were acquired for MS and MS/MS mode, and spectra were obtained at a laser power, maximized for the highest possible resolution and peak intensity in each analytical situation. CID spectra were acquired using a dual-timed ion gate at a laser power approximately 20% higher than MS acquisition. MS data were acquired in the m/z range from 800 to 3500, with an accelerating voltage of 20 kV and delayed extraction, peak density of maximum 50 peaks per 200 Da, minimal S/N ratio of 10 and maximum peak at 60. MS/MS data were acquired in the mass range from 60 Da up to each precursor mass, with a minimum S/N ratio of 10; a maximum number of peaks set at 65 and peak density of maximum 50 peaks per 200 Da.

Protein Identification

Launchpad 2.8 (Shimadzu) was used to submit the combined MS and MS/MS data to MASCOT protein search engine version 2.2 (<http://www.matrixscience.com>) using the National Center for Biotechnology Information (NCBI) protein database, restricted to the taxa Metazoa. The search parameters were as follows: no restrictions on protein molecular weight, one tryptic missed cleavage allowed; peptide mass tolerance in the search was 0.8 Da for MS spectra and 0.5 Da for MS/MS spectra. Iodoacetamide derivative of cysteine and oxidation of methionine were specified in MASCOT as fixed and variable modifications, respectively. Scaffold (version 2.04.00, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. The identifications were accepted when their score exceeded the specific database search engine thresholds. MASCOT identifications required ion scores greater than the associated identity scores and 20, 30 and 40 for singly, doubly and triply charged peptides. The FDR assess-

ment was estimated using the original decoy FDR approach from Mascot; a separate decoy database was generated from the protein sequence database with the decoy.pl Perl script provided by Matrix Science. This script randomizes each entry, while it retains the average amino acid composition and length of the entries. For protein identification, the maximum protein and peptide FDR rates were set to 0.01 and the maximum peptide FPR to 0.1; FPR was calculated based on the Mascot Score. Proteins were considered identified with at least two peptides assigned to the respective sequence. All peptides/proteins corresponding to toxins were also confirmed by manual examination of the spectra.

Molecular Modeling

For hyaluronidase molecular modeling, the restrained-based modeling approach was used as implemented in the program MODELLER 9v4. MODELLER is an automated approach for comparative modeling by fulfillment of spatial restraints. A total of 1000 models were generated and the final model was selected based on stereochemical quality and MODELLER objective function. The images of three-dimensional structures of the models were generated from the program PyMOL [28].

Analysis of the Model

The program PROCHECK [29] was used to check bond lengths, bond angles, peptide bonds and side-chain ring planarities, chirality, main-chain and side-chain torsion angles. The results of this analysis are shown in Ramachandran diagram and values of the G-factor whole. VERIFY 3D program, which measures the compatibility of a protein model with its sequence using a 3D profile, was also used for model validation [30]. The RMSD differences from ideal geometries for bond lengths and bond angles were calculated with XPLOR [31].

RESULTS

Cation exchange liquid chromatography in CM FF column (GE Healthcare) was carried out with the crude venom of *P. paulista* wasp. A total of 11.54 units were submitted to chromatographic fractionation. The chromatographic profile Fig. (1) resulted in eight protein peaks, assigned from A to H. The assay of hyaluronidase activity was carried out on the chromatographic profile; the results show the hyaluronidase activity associated with the peak F, which contained a total activity of 11.54 units, representing a recovery of 22.2 % in the chromatographic step. Thus, the enzyme was purified five fold in comparison to the crude extract, corresponding to 4.3% of total protein from the venom (Table 1).

The fractions corresponding to peak "F" were collected, pooled and lyophilized, which resulted in a recovery of 1 mg protein. This amount was apparently not enough for amino acid sequencing through the traditional protocols of Edman degradation chemistry. Consequently, a proteomic approach was chosen, combining 2-D SDS PAGE, with *in-gel* digestion and mass spectrometric-based amino acid sequencing.

About 200 µg of protein from peak "F" was submitted to 2-D SDS-PAGE; the electrophoretic profile revealed four protein spots, which were assigned from I to IV Fig. (2A).

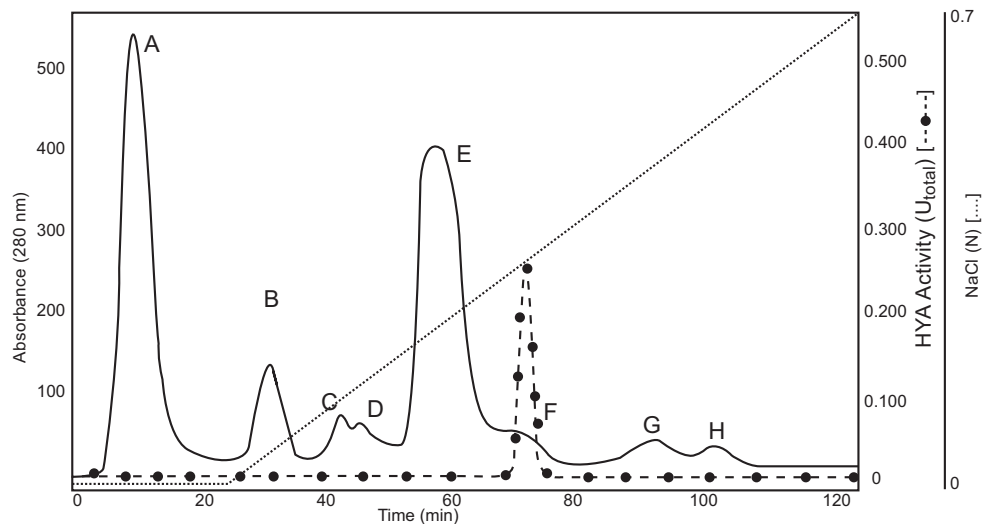


Figure 1. Chromatographic profile of *P. paulista* crude venom (23.4 mg) obtained in a Hiprep FF CM column (160 mm x 10 mm, 20 mL – GE Healthcare) coupled to an Akta-FPLC system. The column was previously equilibrated with the same solubilization buffer and the elution was performed under isocratic elution with 50 mM sodium acetate buffer, pH 5.2, during the first 20 min, and then under a linear gradient from 0 to 1 M NaCl in the same buffer from 20.1 to 180 min at a flow rate of 2 mL/min; fractions of 5 mL were collected. The elution was monitored by measuring the absorbance at 280 nm and the hyaluronidase activity was assayed. The elution of proteins is represented by continuous line (—), while the dashed line (---) represents the hyaluronidase activity.

Table 1. Records of Protein and Hyaluronidase Activity Recovery at the Different Steps of Purification Protocol

Steps	Vol (mL)	Total Protein (mg)	HYA Activity (U/mL)	Total Activity (U _{Total})*	Recovery of Activity (%)	Specific Activity (U/mg)	Purification
Crude Venom	2	23.4	5.77	11.54	100	0.493	1.0
Peak F	40	1	0.064	2.566	22.23	2.566	5.2

*U_{Total} = μmol/mL

The proteomic analysis of these protein spots corroborated the identification of all proteins as hyaluronidases, with molecular weight of 44.340 Da and pI values from 9.29 to 9.65. The proteomic sequence was deposited in GenBank with the following accession number: Q9U6V9 for all the forms (Table 2). The protein identification presented MASCOT score from 110 to 352, with sequence coverage from 10 to 19% (Table 2). The *in-gel* reaction with Pro-Q Emerald 300 dye revealed that the four different forms of *P. paulista* hyaluronidases were glycoproteins Fig. (2B).

In view of the reduced amount of hyaluronidase in wasp venoms, particularly in the present study, and that the spot III from the electrophoretic profile Fig. (2A) was the most abundant amongst the four different forms of hyaluronidase, the amino acid sequence of the hyaluronidase present in this spot was determined using mass spectrometry analysis of the proteolytic peptides generated by digestion of spot III with trypsin, chymotrypsin and V8 protease. Four different gels were run in parallel (200 μg/gel) in order to get three times the spot III for *in-gel* digestion with the three proteolytic enzymes, and one gel to be submitted to the reaction with Pro-Q Emerald 300 dye solution for detection of glycoproteins Fig. (2B).

MS spectra of the digests of spot III for each proteolytic enzyme are presented in Fig. (3), where it is possible to observe nine major peaks of tryptic peptides (corresponding to the ions of m/z 1342.65, m/z 1589.71, m/z 1913.84, m/z 2099.12, m/z 2052.08, m/z 2151.21, m/z 2259.26, m/z 2363.32 and m/z 2805.11 in Fig. (3A)), seven major peaks of chymotryptic peptides (corresponding to the ions of m/z 1566.76, m/z 1924.89, m/z 2117.14, m/z 2444.21, m/z 2627.26, m/z 2663.36 and m/z 2849.67 in Fig. (3B)), as well the six proteolytic peptides from V8 protease (corresponding to the ions of m/z 1198.64, m/z 1359.72, m/z 1890.95, m/z 2108.10, m/z 2278.36 and m/z 2448.41 in Fig. (3C)).

CID spectra were acquired and interpreted for the 22 major peaks assigned in Figs. (3A to 3C), to get the sequence of each corresponding proteolytic peptide obtained from *P. paulista* hyaluronidase-III. Considering that it would not be possible to include all these spectra in the body of the present manuscript, it was decided to show only one CID spectra of a single proteolytic peptide obtained for each type of protease used to digest the spot III, only as an example. Therefore, Fig. (4) is showing the CID spectra and sequence assignment for the tryptic peptide of m/z 1589.71 Fig. (4A), for the

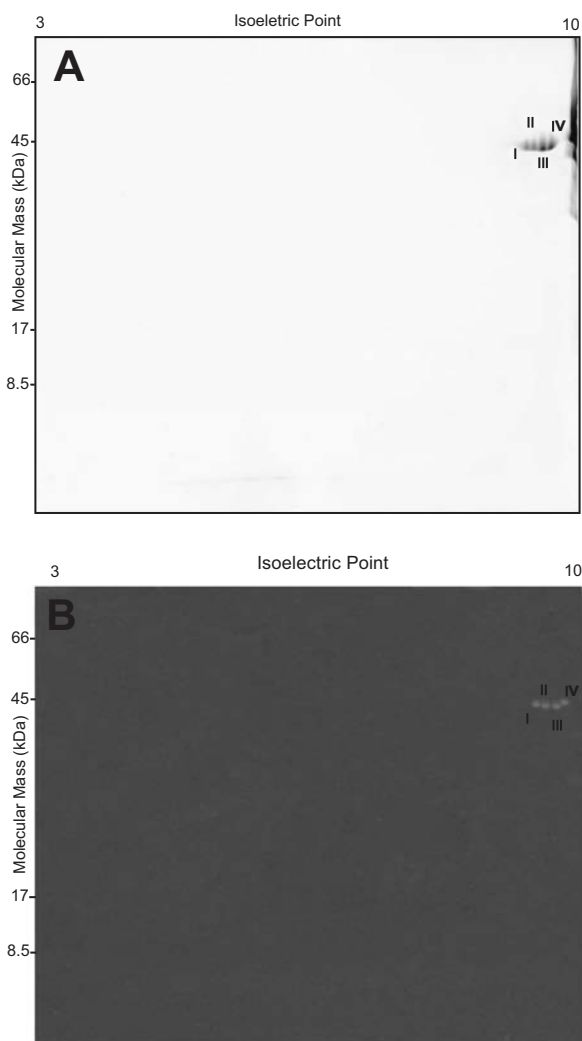


Figure 2. Two-dimensional SDS-PAGE profile of *P. paulista* venom hyaluronidases (peak F from cation exchange chromatography): CBB-stained (**A**) and after reaction with Pro-Q Emerald 300 (**B**).

chymotryptic peptide m/z 1566.76 Fig. (4B) and for the V8 protease proteolytic peptide of m/z 1359.72 Fig. (4C). The CID spectra of the remaining peptides were not shown; however, their sequences are displayed in (Table 3).

Taking into account a partial overlapping due to complementarity and/or sharing of small common sequences of the 22 proteolytic peptides, the sequence of *P. paulista* venom hyaluronidase-III was completely assigned Fig. (5). The enzyme presented 288 amino acid residues in its sequence, consistent with a theoretical molecular mass of 33,716 Da.

The sequence of *P. paulista* hyaluronidase-III (HUGA_POLPI) was aligned with the sequences of other Hymenoptera venom hyaluronidases, as shown in Fig. (6), which included *Polistes annularis* (HUGA_POLAN), *Dolichovespula maculata* (HUGA_DOLMA), *Vespula vulgaris* (HUGA_VESVU), *Vespula germanica* (HUGA_VESGE) and *Apis mellifera* (HUGA_APIME). Identity and similarity values among primary sequences of these proteins were 95.5% and 97.9%, respectively, in relation to HUGA_POLAN; 78.8% and 93.4%, respectively, in relation to HUGA_DOLMA; 75.3% and 94.1%, respectively, in relation to HUGA_VESVU; 75.3% and 93.8%, respectively, in relation to HUGA_VESGE; and 55.6% and 79.9% in relation to HUGA_APIME. It is important to emphasize that in the sequence of *P. paulista* venom hyaluronidase-III, the first 47 amino acid residues from the N-terminal region are missing, a common situation in other Hymenoptera venom hyaluronidases Fig. (6).

Hyaluronidases constitute important allergens from vespid venoms. For that reason, the molecular modeling of *P. paulista* hyaluronidase-III was performed in order to make its complete structural characterization for future understanding of its interaction with IgE. The primary sequence of *P. paulista* venom hyaluronidase-III presented 75.3 % identity with the selected template – a recombinant allergen Ves v 2 corresponding to *V. vulgaris* venom hyaluronidase (PDB ID: 2ATM), which was resolved by X-ray crystallography with a resolution of 2.0 Å. The structural model for *P. paulista* venom hyaluronidase-III was analyzed using the

Table 2. Proteomic Identification of the Peak “F” Obtained in the Cation Exchange Chromatography

Spot	Protein	Accession Code	Protein Score ^a	Sequence Coverage %	MW (Da)	pI	Peptide Sequences
I	Hyaluronidase	Q9U6V9	146	10	44 340	9.29	NFGGIGVIDFER, NGNYEERNGG, NGGVPQR, YSIELVR
II	Hyaluronidase	Q9U6V9	256	15	44 340	9.39	DNFRGEK, KYSIELVR, YFMEEILK, NFGGIGVIDFER, WSESMIEAEATKKFEK
III	Hyaluronidase	Q9U6V9	352	19	44 340	9.50	DNFRGEK, KYSIELVR, NFGGIGVIDFER, GETISIIYYDPGKFPALMPLK, LSWMYNNQEILFPSVYVR
IV	Hyaluronidase	Q9U6V9	110	12	44 340	9.65	DNFRGEK, WKPIFR, NFGGIGVIDFER, LSWMYNNQEILFPSVYVR

^a Mascot Score: Protein Score greater than 81 is significant ($p < 0.05$)

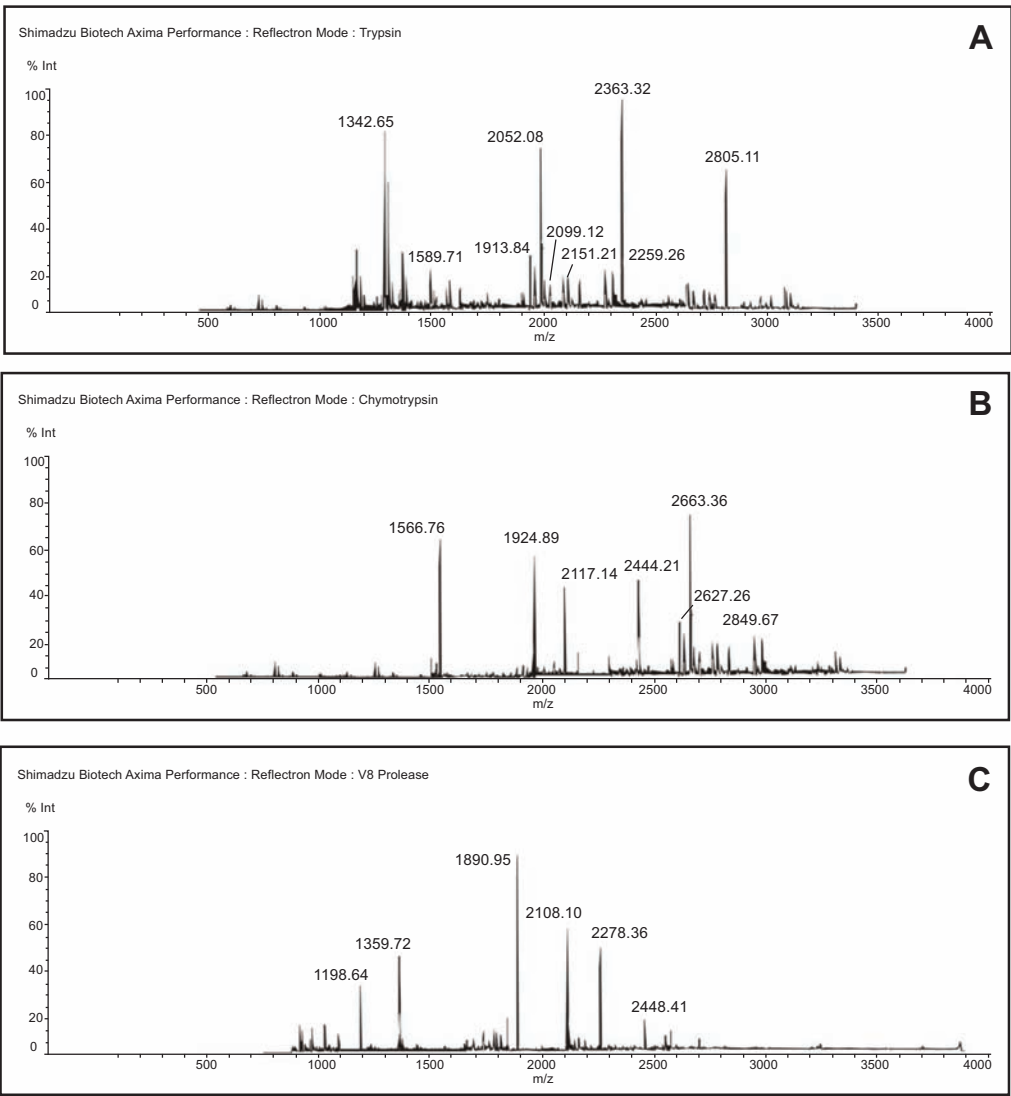


Figure 3. MALDI-ToF-MS spectra of *P. paulista* venom hyaluronidase-III digested with trypsin (A), chymotrypsin (B) and V8 protease (C).

Table 3. Amino acid sequence of proteolytic peptides obtained by digestion of the spot-III of Fig. (2B) with trypsin, chymotrypsin and V8 protease, after mass spectrometry analysis under CID conditions; their position in hyaluronidase-III sequence, their experimental and theoretical molecular masses, and the values of ion score for each peptide sequence in tandem mass spectrometry analysis

Enzyme	Start - End	Mr (expt)	Mr (calc)	Sequence	Ion Score
Trypsin	1 - 12	1342.65	1341.54	GETISIIYDPGK	35
	21 - 34	1589.71	1588.68	NGNYEERNGGVPQR	42
	56 - 73	2151.21	2150.27	NFGGIGVIDFERWKPIFR	72
	97 - 112	1913.84	1912.78	WSESMIEAEATKKFEK	64
	134 - 157	2805.11	2804.16	WGYYGFPPYCNYTPNNPGPDCDAK	53
	165 - 182	2259.26	2258.31	LSWMYNNQEILFPSVYVR	68
	183 - 199	2052.08	2051.03	HELTPDQRVYLVQGRIK	75
	210 - 225	2099.12	2098.16	HSPKVLSYWWVYVYQDK	39
	268 - 288	2363.32	2362.28	LREYLLNTLGPFVAVNTETVN	47

(Table 3) Contd....

Enzyme	Start - End	Mr (expt)	Mr (calc)	Sequence	Ion Score
Chymotrypsin	8 - 24	1924.89	1923.95	YDPGKFPALMPLKNGNY	38
	45 - 57	1566.76	1565.70	NEDLDKMTDPKNF	62
	77 - 97	2627.26	2626.21	GNTIEHKKYSIELVRYEHPKW	46
	118 - 134	2117.14	2116.22	MEETLKLAKKTRKRAKW	29
	146 - 167	2444.21	2443.14	TPNNPGPDCDAKAMIENDRLSW	55
	193 - 217	2849.67	2848.74	LVQGRIKEAVRISNNLKHSPKVLSY	49
	230 - 253	2663.36	2662.32	LSETDVKKTFQEIVTNGGDGHIIW	39
Enzyme	Start - End	Mr (expt)	Mr (calc)	Sequence	Ion Score
V8 Protease	27 - 46	2278.36	2277.43	RNGGVPRGNITIHLQQFNE	66
	65 - 80	2108.10	2107.13	FERWKPIFRQNWGNT	37
	106 - 120	1890.95	1889.92	ATKKFEKYARLFMEE	73
	174 - 184	1359.72	1358.68	ILFPSVYVRHE	52
	225 - 234	1198.64	1197.67	KMDIFLSETD	48
	249 - 270	2448.41	2447.53	GHIIWGSSSDVNSLSKCKRLRE	69

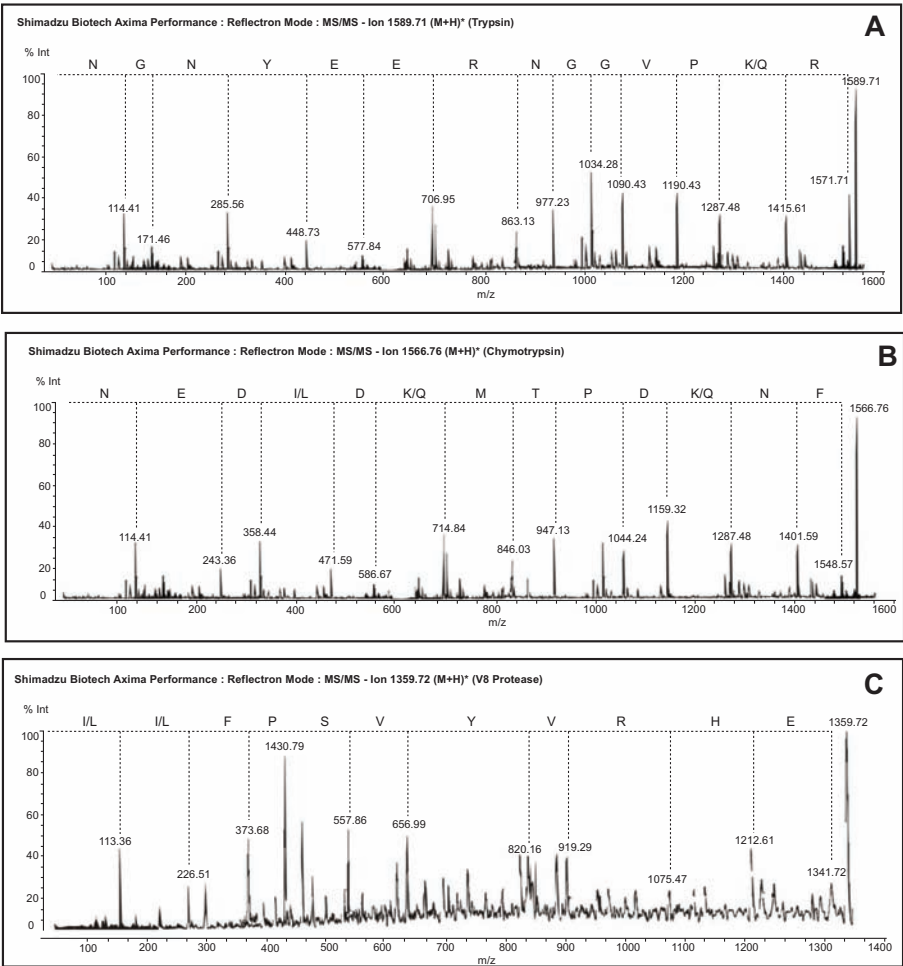


Figure 4. CID spectra of the ions of m/z 1589.71 as [M+H]⁺ (tryptic peptide) (A), m/z 1566.76 as [M+H]⁺ (chymotryptic peptide) (B) and m/z 1359.72 as [M+H]⁺ (C) (protease V8 proteolytic peptide).

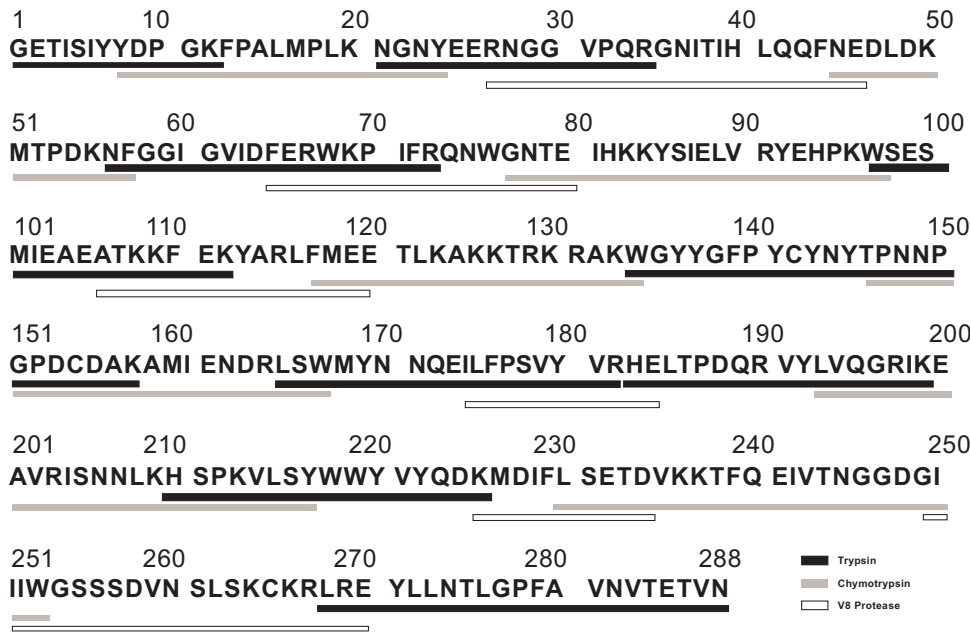


Figure 5. Sequence of *P. paulista* venom hyaluronidase-III obtained from the interpretation of the CID spectra of the peptides generated by digestion with trypsin (■), chymotrypsin (■), protease V8 (□), and overlapping of some individual peptide sequences.

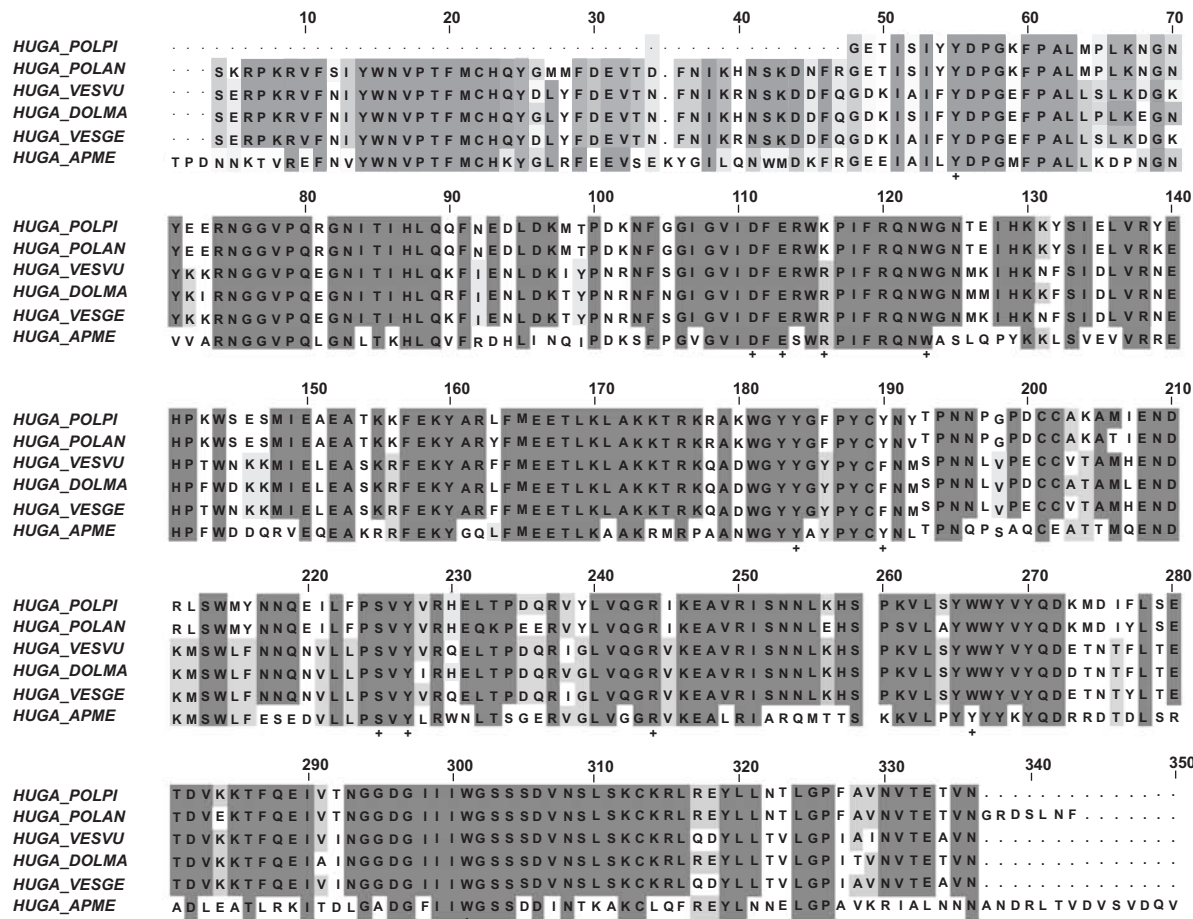


Figure 6. Multiple alignments amongst the primary sequence of the hyaluronidase-III from the venom of the social wasp *P. paulista* (HUGA_POLPI), with the primary sequences of other vespid venoms: *P. annularis* (HUGA_POLAN), *V. vulgaris* (HUGA_VESVU), *D. maculata* (HUGA_DOLMA), *V. germanica* (HUGA_VESGE) and of the bee *A. mellifera* (HUGA_APIME). The most important regions of identity are marked in dark grey, highlighting the highly conserved residues in this type of alignment. The residues marked with an asterisk belong to the catalytic site.

PROCHECK software to estimate the secondary structure content: 44% α -helix, 11% β -sheets and 45% turns and random structures Figs. (7 and 8). The Ramachandran diagram plot (data not shown) indicates that over 94.5% of the residues are in the most favorable regions. The analysis of the structural quality of the molecular model was performed by using PROCHECK [for Ramachandran diagram F-C plots and G-factor (torsion angles = 0.12; covalent geometry = -0.08; global G-factor = 0.05)], XPLOR [for RMSD from ideal geometry (bond length = 0.018 Å; bond angles = 2.029 Å)] and VERIFY-3D (Quality = 76%), which strongly indicated that the model was good enough for structural studies.

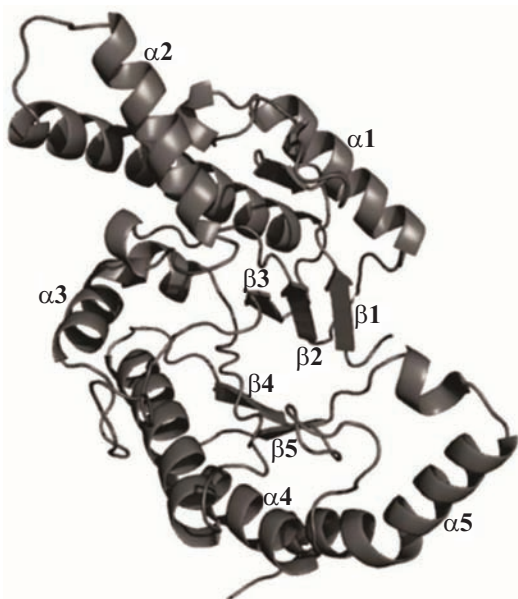


Figure 7. Theoretical model of the three-dimensional structure of the allergen hyaluronidase from the venom of the social wasp *P. paulista*. The β -sheets ($\beta 1$ - $\beta 5$) and the α -helix ($\alpha 1$ - $\alpha 5$) refer to the $(\alpha/\beta)_5$ formation.

DISCUSSION

Hyaluronidases from animal venoms act on the hydrolysis of glycosaminoglycans of cellular interstices by converting them into small fragments, which reduces the viscosity of the interstices, and facilitates the diffusion of venom components through tissues [32]. In addition, hyaluronidases and phospholipases represent important allergens in the venoms of social Hymenoptera insects [2, 3, 4, 21]; consequently, the structural characterization of *P. paulista* venom hyaluronidase-III is essential for the future development of epitope mapping of this allergenic protein.

The results of the present investigation identified four different forms of *P. paulista* venom hyaluronidases, with experimental molecular weight of 44,340 Da and pI values from 9.29 to 9.65. The combination of different proteomic protocols such as 2-D SDS-PAGE with *in-gel* digestions (using three different proteolytic enzymes), followed by MALDI-TOF/MS and CID analysis with manual spectra interpretation, permitted the complete assignment of the amino acid sequence of *P. paulista* venom hyaluronidase-III from a very reduced amount of protein (about 600 μ g of a mixture of four different forms).



Figure 8. Ribbon diagrams of a three-dimensional structural model of *P. paulista* hyaluronidase. The characteristic $(\beta/\alpha)_5$ TIM barrel fold of the main domain is the catalytic domain that supports the binding to the substrate.

The occurrence of different forms of proteins in the venoms of social Hymenoptera is relatively common, and seems to be attributed to the occurrence of different genes, or due to single nucleotide polymorphisms [33, 34], or to alternative gene splicing, or to post-translational modifications, or even by the combination of a few of these alternatives [4]. The ability of venomous organisms to produce different forms of the same protein in their venoms increases the diversity of their proteome; however, it is difficult to know the exact biological function of this mechanism in animal venoms. If it is considered that all the forms were identified with the same accession code (Q9U6V9) in (Table 2), this may indicate that these forms are expression products of the same gene. Furthermore, all the forms presented the same molecular weight and small differences in their pI values, which suggests that they differentiate from each other by a reduced polymorphism and/or due to small differences of post-translational modifications. The occurrence of different forms of a venom protein, especially in allergenic ones, may be related to their transport to distinct cellular destinations, divergent molecular targets and/or to regulate different levels of affinity by IgE [4, 33, 34].

In Hymenoptera venoms, the hyaluronidase-III was described with molecular weights from 33 to 44 kDa [2, 4, 22]. It is believed that the relatively large differences in molecular weights observed amongst hyaluronidases from animal venoms may be due to the extensive glycosylation of these proteins [3]. The great differences between the theoretical molecular weight obtained for *P. paulista* venom hyaluronidase-III (33,716 Da) and the experimental value obtained in the 2-D SDS-PAGE (44,340 Da) suggests that the enzyme may have suffered extensive post-translational modifications, such as glycosylation. This hypothesis was corroborated by the positive reaction of the proteins from gel spots with Pro-Q Emerald 300 dye, indicating that all the hyaluronidase forms are glycoproteins. Skov *et al.* (2006)

[24] identified four glycosylation sites (Asn⁷⁹, Asn⁹⁹, Asn¹²⁷ and Asn³²⁵) in *V. vulgaris* venom hyaluronidase. A simulation of the putative sites of glycosylation for *P. paulista* venom hyaluronidase-III with NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>) revealed two potential sites for N-linked carbohydrates at Asn⁸³ and Asn³³⁰. The role of the carbohydrates attached to the wasp venom hyaluronidases is not clear, but it has been proposed that they may constitute secondary immunogenic determinants [3].

It is important to emphasize that venom extracts were obtained and manipulated all the time in the presence of a cocktail of proteinase inhibitors. Thus, the missing of the first 47 amino acid residues from the N-terminal region of *P. paulista* hyaluronidase-III, in comparison with the known sequences of hyaluronidases from the venoms of Northern vespids, apparently was not resulting of any artifact of sample manipulation, such as uncontrolled proteolysis in the wasp venom. Therefore, the observed sequence of this enzyme seems to be a natural product of *P. paulista* venom.

Since the sequence of *P. paulista* hyaluronidase-III was completely assigned, it was possible to make the molecular modeling of the three-dimensional structure of this form of hyaluronidase. The molecular model revealed that the enzyme has a single polypeptide chain, folded into a tertiary structure, presenting a central (β/α)₅ core with alternation of β-strands and α-helices Fig. (7). Thus, it is missing two elements of strand-helix in the molecule of this enzyme in relation to the central (β/α)₇ core of the hyaluronidases from the venoms of vespid species of Northern Hemisphere. This central core is stabilized by two disulfide bridges between the residues Cys²²-Cys³¹³ and Cys¹⁸⁹-Cys²⁰¹ in the enzymes from vespid venoms of Northern Hemisphere [24]. However, *P. paulista* venom hyaluronidase-III has no Cys residue at the original position 22 (in fact, the first 47 residues are missing) Fig. (6); therefore, it appears that Cys³¹³ presents its thiol group free in *P. paulista* enzyme, while the disulfide bridge seems to occur only between the residues Cys¹⁸⁹-Cys²⁰¹.

The structure of the hyaluronic acid-*P. paulista* venom hyaluronidase-III complex is displayed in Fig. (8). The comparison between the 3-D structure of this protein with its template (recombinant hyaluronidase from *V. vulgaris* venom) reveals that the catalytic site is composed of twelve residues (Tyr⁵⁵, Asp¹¹¹, Glu¹¹³, Lys¹¹⁶, Trp¹²³, Tyr¹⁸⁴, Tyr¹⁹⁰, Ser²²⁵, Tyr²²⁷, Arg²⁴⁴, Trp²⁶⁶ e Trp³⁰¹), which are located within the substrate binding cleft that traverses the wider end of the barrel structure of the catalytic domain. These twelve residues were emphasized with an asterisk in the sequence alignment of *P. paulista* venom hyaluronidase-III with five other known hyaluronidases in Fig. (6). The presentation of a single type of glycosidase activity – hyaluronidase (E.C. 3.2.1.35), with the structural pattern reported above is compatible with the classification of this enzyme as member of the family 56 of glycosidase hydrolases [32].

CONCLUSION

Polybia paulista is an extremely aggressive social wasp found in Southeast Brazil that provokes several stinging accidents all over the seasons, especially in urban areas. In the present study, a combination of different experimental proto-

cols, typical of proteomic analysis, was employed in order to make the separation of different forms of *P. paulista* venom hyaluronidase, and to assign the complete sequence of the most abundant of these forms. The glycoprotein nature of these hyaluronidases was determined, and a three dimensional model was developed and proposed for the sequenced protein. The proteomic strategy used in the current investigation may be easily applied to identify, separate and elucidate the structures of other low-abundant proteins.

This was the first report of a sequence and molecular structure of a hyaluronidase from the venom of social wasps of the Neotropical region of the planet. Despite to be relatively well conserved in relation to the sequences of the congener enzymes of the Northern vespids, the amino acid sequence of *P. paulista* venom hyaluronidase-III congener enzymes seems to be shorter and the tertiary structure of this protein does not contain two α/β elements typical of hyaluronidases from venoms of social Hymenoptera.

The sequencing and three dimensional structure assignment of this enzyme may contribute to improve our knowledge on the structural biology of one of the most the important allergens of Neotropical social wasps, opening possibilities to use this protein as model for the future development of “component resolved diagnosis”.

ACKNOWLEDGMENTS

This work was supported by grants from FAPESP (Proc. 2011/51684-1), INCT-iii/CNPq and CAPES. M.S.P. is a researcher from the National Research Council for Scientific and Technological Development (CNPq).

CONFLICT OF INTEREST

There is no conflict of interest.

REFERENCES

- [1] Aalberse, R.C. Structural Biology of Allergens. *J. Allergy Clin. Immunol.*, **2000**, 106, 228-238.
- [2] King, T.P.; Lu, G.; Gozalez, M.; Qian, N.F.; Soldatova, L. Yellow jacket venom allergens, hyaluronidase and phospholipase: sequence similarity and antigenic-cross reactivity with their hornet and wasp homologs and possible implications for clinical allergy. *J. Allergy Clin. Immunol.*, **1996**, 98, 588-600.
- [3] Kolarich, D.; Léonard, R.; Hermmer, W.; Altmann, F. The N-glycans of yellow jacket venom hyaluronidases and the protein sequence of its major isoform in *Vespula vulgaris*. *FEBS J.*, **2005**, 272, 5182-5190.
- [4] Santos, L.D.; Santos, K.S.; Pinto, J.R.A.S.; Dias, N.B.; Souza, B.M.; Dos Santos, M.F.; Perales, J.; Domont, G.B.; Castro, F.M.; Kalil, J.E.; Palma, M.S. Profiling the Proteome of the Venom from the Social Wasp *Polybia paulista*: A Clue to Understand the Envenoming Mechanism. *J. Proteome Res.*, **2010**, 9, 3867-3877.
- [5] McNairy, M.M.; Gastmeyer, J.; Pantera, B.; Hoffman, D.R. Isolation of paper wasp venom proteases by affinity chromatography. *J. Allergy Clin. Immunol.*, **2000**, 105, S57.
- [6] Kemparaju, K.; Girish, K.S. Snake venom hyaluronidase: a therapeutic target. *Cell Biochemmistry and Function*, **2006**, 24, 7-12.
- [7] Laurent, T.C.; Fraser, J.R.E. Hyaluronan. *FASEB J.* **1992**, 6, 2397-2404.
- [8] Stern, R.; Jedrzejewski, M. Hyaluronidases: their genomics, structures, and mechanisms of action. *Chem. Rev.*, **2006**, 106, 818-839.
- [9] Pukrittayakamee, S.; Warrel, D.A.; Desakorn, V.; McMichael, A.J.; White, N.J.; Bunnag, D. The hyaluronidase activities of some Southeast Asian snake venoms. *Toxicon*, **1988**, 26, 629-637.

- [10] Rash, L.D.; Hodgson, W.C. Pharmacology and biochemistry of spider venoms. *Toxicon*, **2002**, *40*, 225-254.
- [11] Nagaraju, S.; Devaraja, S.; Kemparaju, K. Purification and properties of hyaluronidase from *Hippasa partita* (funnel web spider) venom gland extract. *Toxicon*, **2007**, *50*, 383-393.
- [12] Barbaro, K.C.; Knysaky, I.; Martins, R.; Hogan, C.; Winkel, K. Enzymatic characterization, antigenic cross-reactivity and neutralization of dermonecrotic activity of five *Loxosceles* spider venoms of medical importance in the Americas. *Toxicon*, **2005**, *45*, 489-499.
- [13] Da Silveira, R.B.; Chaim, O.M.; Mangili, O.C.; Gremski, W.; Dietrich, C.P.; Nader, H.B.; Veiga, S.S. Hyaluronidases in *Loxosceles intermedia* (brown spider) venom are endo- β -N-acetyl-d-hexosaminidases hydrolases. *Toxicon*, **2007**, *49*, 758-768.
- [14] Pessini, A.C.; Takao, T.T.; Cavaleiro, E.C. A hyaluronidase from *Tityus serrulatus* scorpion venom: isolation, characterization and inhibition by flavonoids. *Toxicon*, **2001**, *39*, 1495-1504.
- [15] Feng, L.; Gao, R.; Gopalakrishnakone, P. Isolation and characterization of a hyaluronidase from the venom of Chinese red scorpion *Buthus martensi*. *Comp. Biochem. Physiol.*, **2008**, *148*, 250-257.
- [16] Girish, K.S.; Jagadeesha, D.K.; Rajeev, K.B.; Kemparaju, K. Snake venom hyaluronidase: an evidence for isoforms and extracellular matrix degradation. *Mol. Cell. Biochem.*, **2002**, *240*, 105-110.
- [17] Mueller, U.; Reisman, R.; Wypych, J.; Elliot, W.; Steger, R.; Walsh, S. Comparison of vespid venoms collected by electrostimulation and by venom sac extraction. *J. Allergy Clin. Immunol.*, **1981**, *68*, 254-261.
- [18] Hoffman, D.R. Allergens in Hymenoptera venom XIII: Isolation and purification of protein components from three species of vespid venoms. *J. Allergy Clin. Immunol.*, **1985**, *75*, 599-605.
- [19] Lu, G.; Kochoumian, L.; King, T.P. Sequence identity and antigenic cross-reactivity of white face hornet venom allergen, also a hyaluronidase, with other proteins. *J. Biol. Chem.*, **1995**, *270*, 4457-4465.
- [20] Hoffman, D.R.; Dove, D.E.; Jacobson, R.S. Allergens in Hymenoptera venom. XX. Isolation of four allergens from imported fire ant (*Solenopsis invicta*) venom. *J. Allergy Clin. Immunol.*, **1988**, *82*, 818-827.
- [21] Hoffman, D.R. Hymenoptera Venom Allergens. *Clin. Rev. Allergy Immunol.*, **2006**, *30*, 109-128.
- [22] Markovic-Housley, Z.; Miglierini, G.; Soldatova, L.; Rizkallah, P.J.; Muller, U.; Schirmer, T. Crystal structure of hyaluronidase, a major allergen of bee venom. *Structure*, **2000**, *8*, 1025-1035.
- [23] Kubelka, V.; Altmann, F.; Marz, L. The asparagine-linked carbohydrate of honeybee venom hyaluronidase. *Glycoconjugate J.*, **1995**, *12*, 77-83.
- [24] Skov, L.K.; Seppl, U.; Coen, J.J.F.; Crickmore, N.; King, T.P.; Monsalve, R. Structure of recombinant Ves v 2 at 2.0 Angstrom resolution: structural analysis of an allergenic hyaluronidase from wasp venom. *Acta Crystallogr. D Biol. Crystallogr.*, **2006**, *62*, 595-604.
- [25] Markovic-Housley, Z.; Padavattan, S.; Schirmer, T.; Schmidt, M.; Akdis, C.; Valenta, R.; Mittermann, I.; Soldatova, L.; Slater, J.; Muller, U. Identification of a B-cell epitope of hyaluronidase, a major bee venom allergen, from crystal structure in complex with a specific Fab. *J. Mol. Biol.*, **2007**, *368*, 742-752.
- [26] De Souza, B.M.; Palma, M.S. In: *Animal Toxins: The State of Art. Perspectives on Health and Biotechnology* De Lima, M.E.; Pimenta, A.M.C.; Martin-Euclaire, M.F.; Zingali, R.B., Eds., UFMG Printing Co., **2009**; p. 273-297.
- [27] Long-Rowe, K.O.; Burnett, J.W. Characteristics of hyaluronidase and hemolytic activity in fishing tentacle nematocyst venom of *Chrysaora quinquecirrha*. *Toxicon*, **1994**, *32*, 165-174.
- [28] DeLano, W.L. *The PyMOL Molecular Graphics System*, DeLano Scientific LLC, Palo Alto, CA, USA, **2002**.
- [29] Laskowski, R.A.; MacArthur, M.W.; Moss, D.S.; Thornton, J.M. PROCHECK - A program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.*, **1993**, *26*, 283-291.
- [30] Luthy, R.; Bowie, J.; Eisenberg, D. Assessment of protein models with three-dimensional profiles. *Nature*, **1992**, *356*, 83-85.
- [31] Schwieters, C.D. The Xplor-NIH NMR Molecular Structure Determination Package. *J. Magn. Reson.*, **2003**, *160*, 66-74.
- [32] Henrissat, B.; Bairoch, A. Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J.*, **1996**, *316*, 695-696.
- [33] Santos, L.D.; Menegasso, A.R.S.; Pinto, J.R.S.P.; Santos, K.S.; Castro, F.M.; Jorge Elias Kalil, J.E.; Palma, M.S. Proteomic characterization of the multiple forms of the PLAs from the venom of the social wasp *Polybia paulista*. *Proteomics*, **2011**, *11*, 1403-1412.
- [34] Santos, L.D.; Santos, K.S.; Souza, B.M.; Arcuri, H.A.; Castro, F.M.; Kalil, J.E.; Palma, M.S. Purification, sequencing and structural characterization of the phospholipase A1 from the venom of the social wasp *Polybia paulista* (Hymenoptera, Vespidae). *Toxicon*, **2007**, *50*, 923-937.