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Profiling the Proteome of the Venom from the Social Wasp *Polybia paulista*: A Clue to Understand the Envenoming Mechanism

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The study reported here is a classical bottom-up proteomic approach where proteins from wasp venom were extracted and separated by 2-DE; the individual protein spots were proteolytically digested and subsequently identified by using tandem mass spectrometry and database query with the protein search engine MASCOT. Eighty-four venom proteins belonging to 12 different molecular functions were identified. These proteins were classified into three groups; the first is constituted of typical venom proteins: antigens-5, hyaluronidases, phospholipases, heat shock proteins, metalloproteinases, metalloproteinase-desintegrin like proteins, serine proteinases, proteinase inhibitors, vascular endothelial growth factor-related protein, arginine kinases, Sol i-II and -II like proteins, alpha-glucosidase, and superoxide dismutases. The second contained proteins structurally related to the muscles that involves the venom reservoir. The third group, associated with the housekeeping of cells from venom glands, was composed of enzymes, membrane proteins of different types, and transcriptional factors. The composition of *P. paulista* venom permits us to hypothesize about a general envenoming mechanism based on five actions: (i) diffusion of venom through the tissues and to the blood, (ii) tissue, (iii) hemolysis, (iv) inflammation, and (v) allergy—played by antigen-5, PLA1, hyaluronidase, HSP 60, HSP 90, and arginine kinases.

Keywords: social wasp venom • *Polybia paulista* • Hymenoptera • allergy • immunoreactivity • 2-D electrophoresis • mass spectrometry • envenoming mechanism

Introduction

Venoms from social Hymenoptera (wasps, bees and ants) are important instruments in the defense of individuals or of the colony.[1] Stinging events involving wasps, honeybees and ants are frequent worldwide; massive envenoming can result both in immediate and delayed reactions in man,[2] sometimes causing death.[3] However, most deaths or clinically important incidents generally involve a reduced number of stings and are more frequently related to allergic responses of the victims than to massive toxic reactions. Mass stinging events may be life-threatening via the toxic action of the venom when injected in large amounts.[4]

A single sting may cause a transient local inflammation characterized by pain, redness and swelling in humans.[5] About 20.7% of the population in general develops a hypersensitivity reaction of type 1,[6] which results in a series of clinical signs, such as edema, fatigue, dizziness, nausea, fever and unconsciousness as well as urticaria, itching, malaise, angioedema, chest constriction, diarrhea, abdominal pain, dyspnea, wheezing, weakness, drop in blood pressure, collapse, incontinence, cyanosis, cardiovascular and/or gastrointestinal symptoms, up to a life threatening systemic anaphylactic shock.[7]

The diagnosis and therapy of allergies to social wasp venoms in tropical countries is very difficult, because there is neither a commercial standardized venom extract nor a kit for diagnosis; in addition, there is a reduced cross reaction between these venoms and those from the species of the North Hemisphere.[8] In tropical countries, like Brazil, about 500 species of known social wasps represent a potential danger to humans.[1] Thus, identifying the stinging insect is a challenge that demands efforts of physicians, biologists and patients. This is a crucial

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step to decide which life-saving venom-specific immunotherapy should be adopted.

The Hymenopteran venoms are a complex mixture of low molecular mass toxins and a series of polycationic peptides[9,10] and proteins, which are recognized as important allergens.[11] However, the venom of social wasps is poorly characterized by proteomics strategies and techniques.[12] The major allergens of wasps and honeybee venoms have been identified as phospholipase-A_1 and -A_2, antigen-5, hyaluronidase, major royal jelly proteins (MRJPs) and acid phosphatases.[11,13,14] Among the stinging Hymenoptera, only the honeybee (*Apis mellifera carnica)* venom has been subjected to proteomic investigation.[12,15,16] A total of 39 proteins were identified, including some of the classical allergens reported above.[15] In addition, a series of proteins related to the protection of venom against the oxidative stress like superoxide dismutase, glutathione-*S*-transferase sigma 1 isoform A, peroxiredoxin and thioredoxin peroxidase 1 isoform A were also reported.[16]

Polybia paulista is a very aggressive social wasp endemic in Southeast Brazil, where it causes hundreds of stinging accidents of medical importance for humans every year.[13] Thus, in this study, 2-DE and MALDI TOF/TOF mass spectrometry were used to identify new toxic components of the venom of *P. paulista*. The diagnosis of allergy is frequently based on the use of natural allergen extracts, generally composed of relatively poor-defined mixtures of nonallergenic, allergenic and cross reactive molecules, which makes it difficult to precisely identify the allergen eliciting the disease, in patients sensitized by two or more allergen sources.[17] The diagnosis of insect venom allergy may be included in this situation, and the proteomic investigation of the venoms from social insects, in parallel with the immunoblottings performed with the sera of venom-sensitive patients, will be important to help in the development of a component resolved analysis, since the discovery of species-specific venom components can help in the differentiation between the species involved in sting allergy.

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 μM Aprotinin, 130 μM Bestatin, 1 mM EDTA, 14 μM E-64 and 1 μM Leupeptin, Sigma-Aldrich) thawed, punctured, washed three times with the protease inhibitors 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.

Protein Assay. Protein concentration was determined by the method of Bradford, using bovine albumin (BSA) as standard.[18]

Two-Dimensional Gel Electrophoresis. Samples (700 μg protein) were applied by rehydration to 13 cm IPG strips, pH 3−10. Isoelectric focusing (IEF) was carried out on a Multiphor II System (GE Healthcare) at 3500 V for 17.000 Vh. IPG strips were incubated in 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 equilibration buffer containing 4% (w/v) iodoacetamide for 15 min. The second dimension was run on self-cast SDS-PAGE gels [15% (w/v) polyacrylamide and 0.8% (w/v) bis (*N,N*′-methylenebisacrylamide)] at 15 mA/gel for 15 min and 30 mA/gel for 3 h, at 10 °C in a Ruby Red system (GE Healthcare). Gels were stained overnight with Coomassie Brilliant Blue R-250 (CBB) as reported elsewhere[19] and stored at 21 °C in preserving solution (7% (v/v) acetic acid).

Image Acquisition. 2-DE gels stained with CBB were scanned and digitized (BioImage, GE Healthcare) in the transparency mode at 24-bit red-green-blue colors and 400 dpi resolutions for documentation. Images were analyzed using Image Master Platinum software v.7 (GE Healthcare).

In-Gel Digestion. The protocol for in-gel digestion was based on a previous publication.[20] Briefly, gel pieces were destained twice for 30 min at 25 °C with 50 mM ammonium bicarbonate/50% acetonitrile, dehydrated in acetonitrile, dried, treated with trypsin (20 μg/mL, Promega, Madison, WI) in 50 mM ammonium bicarbonate pH 7.9 at 37 °C, during 18 h). 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, Hamburg, Germany) according to the manufacturer's instructions and vacuum-dried. The concentrated digests were mixed with 0,6 uL of matrix (10 mg/mL α-cyano-4-hydroxycinnamic acid in methanol/acetonitrile (1:1, v/v) mixed with an equal volume of 0.2% (v/v) aqueous TFA) and spotted onto a MALDI plate target.

MALDI-TOF/TOF Mass Spectrometry Data. Mass spectrometric analysis was performed by MALDI TOF/TOF-MS/MS (matrix-assisted laser desorption ionization time-of-flight/time-of-flight-mass spectrometry) on a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, USA). MS data were acquired in the m/z range 800 to 4000, 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 until each precursor mass, with a minimum S/N ratio of 10; a maximum number of peak set at 65 and peak density of maximum 50 peaks per 200 Da.

Protein Identification. GPS Explorer (Applied Biosystems) 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 searches 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 both the associated identity scores and 20, 30, 40, and 40 for singly, doubly, triply, and quadruply charged peptides. The FDR assessment 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

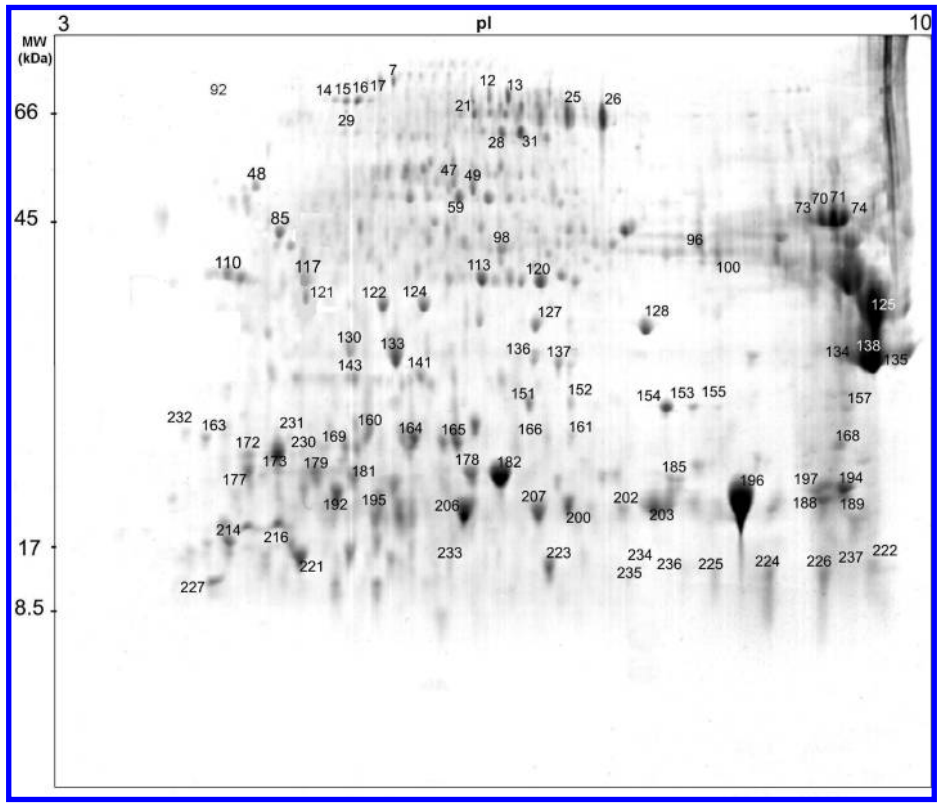

Figure 1. Representative 2D-gel of *P. paulista* wasp venom, stained with Coomassie Brilliant Blue G-250. Detailed information about the different spots can be found in Table 1.

least two peptides assigned to the respective sequence. All peptides/proteins corresponding to toxins were also confirmed by manual examination of the spectra.

Immunoblotting. Sera from five *P. paulista*-sensitized patients were obtained in the Division of Clinical Immunology and Allergy from the Clinics Hospital, University of São Paulo Medical School, São Paulo, Brazil, with the approval of the University Ethical Board. Sera were collected before starting the specific immunotherapy treatment of each patient and stored at −20 °C until used. The protein profiles obtained as described above were electrotransferred to nitrocellulose membranes (Hybond-C Extra, Amersham) for 1 h at room temperature. The binding of IgE antibodies to the membrane-immobilized allergens was analyzed by Western Blot using individual sera from five *P. paulista*-allergic patients. The membranes were blocked with PBS containing 0.5% Tween 20 and incubated with sera from each patient, diluted 1:10 in blocking buffer overnight at 8 °C. After washing with PBS-0.1% Tween 20 the membranes were incubated with a secondary antibody (mouse IgG antihuman IgE) conjugated to peroxidase (1:10 000, Zymed) for 1 h at room temperature. Chemiluminescence detection reagents (ECL Chemiluminescence Reagent Plus Western - GE Healthcare) were added to the membrane according to the manufacturer's instructions. The membrane was incubated with Hyperfilm film (GE Healthcare) in a X-ray cassette and the film was submitted to conventional photographic film development.

Protein Glycosylation Detection. Gels were incubated in 50% (v/v) methanol for 30−45 min, washed twice in 3% (v/v) acetic acid for 5−10 min each, and the glycans were oxidized by incubation in 1% (v/v) periodic acid, 3% (v/v) acetic acid for 20−30 min at room temperature. The gel was washed four times for 5−10 min each in 3% (v/v) acetic acid to remove residual periodate and incubated in Pro-Q Emerald 300 dye solution (GE Healthcare) for 30−120 min following the manufacturer instructions. Afterward, the gel was incubated in 3% (v/v) acetic acid twice for 5−10 min each. Glycoproteins were visualized by scanning the gel at 300 nm in a photo documentation system through the VDS Image Master (GE Healthcare).

Results and Discussion

Patterns of venoms from three different colonies were analyzed and three replicate 2-DE gels per collection were used. The nine gels showed a high degree of identity, reflected by the high scatter plot correlation coefficient (>85%) between all gels. Figure 1 shows a representative 2-DE gel. Image analysis showed 237 ± 36 spots in *P. paulista* venom, in the MW range from 8 to 96 kDa and p*I* from 3 to 10. For identification by mass spectrometry, the 180 most dominant spots were selected since they were present in all venom profiling across the gels. It must be considered that there is no genomic information available in data banks for social wasps; however, wasp venom proteins still may be identified through cross-species data.[21]

Table 1 shows the identification of 84 proteins from *P. paulista* venom with MASCOT score higher than 76 and sequence coverage from 5 to 35%. The proteins identified were classified into three groups: venom proteins, muscle proteins and housekeeping proteins from the venom glands (Table 1). The first comprised 53 typical venom proteins:, from which 23 were identified as being similar to social wasps venom proteins, mainly to those related to allergenicity, such as antigen-5 (spots 125, 134, 194, 188, 189, 236 and 161), hyaluronidases (spots 70, 71, 73 and 74), PLAs (spots 207, 179, 203, 192, 196, 222, 234, 235, 177 and 121) and serine proteinases (spots 172 and 206). Other proteins were included in this group because they have

Table 1. Protein Identification on the 2-D Gels of *P. paulista* Venom

spot no	protein	acession code	MASCOT score[a]	% coverage	peptides sequences
		Polybia paulista venom toxins			
125	Antigen 5	Q7Z156	116	13	CVAHTVCQTGESTK, EVGCGSIK, LIVDEHNR, EVGCGSIK, DFNYNTGITK
134	Antigen 5	Q7Z156	121	18	CVAHTVCQTGESTK, VAHTVCQTGESTKPSSK, EVGCGSIK, LIVDEHNR, DFNYNTGITK
189	Antigen 5	Q7Z156	150	26	CVAHTVCQTGESTK, VSITSVGVTEEEK, VAHTVCQTGESTKPSSK, EVGCGSIK, LIVDEHNRENFAK, VAHTVCQTGESTK, DFNYNTGITK
194	Antigen 5	P35759	77	14	PSSKNCAGK, VAQGLETR, LIVDEHNR
188	Antigen 5	Q7Z156	119	19	CVAHTVCQTGESTK, VAQGLETR, VAQGLETR, EVGCGSIK, YCNIK, DFNYNTGITK
236	Antigen 5	Q7Z156	107	27	CVAHTVCQTGESTK, VGHYTQVVWAK, EVGCGSIK, LIVLWENEVK, DFNYNTGITK
161	Allergen Sol i III	P35779	154	19	PAGNVLGAQIYEIK, FAVGQNIAATSSSGKNK, EVGCGSIK, IMFK
70	Hyaluronidase	Q9U6 V9	146	13	NFGGIGVIDFER, SWMYNNQEILFPSVYVR, YSIELVR, WSESMIEAEATK
71	Hyaluronidase	Q9U6 V9	256	15	NFGGIGVIDFER, SWMYNNQEILFPSVYVR, YSIELVR, WSESMIEAEATK, QNWGNTEIHK
73	Hyaluronidase	Q9U6 V9	352	19	NFGGIGVIDFER, YSIELVR, SWMYNNQEILFPSVYVR, WSESMIEAEATK, QNWGNTEIHK, VYLVQGRIK
74	Hyaluronidase	Q9U6 V9	110	19	NFGGIGVIDFER, YSIELVR, LRSWMYNNQEILFPSVYVR, WSESMIEAEATK, QNWGNTEIHK, VYLVQGRIK
197	Allergen Sol i II	P35776	150	26	GVYDNPDPAVVK, DIAECARTLPK, NSKMCPK
207	Phospholipase A_2	AF438408	131	29	WCGHGNK, SSGPNELGR, IIYPGTLWCGHGNK, IGDNELEER, FKHTDACCR
179	Phospholipase A_2	AF438408	110	25	WCGHGNK, SSGPNELGR, IIYPGTLWCGHGNK, YFNLIDTKCYK
203	Phospholipase A_1	A2VBC4	105	17	DGIVLTEETLQNYDLFK, YVADFTK, DNFLVISVDWK
192	Phospholipase A_1	A2VBC4	108	20	DGIVLTEETLQNYDLFK, DNFLVISVDWK, LLVEQYK
196	Phospholipase A_1	A2VBC4	84	16	DGIVLTEETLQNYDLFK, YVADFTK, LCETDAEYVQIIHTSNILGVYSK, DTCVCVGLNAK
222	Phospholipase A_1	A2VBC4	114	26	DGIVLTEETLQNYDLFK, DNFLVISVDWK, VSMSNIRLIGHSLGAHT, LLVEQYK, LIGHSLGAHTSGFAGK
234	Phospholipase A_1	A2VBC4	90	17	DGIVLTEETLQNYDLFK, YVADFTK, IGTVDFYMNYGSHQPGCGR, FFSPSCSHTK
235	Phospholipase A_1	A2VBC4	89	18	DGIVLTEETLQNYDLFK, YVADFTK, DNFLVISVDWK, YVADFTK, LLVEQYKVSMSNIR
177	Phospholipase A_1	A2VBC4	89	18	DGIVLTEETLQNYDLFK, YVADFTK, YLTECIK, FFPSPSCSHTK, YFSTPKPISQCTK, IGTVDFYMNYGSHQPGCGR
121	Phospholipase A_1	A2VBC4	76	15	DGIVLTEETLQNYDLFK, YVADFTK, GLIPECPFNEYDILFFVYTR, DGIVLTEETLQNYDLFK
59	Heat Shock Protein 60	Q8INI8	340	12	TTPSYVAFTDSER, NALESYVFNVK, LVTHLAEEFK, FAPEEISSMVLTTK, DNNALGTFDLSGIPPAPR, YADEDEK
110	Heat Shock Protein 60	Q9VPS5	125	13	DELNIIQGLR, GVMLAVDVVK, GIIDPTK, AIPGMEQVEVR, DVRFGSGVR, ITGLCQIVK, FDNGYVSPFFVNSSK, SPSYGHHR
138	Heat Shock Protein 70	P29844	109	15	VEIIANDQGNR, VLEDADMNK, IVITNDQNR, ETAEAYLGK, DAGVIAGLQVMR, DVHEIVLVGGSTR, IINEPTAAAIAYGLDK, VFAPEEISAMVLGK
168	Heat Shock Protein 90	C1JYH6	122	18	TLTILDSGIGMTK, IILHIK, ELFIK, EMLQQNK, LGIHEDSQNR, IEEVGGDEDEDK, TLTIDSGIGMTK, ELISNASDALDK, YESLTDPSK, LSESSR, YHTSASGDEMCSLK
21	Snake venom metalloproteinase-like protein	P15167	143	25	YEDAMQYELK, YNSDLNTIR, YIELVVVADHR, DYSETHYSPDGR, VHEIVNFINGFYR, ASDLNLPDQQNLPQR, SYEFSDDSMHYYER

Table 1. Continued

spot no	protein	acession code	MASCOT score[a]	% coverage	peptides sequences
133	Snake venom metalloproteinase-like protein	Q2UXQ0	76	19	RDLINVTFTADDTMDSFGEWR, INNDSTAVR, IPCAPQDVK, ASGLIVPSQK, ESDEPIK, GEPVVLHLEK, VPLVGIVFWSNR, YENIEEEDEAPKMCGVK, EITTKPSVEDHCYYHGR
165	Snake venom metalloproteinase-like protein	Q2UXQ3	116	17	MIQVLLVTICLAVFPYQGSSK, QRDLVNR, IQNDADSTASISACNGLK, GETYLIEPLK, ASHLVATSEQQHFDPR
223	Snake venom metalloproteinase-like protein	Q2UXQ3	81	24	MIQVLLVTICLAVFPYQGSSK, DLVNR, VPDSESHAVYK, IQNDADSTASISACNGLK, GETYLIEPLK, ASHLVATSEQQHFDPR, ITHDNAQLLTAVNLNGDTIGR
15	Zinc metalloproteinase-disintegrin-like protein	P15503	84	16	DHNAIVFVVAVTMTHEMGR, APVGGMCDPK, GAVQQK, VNGEPVVLHLEK, GDDLDDYCNGR, VLSRQPSK
29	Zinc metalloproteinase-disintegrin-like protein	P15503	125	23	DHNAIVFVVAVTMTHEMGR, APVGGMCDPK, GAVQQK, VNGEPVVLHLEK, ETVLLNR, GDDLDDYCNGR, QPSK, YENVEK, EDEPPK, ALNIVTTLSVLEIWSEK
232	Zinc metalloproteinase acutolysin-like protein	P30431	76	14	YEDAMQYEFK, SGTECR, IPCAPEDVK, DNSPGQNNPCK, ASQLAFTAEQQR, MCGVTQNWK
214	Zinc metalloproteinase-disintegrin-like protein	P60244	103	15	TATNFNGNTVGLAYLK, LFASWR, ETDLLK; HDYQSFLTIHK
172	Venom serine proteinase	Q7Z269	81	16	VDLHVITR, YNGQNSK, IILLFITIIGVAK, DACQNDSGGPILWR
206	Venom serine proteinase	B7SD94	82	17	TCADEAPGVNLR, YHFLATCK, QLCTFDIGK, VTSYLDFIR, YNTYGGK
216	Venom serine proteinase	O13057	143	19	PVPGSYFVAGWGR, FFCLSSK, VFDHLDWIK, TLCAGILEGGK
233	Venom serine-proteinase	Q8MQS8	140	17	DSTNCNCGWK, MTVILTPPGR, CSLVEFSENK, LAIVVGEHDWSSK, LVNIGIISWGAECGK
202	Venom serine-proteinase	P33589	103	16	SLMNIYLGMHNK, GAYPRMPTK, WDEDIR, FSAHIEPLSLPSNPPSEDSVCR
124	Chymotrypsin-like proteinase	Q5I029	247	28	VILGEYDR, LSSTASFNSR, YWGNK, VSTLR, HPNYNTNTMINDITLLK, LQQVTLPLLSNTECQR
12	Serine Proteinase Inhibitor	Q8T0W2	77	13	YYCNSCTCGAEGK, NDEPCTPGENFK
128	Serine Proteinase Inhibitor	Q8T0W2	81	12	YYCNSCTCGAEGK, SDESCAPGASFK
137	Cysteine Proteinase Inhibitor	P84032	114	25	GHAASPISTKVKECGCYLK, ESAIIPQCEEDGK
16	VEGF	Q90×23	106	17	CGGCCTDESLECTATGK, NPEEGEPR
17	VEGF	Q90×23	105	17	CGGCCTDESLECTATGK, NPEEGEPR
200	VEGF	P67862	101	28	CGGCCSDESLTCTATGK, SACQTR, ETLVPILK
113	VEGF	P67862	110	21	CGGCCSDESLTCTATGK, EIMR, VDPHK, SPGDVNNGK
226	Arginine Kinase	Q9U9J4	82	28	LEAATDCK, SLLK, SVFDQLK, LVTAVNDIEK, FLQAANACR, GEHTEAEGGVYDISNK, LIDDHFLFK, EMESK, VSSTLSNLEGELK
237	Arginine Kinase	B3VUH4	94	14	VSSTLSGLTGELK, LVESDS, KSLLK, FGFLTFCPTNLGTTVR
26	Alpha-glucosidase	Q17058	100	15	PYDEYYVWR, ENYQTMSR, NSFFNMFK, ENYQTMSR, DSNGDGIGDIEGIK, DVLDEFPQPK, LNMFYBBFNSDIK, FGEEK, DSNSSDFK
	Proteins from the muscles involving the venom reservoir				
14	Nebulin	Q80XB4	95	14	HQYTMTLGLPEFVR, LDAIPFQTAR, TNAANLSEAK, ASGELASSVK, CGQVYSEECDEPR, GHSINYCETPQFR, LHDYTVLPEDMK, GVPCVVPGTLEIEGR, GTGWLALQSPQIESAK
15	Nebulin	Q80XB4	112	8	AGGQLQSDVR, HQYTMTLGLPEFVR, LDAIPFQTAR, TNAANLSEAK, ASGELASSVK, CGQVYSEECDEPR, GHSINYCETPQFR, LHDYTVLPEDMK, GVPCVVPGTLEIEGR, GTGWLALQSPQIESAK
28	Filament B (Actine)	Q80×90	212	10	VHAGGPGLER, GEQGEPCEFNIWTR, IAGPGLSSCVR, LDVTILSPSR, DLAEDAPWK, AWGPGLHGGIVGR, LIALLEVLSQK, LPNNHIGISFIPR, IGNLQTDLSDGLR, VMYTPMAPGNYLIGVK
31	Filament B (Actine)	Q80×90	193	11	VHAGGPGLER, GEQGEPCEFNIWTR, IAGPGLSSCVR, LDVTILSPSR, DLAEDAPWK, AWGPGLHGGIVGR, LIALLEVLSQK, LPNNHIGISFIPR, IGNLQTDLSDGLR, VMYTPMAPGNYLIGVK

Table 1. Continued

spot no	protein	acession code	MASCOT score[a]	% coverage	peptides sequences
49	Plectine	Q15149	108	5	LFDEEMNEILTDPSDDTK, VSITEAMHR, LAEVEAALEK, QAEVELASR, PVAMVMPAR, QEELYSELQAR, GPLPTEEQR, EMELPAK
117	Tropomyosin (Lep d 10)	Q9NFZ4	86	18	EQVQCAEVASLNR, MEGLESQLK, IQLIEEDLER, SLQTAEGDVAALNR
122	Myosin-like Antigen	P21249	101	6	VNAVQALEARK, ANDNNVLQR, ADIVALNDR, SQEEALK, SEVEK, ENNDQK, FDISDLDTNIQK, ILSGEVNK, LYDMTYSYEINAEK, ILYEHPR, DELINYR, HLDEIDNFK
181	Titin	Q9I7U4	125	5	APVFTVPLSNIDGLR, ATAADSGEYTVR, YVNPEDSGTYTCR, AINEYGEAVTTATMK, QHDFGFVSLDISHIR
225	Calponin	P14318	136	17	HADFK, LINVLSPNAVPK, DIANVTNTIFALGR, IASK
		Housekeeping proteins of the venom gland			
166	Superoxide Dismutase	Q00637	201	24	HTLPDLPYDYR, LIQLAPALR, ISPNCK, ELTTLTVAVQGSWGWLGFNK
182	Superoxide Dismutase	P81926	203	32	AVCVLQGESVK, LSCGVIGINHL, GGHEDSK, TTGHAGGR, AVVVHAGEDDLGK
47	Aminoacylase-1	P37111	119	29	CVGIQYLEAVR, QLGLGCQK, PEFQALR, EGEHPSVTLFR, GAQDMK, CVSIQYLEAR, LALELEICPASTDAR, VTSTGK, PGHGSR, FIEDTAAEK, TVQPEPDYGAAVAFLEER
221	Apolipoprotein B-100	P04114	88	7	GMALFGEGK, VQGVEFSHR, ENLCLNLHK, LEDTPK, IDDIWNLEVK, VAWHYDEEK, AHLDIAGSLEGHLR, HEQDMVNGIMLSVEK
120	Calcium-transporting Protein	P22700	76	12	DLTFIGVVGMLDPPR, LDEFGEQLSK, EFDDLSPTEQK, AEIGIAMGSGTAVAK, IDQSILTGESVSVIK, VIVITGDNK, ATAEAR, IPADIR, ITHIYSTTLR, IGVFAEDEDTTGK
100	Ferredoxin III	P46036	131	9	GCIGCGPCGTGANDGTFFK, VMTIANR
231	Guanine nucleotide exchange factor	Q92888	114	10	MEDFAR, GAASPGPSR, GLSSILDAAR, ADLISEDVQR, EILHHVNQAVR, ASYEAR, SAAVVNAIGLYMR, AFLDFYHSFLEK
160	kinase C inhibitor protein	P68252	140	24	YLAEVATGETR, TSADGNEK, ATVVESSEK, GDYYR, YLAEVATGEK, DSTLIMQLLR
7	Membrane protein	P64025	109	21	AYDQIDAAPEER, AEAYILTK, EDVER, GSALAALEDSSK, FFGEFK, QVGVPAIVVFLNK, LLDQGQAGDNIGALIR
98	Membrane protein	Q8SBT6	90	19	LPGGQNLGEMTDVEYLLR, DSYFSELK, IDMAAALDPFVGK
157	Membrane protein	Q6MLR4	185	32	GASVIK, QIGMEAR, ELGYVVDSK, ILGIWSTEK, IYAEIVVSK, DSHK, PWIAVITK, TDIEEK, EQCFEALHHEIPYSIAVR, EIEKLMGEK
195	Membrane protein	Q00179	140	15	IACGSGTTVR, QAFDDMIK, EQQLAAMK, INAAVNDLTR, AGAFDQLKQNATK, LLGMDMAGLVEHVQAVTK, AGAFDQLK
169	*N*-acetylglucosamine-1-phosphate URD	Q5FMG0	77	12	NTDIGPNSHLR, PKALIK, DGNPEELK, GAHIGNFVEVR, DINIGCGTIFSNYDGVK
185	Nucleoside diphosphate kinase	P08879	98	14	TFIMIK, PDGVQR, GDFCIQVGR
224	Pyruvate dehydrogenase	P60090	110	20	EATK, FAAEHCR, DPLVR, EDYILPGYR, TSDEDAEWEK, YGPHTMAGDDPTR, ENEVIERAK, GLWNEDK, ENEVIER
227	Ribosomal protein	P41094	103	35	HYWGLR, VGIAMTAIK, VVTIISNPLQYK, YWQLTSSNLDSK, DDLER, ADVDLTK
141	Succinate dehydrogenase	Q94523	239	10	ELLPR, DEVAR, AINAEVK, VAVASMQR, WHMYDTVK, HANGQITTADLR, LGANSLLDLVVFGR, AVIELENYGMPFSR
13	Zinc finger protein (transcription repressor)	Q12986	77	10	KVWTCDSCFTSLHLQCIQK, DVLCGTDVGK, NSGLNCGTQR, SSGSK, FSDSLK, FVSDVEK, ELPCTSLK, NPGSSNLQK, ATQFVYSYGR, FNTDAAEFIPQEK, EHSPSESEKEVVGADPR
151	Transcriptional regulation protein	Q9W0K7	114	11	SSPTQQEEK, GTGSGADSPK, DVNWSDLK, TSDWDPAELR, ELSGLGPGPSAEPR, EAEELLAFMQPEK, LHSPLGDLGLDMASYK, DESLAAHMPPYGR, EGLSLSQAAR
25	Zinc finger protein (transcription repressor)	P52744	150	25	HSALCSR, HEMVVAK, QSSHLTR, AFHQSSILTK, FAQDLWLEQNIK, IIHTEEK, HQIIYTGEEPYK
85	Zinc finger protein	Q05481	85	7	NLAFLGIALSK, AFSHSSALAK, MHTGEKPYK, AFSNSSTLANHK, ECGKAFSNSSTLANHK, HKIIHAGEK, GQEMETILANTVKPLLY

Table 1. Continued

spot no	protein	acession code	MASCOT score[a]	% coverage	peptides sequences
127	Zinc finger protein	Q9BUY5	142	13	CQQCGK, AYSHPR, THSAQK, PYTCK, AFAVSSNLSGHLR, ECGK, AFTQYSGLSMHVR, IFSLTPNIVYQR, TSTQEK
130	Zinc finger protein	Q8NDQ6	97	16	IHTGVKPYNVR, ESIIEK, DIHEISLSK, LSFYLTEHR, LHTGVK, PYECK, TIHTGIK, PFACK, IHSGLKPYDCK, QYSHLYQHQK, LNSHLTEHQR, IHTGEK
153	Transcriptional regulation protein	Q6P9G9	97	17	LIEELQR, SHLIGHQR, QLLDSK, EAAGPHEAFNK, AGLIMHQVTHFR, QFQYR, EAAGPHEAFNK, IGFEIGIENEEDTSK, SQLTGHQR
178	Zinc finger protein	P17040	114	7	APDMGFEMR, NLLR, AAVRAMGTVR, AIAER, LCALGFLR, EQGPEFWGLSLINSGK

[a] Mascot Score: Protein overall scores greater than 65 are significant ($P < 0,05$).

been reported in Hymenopteran venoms:[22] growth factors-like proteins (spots 16, 17, 113 and 200), arginine kinase (spots 226 and 237), superoxide dismutase (spots 166 and 182)[12,15,16] and alpha-glucosidase (spot 26). The remaining proteins from this group were similar to other animal venom proteins, such as metalloproteinases (spots 21, 133, 165 and 223), zinc metalloproteinase-disintegrins (spots 15, 29, 232 and 214), serine proteinases (spots 124, 172, 202, 206, 216 and 233) and proteinases inhibitors (spots 12, 128 and 137), heat shock proteins (59, 110, 138 and 168), vascular endothelial growth factor (VEGF)-related protein (16, 17, 113 and 200), arginine kinases (226 and 237), Sol i-II and -II like proteins (161 and 197) and superoxide dismutases (166 and 182).

The second group included those proteins structurally related to the muscles involving the venom reservoir. Proteins identified were nebulin (spots 14 and 15), actin (spots 28 and 31), plectin (spot 49), tropomyosin (spot 117), myosin (spot 122), titin (spot 181) and calponin (spot 225); generally, all these proteins play some structural role in striated muscles. It is important to emphasize that we used venom obtained by dissection of the venom apparatus; during the dissection, part of the muscles from the venom reservoir may have been cut or disrupted, leaking the content of the myocytes to the venom solution.

The third group of proteins shown in Table 1 probably originated from the cells of venom glands and do not constitute true venom toxins. They may belong to the group of housekeeping proteins that play metabolic functions in secretory cells of venom glands. Apparently, these proteins do not play any functional role in the envenoming mechanism. Proteins identified in this group are aminoacylase (spot 47), apolipoprotein B-100 (spot 221), calcium-transport protein (spot 120), ferrodoxin III (spot spot 100), *N*-acetylglucosamine-1-phosphate URD (spot 169), pyruvate dehydrogenase (spot 224), succinate dehyrogenase (spot 141), a series of different transcription factors (spots 13, 25, 85, 127, 130, 151, 153 and 178) and membrane proteins (spots 7, 98, 157 and 195). Among these, *N*-acetylglucosamine-1-phosphate URD (spot 169), one of the membrane proteins (spot 195) and the apolipoprotein B-100 (spot 221) were glycosylated (Figure 2A) and, apparently, also do not play any role in envenoming.

The protein profile obtained for the crude venom of *P. paulista* by 2-DE was also detected for glycosylation with Pro-Q Emerald 300 dye. Results shown in Figure 2A reveal fourteen glycosylated proteins (16, 17, 26, 125, 134, 169, 179, 195, 196, 207, 214, 216, 221 and 234).

Considering the well-known allergenicity of the wasp venom proteins to humans, the protein profile obtained for the crude venom of *P. paulista* in the 2-DE, was also immunoblotted using the sera from *P. paulista*-allergic patients. Results shown in Figure 2B indicate that 16 proteins were reactive to human specific-IgE (70, 71, 73, 74, 121, 125, 134, 138, 168, 177, 189, 194, 222, 226, 234 and 237), revealing the high allergenic potential of this venom. No protein from the last two groups were immunoreative to human specific-IgE (Figure 2B).

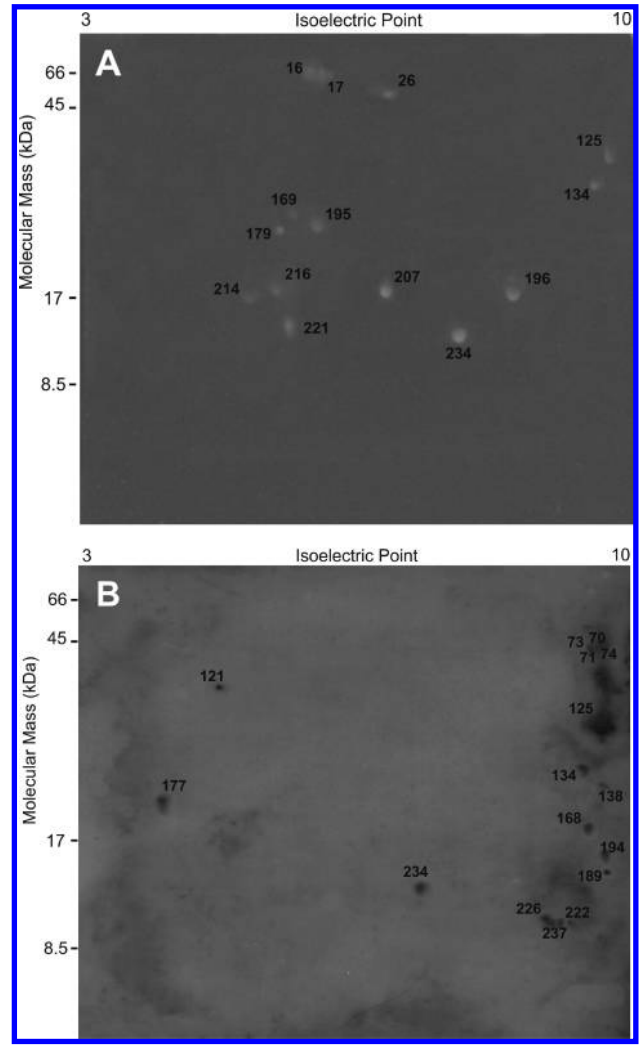

Figure 2. Representative 2D-gel of *P. paulista* wasp venom: (A) stained by reaction with the dye Pro-Q Emerald 300, revealing the profile of glycoproteins; (B) submitted to immunoblotting with the sera of patients sensitive to *P. paulista* venom, revealing the protein spots reactive to human specific-IgE.

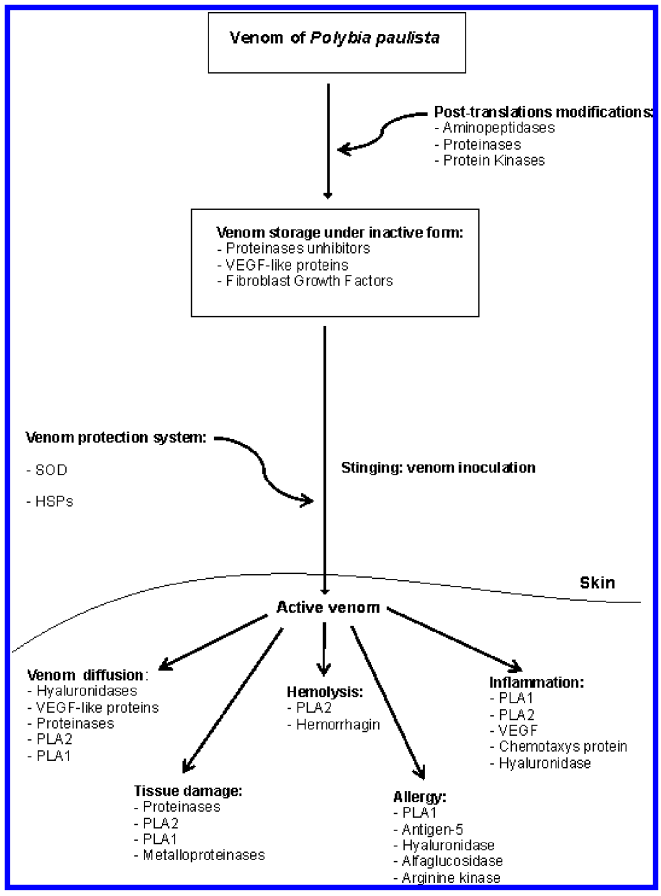

Figure 3. General mechanism of actions proposed for the venom of the social wasp *P. paulista* venom, involving of the proteins identified in the present investigation.

To establish a possible relationship between the putative molecular function of proteins from *P. paulista* venom (Table 1) with the known local and systemic symptoms/effects of stinging accidents in humans by social wasps, a general envenoming mechanism for these toxins (Figure 3) is proposed below.

P. paulista represents a latent risk for humans allergic to venom proteins of social wasps, for which antigen-5, hyaluronidase and PLAs are recognized as the major allergens.[14] As may be observed in Table 1, these proteins occurred in different molecular forms in *P. paulista* venom. Antigen-5 represents an important and very common allergen and has no known physiological role in wasp venoms;[23] seven different forms of this protein were identified with M_r ranging from 19.5 to 26.5 kDa and p*I* from 7.2 to 9.7 (spots 125, 134, 188, 189, 194, 236 and 161). It is important to emphasize that five of these forms have the same accession number (Q7Z156), suggesting that they may represent either truncated forms of a common larger protein, such as spot 125 (26.5 kDa), or even isoforms of antigen-5. The protein identified in Table 1 as Sol i-III (spot 161) is similar to the allergen reported in the venom of the ant *Solenopsis invicta*, which in turn has a highly conserved structure associated with antigen-5 from the venom of social wasps.[14] Proteins corresponding to the spots 125 and 134 were glycosylated (Figure 2A), indicating that they had undergone post-translational modifications. The immunoblotting assays revealed that the proteins corresponding to spots 125, 134, 189, and 194 were immunoreactive to human specific-IgE (Figure 2B). Structural studies of antigen-5 showed that this protein has structural similarities to chemokines.[24] In addition, it has been recognized that structurally, antigen-5 belongs to the cysteine-rich secretory proteins (CRISP) family, which is characterized by the presence of many cysteine residues in their sequences, generally forming several disulfide bridges;[25] in this protein family are included a series of cancer-related antigens and potent inflammatory proteins from sandflly saliva.[26] Thus, the four different forms of antigen-5 and the protein identified as Sol i-III-like (similar to the antigen-5) seem to constitute important allergens in the venom of the social wasp *P. paulista*.

Hyaluronidase is a endo-*N*-acetylhexosaminidase, acting as a "venom spreading factor", cleaving the β-1,4 glycosidic bond between *N*-acetyl-glucosamine and D-glucuronic acid of hyaluronic acid of the extracellular matrix, resulting in an increase in tissue permeability and facilitating the diffusion of the toxic venom substances.[14] This protein is commonly found in the venoms of social wasps and bees, being identified as an important allergen that sometimes causes crossed-reactivity among the venoms of different wasps species, and even with honeybee venom proteins.[25] This enzyme also causes potent inflammation around the stinging site. Four different forms of this enzyme (spots 70, 71, 73 and 74; Table 1) were identified in the venom of *P. paulista* with the same accession number (Q9U6V9), suggesting that these proteins may have originated due to some type of PTMs of a precursor protein. Figure 2B shows that all these hyaluronidases are immunoreactive to human specific-IgE, confirming their allergenic potential.

PLAs are relatively common in Hymenopteran venoms, occurring mainly as -A_2 and -B types.[27] The PLA_1 and PLA_2 catalyze the specific hydrolysis of ester bonds of 1,2-diacyl-3-snglycerophospholipids, at the positions sn-1 and sn-2, respectively, converting these substrates into their corresponding lyso compounds with the release of fatty acids. Thus, the PLAs are able to disrupt the phospholipid packings from several types of biological membranes, leading to pore formation, cell lysis, inflammation and tissues damage.[28] When compared to each other, PLA_1s and PLA_2s present no sequence homology; apparently, these proteins have distinct functions. In the present investigation, eight different forms of PLA_1 were identified (spots 121, 177, 192, 196, 203, 222, 234 and 235; Figure 1 and Table 1) and two forms of PLA_2 (spots 179 and 207; Figure 1 and Table 1) in *P. paulista* venom. PLA_2 was previously reported in social wasps venoms and in honeybee venom, being described as highly hemolytic.[13] Meanwhile, PLA_1 have been partially characterized in the venoms of some wasp species from the northern hemisphere,[29] in ants[14] and in the neotropical social wasp *P. paulista*.[27]

The reaction with Pro-Q Emerald 300 dye (Figure 2A) indicates that the PLA_1s (spots 196 and 234) are glycoproteins, as well as both PLA_2 (spots 179 and 207), confirming a previous report that PLA_2s from social wasp venoms are glycoproteins;[13] meanwhile, the PLA_1s are generally associated to allergic and inflammatory processes.[29] Immunoblot data (Figure 2B) showed that the PLA_1s corresponding to the spots 121, 177, 222, and 234 are immunoreative to human specific-IgE; therefore, these forms of PLA_1 are allergenic proteins.

As already reported above for antigen-5 and hyaluronidase, the enzymes PLA_1 and PLA_2 occurred as series of different proteins, identified with same accession code for each enzyme, A2VBC4 and AF38408, respectively, suggesting that these enzymes occur as a series of different isoforms and/or post-translationally modified forms.

It is surprising to observe that some proteases identified in *P. paulista* venom look like those so far isolated from snake venoms. However, it must be considered that there is no genomic information available in data banks for social wasps; because of this, the wasp venom proteins were identified based on cross-species data. The method of venom collection in the present investigation leaves the possibility that some cellular proteins may be mixed to the true venom components. Therefore, it is not possible to be sure if the snake venom-like proteins (as well those identified by homology with cross-species) represent true wasp venom proteins or if they are cellular proteins from the reservoir/gland tissue compartment. However, taking into account that the antibody response in venom-sensitive patients is expected to be raised only against venom proteins, the HSPs (spots 138 and 168) and the arginine kinases (spots 226 and 237), which were immunoreactive to human specific-IgE from *P. paulista* venom-sensitive patients (Figure 2B), may be considered to be from venom itself.

Among the serine proteinases found in *P. paulista* venom, two of them are similar to those already reported in other wasp venoms (spots 172 and 206), one is similar to the enzyme reported in honeybee venom (spot 233), while the others are similar to those already reported in snake venoms (spots 216 and 202). Spot 124 corresponds to a chymotrypsin-like proteinase, which also belongs to the family of serine-proteinases. Spot 216 was detected in a glycosylated form (Figure 2A). Some of these proteinases seem to degrade tissue proteins in the site of bites/stinging in a nonspecific manner, while others cleave plasma proteins of the victims in a relatively specific manner, causing potent effects. These proteins may act either as activators or inhibitors of hemostasis and thrombosis processes, such as blood coagulation, fibrinolysis and platelet aggregation,[30] which cause a cascade of systemic effects leading to multiple organ disfunction syndrome, sometimes observed after an incident of wasp stinging.[31]

In the venom of *P. paulista*, four metalloproteinases similar to those reported in snake venoms (spots 21, 133, 165 and 223) were identified. The actions of these enzymes and those from snakes venoms in the pathogenesis of venom-induced tissue damage are comparable; they have a putative involvement in myonecrosis, skin damage, edema, among other inflammation-related reactions.[32] Some of these manifestations have been already reported in patients who suffered massive attacks of honeybees or wasps.[33]

The typical symptoms caused by the action of zinc metalloproteinase-disintegrins in the envenoming are hemorrhage and edema around the site of the bite/stinging, which lead to local necrosis and tissue damage, hemorrhage, hypovolemia, shock, and some types of coagulopathies.[34] In addition to this, some metalloproteinases may degrade the extracellular matrix and capillary basement membranes, contributing to the disruption of local capillary networks, resulting in hemorrhage and edema.[34] In the venom of *P. paulista* four zinc metalloproteinase-disintegrin-like proteins were identified [spots 15, 29, 214 (glycosylated) and 232] (Figure 2A). Systemic toxic reactions are frequently observed in victims of mass attacks of social wasps, which result in hemolysis, coagulopathy, rhabdomyolysis, acute renal failure[35] and hypovolemia.[36] Thus, some of the characteristic effects caused by the presence of zinc metalloproteinases-disintegrins are also observed in the victims of wasp stinging envenomation. However, since this is the first report about the presence of zinc metalloproteinase-disintegrin-like proteins in Hymenopteran venom, these enzymes must be individually characterized; as well, the clinical manifestations must be observed in more details.

Two arginine kinases were identified in the venom of *P. paulista* (spots 226 and 237). This enzyme, previously detected in the venom of the honeybee (*Apis mellifera carnica*), was proposed to play a putative role in protein phosphorylation;[16] however, it was not determined if the targets are the self-venom toxins, the proteins from the victims of envenomation, or both. The two forms of arginine kinase present in *P. paulista* venom were glycosylated and immunoreative to human specific-IgE (Figure 2B), indicating their potential action as allergens. Arginine kinase from shrimp muscles has been recognized as potent food allergen, known as Pen m2.[37]

It was recently reported that honeybee venom contains a series of proteins (superoxide dismutase, glutathione-*S*-transferase, peroxiredoxin and thioredoxin peroxidase) involved with the protection of venom toxins against natural oxidative stress, are synthesized in honey bee venom gland tissue.[16] Among these proteins, superoxide dismutase was identified in the present investigation (spots 166 and 182), being reasonable considering it plays the same role in social wasp venoms. The presence of this protein in *P. paulista* venom may have been due to the rupture of venom apparatus rupture during the venom collection. The storage of venom by the social Hymenoptera within the venom reservoir also exposes toxins to another source of stress—the high temperatures outside the hive, especially in tropical regions. Under this condition, the venom toxins may undergo some degree of thermal denaturation, losing their biological activity. Recently, the presence of heat shock proteins (HSPs) was reported in honeybee venom gland,[16] acting as chaperonins to facilitate the folding of other venom toxins. In the present investigation, four HSPs were identified: spots 59 and 110 (HSP 70), spot 136 (HSP 60) and spot 168 (HSP 90). HSPs may be expressed as a result of different sources of stresses, inclusive the oxidative, in order to protect the cell and the proteins against molecular damages.[38] Thus, the presence of HSPs in *P. paulista* venom may be associated with self-protection of the venom to preserve its activity. In addition, Figure 2B shows that HSP 90 (spot 168) and HSP 60 (spot 168) are immunoreactive to human specific-IgE, suggesting that these proteins also may be important allergens in *P. paulista* venom.

Four different VEGF forms identified in *P. paulista* venom (spots 16, 17, 113 and 200) were also reported in honeybee venom.[15] EGF is well known to promote vascular permeability,[39] which may contribute to venom diffusion. In addition, wasp venom contains a series of cytolytic proteins[13] and peptides,[9] which may damage the venom glands and reservoir. The presence of VEGFs also could contribute to stimulate the growth of cells from the venom glands and reservoir, preventing the rupture of the tissues due to the action of cytolytic components. Data shown in Figure 2B indicate that some VEGF proteins are immunoreactive to human specific-IgE (spots 16 and 17), suggesting that these proteins also may be allergenic.

The venom also showed the presence of serine- and cysteine-proteinase inhibitors (spots 12, 128 and 137); these proteins could act as inhibitors of stored venom proteases to prevent the self-proteolysis of the of the venom proteins.

Concluding Remarks. In the present study, a classical bottom-up proteomic approach was used, where the proteins from *P. paulista* venom were extracted and separated by 2-DE; the individual protein spots were proteolytically digested and

subsequently identified by using tandem mass spectrometry and database query with the protein engine search algorithm MASCOT.

The composition of *P. paulista* venom suggests a the general envenoming mechanism based on five classes of actions: (i) diffusion of venom through the tissues and to the blood—this role probably involves the hyaluronidases, PLA_1, PLA_2, metalloproteinases, zinc metalloproteinases-disintegrins and VEGF-like proteins; (ii) tissue damage—probably due to PLA_1, PLA_2, serine-proteinases and metalloproteinases; (iii) hemolysis—played by PLA_1, PLA_2, serine-proteinases; (iv) inflammation—generally, may be played by PLA_1, PLA_2, VEGF-like proteins, hyaluronidases; and (v) allergy—due to antigen-5, PLA1, hyaluronidase, HSP 60, HSP 90 and arginine kinases. These general roles are summarized in Figure 3.

Another important aspect of protein allergenicity, which currently attracts attention of clinical immunologists, is the discrimination between carbohydrate- and protein-based epitopes, because there is evidence that both types of epitopes may elicit immune responses.[40] Up to now, only hyaluronidases,[14,28,41] PLA2[13,42] and DPP4[43] have been identified as allergenic glycoproteins in the venom of social Hymenoptera. The present study reports for the first time the existence of allergenic forms of antigen-5 glycoprotein.

This manuscript also reports for the first time the presence of metalloproteinases and zinc−metalloproteinases in the venom of social Hymenoptera. Certainly this result will open novel possibilities to investigate some unexplained pathophysiological effects, some times observed after the envenoming caused by wasps stingings.

Thus, results of the proteomic analysis of *P. paulista* venom will contribute to a better understanding of the general envenoming mechanism caused by this insect. On the other hand, identification of the whole allergen panel of this venom may improve the diagnostics of allergies in the near future by indicating novel protein targets to build more complete microarrays or even for the preparation of suitable recombinant proteins to be used in immunotherapy of patients sensitive to wasp venom.

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Supporting Information Available: Supplementary table. This material is available free of charge via the Internet at http://pubs.acs.org.

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