

## A new scenario of bioprospecting of Hymenoptera venoms through proteomic approach

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**Abstract:** Venoms represent a huge and essentially unexplored reservoir of bioactive components that may cure diseases that do not respond to currently available therapies. This review select advances reported in the literature from 2000 to the present about the new scenario of Hymenoptera venom composition. On account of new technologies in the proteomic approach, which presents high resolution and sensitivity, the combination of developments in new instruments, fragmentation methods, strategic analysis, and mass spectrometry have become indispensable tools for interrogation of protein expression, molecule interaction, and post- translational modifications. Thus, the biochemical characterization of Hymenoptera venom has become a major subject of research in the area of allergy and immunology, in which proteomics has been an excellent alternative to assist the development of more specific extracts for diagnosis and treatment of hypersensitive patients to Hymenoptera venoms.

**Key words:** hymenoptera, venoms, allergens, phospholipase A1, wasp venom antigen 5, hyaluronoglucosaminidase, mass spectrometry, proteomics.

### INTRODUCTION

Several venomous organisms such as reptiles, fish, amphibians, mammals, starfish, sea urchins, cone snails, octopi, nemertines, arachnids, insects, myriapods, and some cnidarians are the target of numerous studies in toxinology for their biotechnological and therapeutic potential (1).

Animal-based medicinal products are involved in global trades that worth billions of dollars per year (2). The World Health Organization already observed that the study of traditional medicines through bioprospecting had found several pharmaceutical compounds, 11.1% of them were obtained from plants, and 8.7% from animals. Moreover, of the 150 prescription drugs currently in use in the United States of America, 27 have animal origin (3). After a bradykinin-potentiating peptide was isolated – from the snake venom of

the Brazilian *Bothrops jararaca* in the 1950s – and used to develop the first commercial angiotensin I-converting enzyme (ACE) inhibitor for the treatment of renovascular hypertension, various venom compounds have been investigated as natural sources of more specific/efficient pharmaceutical products (4, 5).

In the early 1980s, a non-local anesthetic drug was obtained from a *Conus magus* cone snail peptide –  $\omega$ -conotoxin M-VII-A, an N-type calcium channel blocker. The US Food and Drug Administration (FDA) approved the production of a synthetic peptide of a conotoxin, ziconotide, under the trade name Prialt®. The ziconotide molecule is used unmodified from the creature's chemistry to treat chronic pain (6). Several studies have investigated specific animal toxins in order to find alternative therapies to various disorders that affect signaling transduction such

as Alzheimer's disease (7). Similarly, in previous studies, researchers showed that the venom of solitary wasps, including *Anoplius samariensis* and *Pseudagenia maculifrons*, may be useful not only for basic neuroscience research but also for the development of therapeutic agents against neurological disorders (8).

Regarding the development of drugs for the treatment of patients sensitive to Hymenoptera venoms, the allergen-specific immunotherapy is highly effective and recommended for patients with a specific Hymenoptera sting anaphylaxis history. However, effective immunotherapy procedures can be hindered by frequent cross-reactions to venoms of different Hymenoptera species, due to difficulties in identifying the responsible species. Thus, a better understanding of the composition of Hymenoptera venoms is a rich subject for many researchers in the allergy and immunology area.

## WHY STUDY HYMENOPTERA VENOMS?

The venom from social Hymenoptera (wasps, bees and ants) is an important defensive weapon. Thus, stinging events involving wasps, honeybees and ants are frequent worldwide and immediate hypersensitivity reactions, such as anaphylaxis, comprise a global cause of death. Moreover, serious envenomations may kill even non-allergic individuals due to multiple stings. The diagnosis of stings by bee, wasp and ant begin from a history of potential contact matched with onset of appropriate clinical signs (9).

It is known that the prevalence of sensitization against Hymenoptera insects was evaluated between 9.3 and 28.5% in world population (10). Anaphylactic reactions to Hymenoptera stings are not dose dependent or related to the number of stings (11). A single sting may cause a transient local inflammation characterized by pain, redness and swelling in humans (12). Studies showed that the general population present type 1 hypersensitivity reaction, which promotes a series of clinical problems, such as urticaria, itching, malaise, angioedema, chest constriction, diarrhea, abdominal pain, dyspnea, wheezing, weakness, edema, fatigue, dizziness, nausea, fever and unconsciousness as well as drop in blood pressure, collapse, cyanosis, cardiovascular symptoms, up to a life threatening systemic anaphylactic shock (13, 14).

Mass stinging events may be life-threatening as a result of venom toxic action when injected in large amounts (4). Systemic reaction is reported to occur in 5% of the general population (5).

The biochemical characterization of Hymenoptera venom has become the focus of much research in the area of allergy and immunology, in which proteomic approach has been an excellent alternative to assist the development of more specific extracts for diagnosis and treatment of hypersensitive patients. Currently, allergy immunotherapy specific to Hymenoptera venoms is carried out with crude venom, which usually presents a complex composition, consisting of a large amount of proteins and peptides. It is not possible to estimate the exact composition of venoms and, moreover, natural factors such as degradation heterogeneity of proteins and the source of allergens may hinder procedures for allergy immunotherapy. Thus, such studies can contribute to increase the knowledge about biochemistry of venoms and offer scientific basis for future development of recombinant allergens for diagnostic and therapeutic use against Hymenoptera venom allergies. Figure 1 shows some Brazilian Hymenoptera insects, which were evidenced by Castro and Palma (15).

## WHAT IS THE COMPOSITION OF THESE VENOMS?

Lima and Brochetto-Braga (16) reported that numerous proteins and peptides from wasp and bee venoms had already been identified and characterized by several laboratories throughout the globe. Hymenoptera venoms are constituted of a complex mixture of proteins, a group of polycationic peptides and low molecular mass toxins, in which the proteins are recognized as important allergens (9, 17-20). Whereas bee and wasp venoms present are composed of protein and peptides and fire ant venoms, alkaloids and few soluble proteins (11). Hereafter, several recent studies will be explored.

### Proteins

Several major allergens, usually glycoproteins with a molecular weight of 10 to 50 kDa, have been identified in venoms of bees, vespids and ants. In general, paper wasp venoms contain phospholipases, antigen 5, hyaluronidase and serinoproteinases. Table 1 presents the classical

**Table 1.** Classical proteins isolated from Hymenoptera venoms by basic analytical techniques

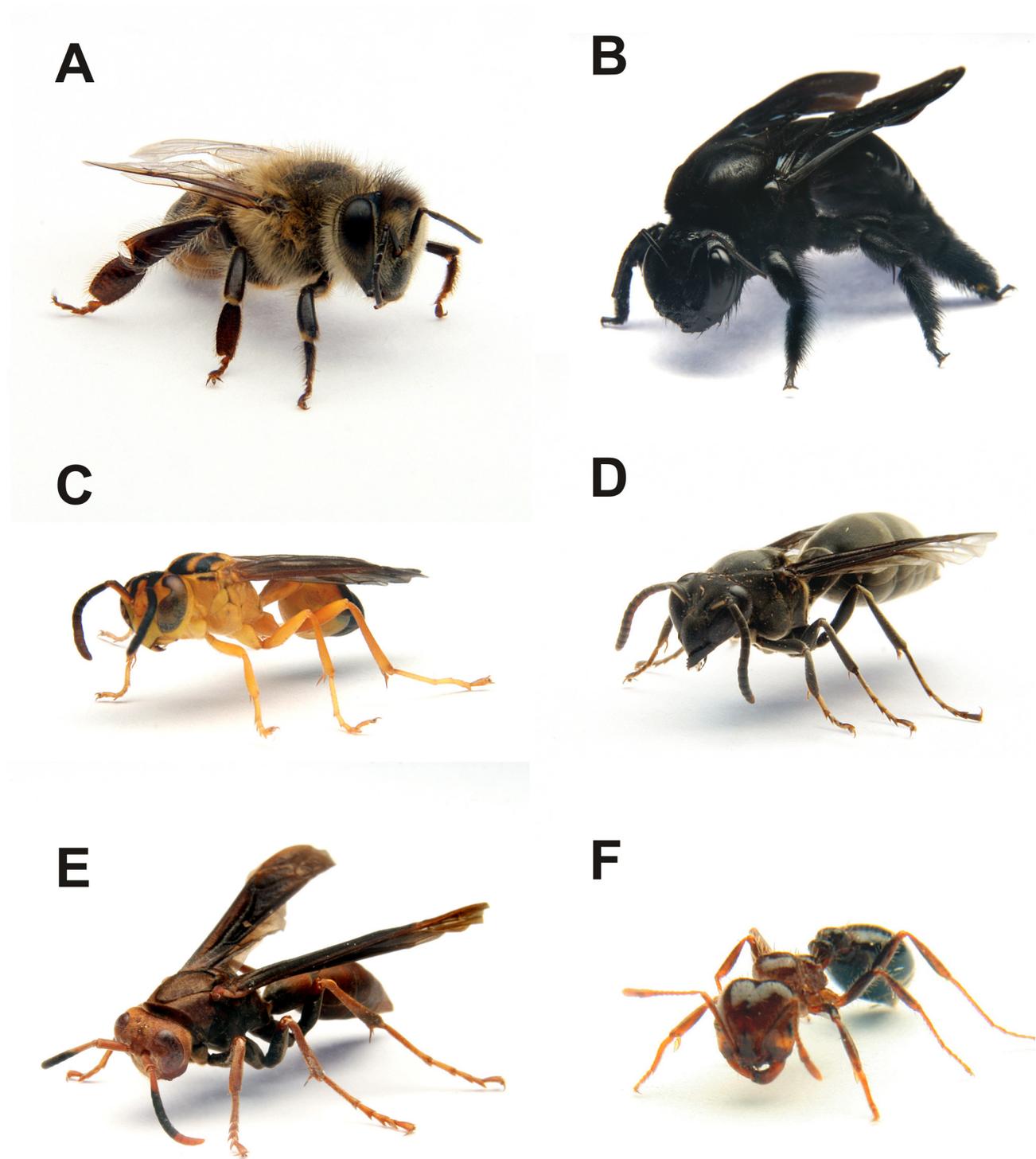
Species	Proteins	References
<i>Apis mellifera</i>	Phospholipases A	21
<i>Solenopsis invicta</i>	Sol i I (phospholipase A <sub>1</sub> )	22
<i>Vespa basalis</i> , <i>V. orientalis</i> and <i>V. flaviavitarus</i>	Phospholipase A1/A2	23, 24
<i>Vespula maculifrons</i> , <i>Vespula maculate</i> , and <i>Vespula arenaria</i>	Phospholipases A and B, hyaluronidase	25
<i>Vespula maculifrons</i>	Phospholipases A and B, hyaluronidase, antigen 5	26
<i>Polybia paulista</i>	Phospholipases A <sub>2</sub>	27, 28
<i>Vespa basalis</i> , <i>V. mandarinia</i> and <i>V. verutina</i>	Phospholipases A <sub>2</sub>	29
<i>Vespa mandarinia</i>	Phospholipases B	30
<i>Dolichovespula maculata</i> , <i>Vespula squamosa</i> , <i>Polistes exclamans</i>	Phospholipases A and B, hyaluronidase and antigen 5	31
<i>Polybia scutellaris</i>	Antigen 5	32, 33
<i>Solenopsis invicta</i>	Sol i III (antigen 5)	22
<i>Apis mellifera</i>	Hyaluronidase	34
<i>Solenopsis invicta</i>	Sol i II (hyaluronidase)	22
<i>Polistes dominulus</i> and <i>Polistes exclamans</i>	Serine protease	35
<i>Bombus pennsylvanicus</i> and <i>Bombus terrestris</i>	Serine protease	36, 37
<i>Bombus pennsylvanicus</i> , <i>P. dominulus</i> and <i>P. exclamans</i>	Serine protease	38
<i>Apis mellifera</i>	Serine proteases	39

proteins found in Hymenoptera venoms by some analytical techniques including liquid chromatography and one electrophoresis.

One of the first studies involving more sophisticated techniques was conducted in the early 1980s. The work showed the complexity of many Hymenoptera venoms and that four proteins were already isolated from *Apis mellifera*, *Polistes fuscatus*, *P. apachus*, *P. metricus*, *P. exclamans*, *P. annularis*, *Vespula flavopilosa*, *V. squamosa*, *V. sulphurea*, *Dolichovespula maculata* and *Vespa cabro* venoms (40). These pioneering findings first characterized Hymenoptera venom protein profile by two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D SDS-PAGE) in the global literature.

The characterization of Hymenoptera venoms has improved since 2000, when the proteomic method was released by academic institutions, as the extracts utilized in the immunotherapeutic process depended on the composition of such venoms and the comprehension of their action mechanisms.

Probably, the best characterized insect venom is the one from *Apis mellifera*. However, little is known about the venoms of the Apidae family, which includes social bees, solitary bees and bumblebees (16). Among the stinging Hymenoptera, the venomics of *Apis mellifera carnica* honeybee was investigated to identify new venom components with allergic potential by Peiren *et al.* (41) through a proteomic



**Figure 1.** Workers of some Brazilian Hymenoptera insects: (A) Africanized *Apis mellifera* bee; (B) *Bombus* sp. bee; (C) *Agelaiia pallipes pallipes* wasp; (D) *Polybia ignobilis* wasp; (E) *Polistes lanio lanio* wasp; (F) *Solenopsis invicta* ant (photos by Martelli Filho).

approach. In that work, honeybee venom was submitted to two-dimensional gel electrophoresis and 49 proteins were elucidated by mass spectrometry (MS), 39 of them were identified as phospholipases A<sub>2</sub>, hyaluronidases, Api m 6, melitin, secapin and acid phosphatases. For the

first time, new venom proteins were described: vascular endothelial growth factor (VEGF) and a protein similar to major royal jelly protein 8, MRJP-8 and a series of proteins related to the protection of venom against oxidative stress such as superoxide dismutase, glutathione-S-

transferase sigma 1 isoform A, peroxiredoxin and thioredoxin peroxidase 1 isoform A were also reported (41). Similarly, Honeybee Genome Sequencing Consortium (42) elucidated the presence of other important proteins in *Apis mellifera* venom including antigen 5, proteinases, heat shock proteins (HSP), disintegrins and metalloproteinases.

The understanding of the mechanisms of action of Hymenoptera venoms is a challenging task. Numerous works were carried out to identify the mechanisms that protect honeybee venom gland secretory cells against harmful toxins present in this venom (43). Such studies showed that the venom of honeybee workers is used to defend the colony or themselves when they are exposed to dangers and predators. The proteomic study by Peiren *et al.* (41) revealed four antioxidant enzymes: thioredoxin peroxidase 1 isoform A (TPX1), CuZn superoxide dismutase (SOD1), peroxiredoxin 2540 (PXR2540) and glutathione-S-transferase sigma 1 isoform A (GSTS1). Although glutathione-S-transferase (GST) has also been associated with xenobiotic detoxification, this protein is known to protect only against oxidative stress. Many proteins involved in other forms of stress were also found in that research. However, the function of these molecules in animal venoms is still unknown (41).

Other important research involved the proteomic analysis of *Polybia paulista* wasp venom (44). Santos *et al.* (44) studied 94 out of 225 proteins present in the *P. paulista* wasp venom and, among them, antigen-5, hyaluronidases and PLAs and serine proteinases. Furthermore, novel proteins were reported in wasp venoms including growth factor-like proteins, arginine kinase, superoxide dismutase,  $\alpha$ -glucosidase, metalloproteinases, zinc metalloproteinase-disintegrins, serine proteinases, proteinase inhibitors, heat shock proteins, Sol i-II and -II like proteins. Through the study, mechanisms of action of Hymenoptera venom were proposed, explaining the action of animal venoms when they are injected into their victims (44). The biochemical characterization of wasp venoms based on protein identification may provide an extensive basis in order to understand their biological mechanisms, which it is an important prerequisite for the development of new drugs.

The proteomic approach also revealed

homologues of some common wasp allergens in *Polistes gallicus* and *Agelaea pallipes pallipes*. In venoms, the classical allergens, such as phospholipase A<sub>1</sub>, antigen 5, hyaluronidase and serine-protease, were identified and characterized by MS and specific binding to IgE (19, 45).

It is necessary to emphasize the importance of proteomic approach in Hymenoptera venom works. A common aspect among studies is the presence of multiple forms of major allergens in Hymenoptera venom (45-47). Some authors suggest that these proteins occur in different molecular forms in Hymenoptera venoms, since they represent important and extremely common allergens, and they may correspond to either truncated forms of a common larger protein or even isoforms. But why would this happen? Would it be a strategy of the nature to develop alternative molecules to escape from the immune system of their victims?

It is known that prediction of risks of anaphylactic reactions is very important, and the correct diagnosis requires the understanding of the most important allergens present in animal toxins. Recently, the major proteins from *Vespula germanica* and four additional *Vespula* species were investigated by proteomic approach. A new hyaluronidase-like protein, proven to be the major component of all *Vespula* species, was studied by Kolarich *et al.* (48) through matrix-assisted laser desorption ionisation/quadrupole-time of flight mass spectrometry (MALDI-Q-TOF-MS). Moreover, sequence comparisons of antigen 5 and phospholipases from *V. vulgaris*, *V. germanica*, *V. maculifrons*, *V. pensylvanica*, *V. flavopilosa* and *V. squamosa* revealed some differences in their amino acid sequences (48).

Recently, many researchers elucidated, by molecular modeling, the tridimensional structure and post-translational modifications of major allergens such as phospholipase A<sub>1</sub> and hyaluronidase from *Polybia paulista* venom, hyaluronidase from *Vespula vulgaris* venom, phospholipase A<sub>2</sub> and Api m7 from *Apis mellifera* (45, 49-51). Additionally, the differences among allergens from Hymenoptera venoms are the focus of several studies. It was demonstrated that the allergen antigen 5 presents differences among *Polistes* venoms, mainly in relation to the amino acids sequences of the allergens from the venoms of North American and European *Polistes* wasps (20). These findings are very important

to pharmaceutical industries since novel drugs and specific treatments can be improved if these allergens are better understood structurally and their linear and conformational epitopes are identified.

Many studies were performed in order to characterize the venoms of social wasps by using SDS-PAGE and MS. Recently, the identification and characterization of venom proteins of two solitary wasps – *Eumenes pomiformis* and *Orancistrocerus drewseni* – elucidated the presence of various immune response-related proteins and antioxidants. It was suggested that solitary wasps might use their own venom to keep their prey fresh to protect it from invasion of microorganisms and physiological stresses (52).

Some works have constructed cDNA libraries of venom glands and, consequently, numerous novel genes were identified, including those that encode venomous proteins of solitary bee venom. The venom gland of *Orancistrocerus drewseni* wasp was used to determine differential gene expression profiles in the venom gland and sac of solitaires hunting wasp species. A total of 498 expressed sequence tags (EST) were gathered into 205 contigs. Among these data, 115 contigs were similar to proteins with assigned molecular function in the Gene Ontology Database. Most contigs of the study were homologous to genes from Hymenoptera, encoding several proteins as hyaluronidase, phospholipase A<sub>2</sub> and zinc-metallopeptidases (53).

Few studies have been carried out using ant venom because of the amount of venom in their glands. It is known the venom from fire ants is composed of 90% of piperidine alkaloids and 10% of allergenic proteins (54). Studies carried out by Chen *et al.* (55, 56) showed that dialkylpiperidines are characteristic of fire ants of the genus *Solenopsis* (Hymenoptera: Formicidae). Workers of the black fire ant, *S. richteri* and *S. invicta*, produce different stereoisomers of 2,6-dialkylpiperidines. These findings are discussed in relation to the evolutionary significance of these piperidines and their possible biosynthetic pathways in *Solenopsis* sp. ants. Moreover, the venom proteins of *Solenopsis invicta* have been the focus of a series of studies, in which three principal allergens were identified (Sol I I, Sol I II and Sol I III) and characterized as phospholipase A<sub>1</sub>, hyaluronidase and antigen 5, classical allergenic proteins from wasp and bee venoms (20).

The identification of novel allergens and different forms of known ones will be useful in helping to build more complete microarrays of proteins for allergy diagnosis. It may even help contribute to the development of suitable protocols for the expression of recombinant forms of proteins, to be used in the immunotherapy of patients sensitive to wasp venom. Besides, the allergenicity of some venom proteins is probably the most well studied action of stinging incidents perpetrated by social wasps. The identification of individual allergens, as well as their molecular characterization, certainly will contribute to the identification of the whole panel of Hymenoptera venom allergens.

### Peptides

The peptide components of venoms from social Hymenoptera are spread over the molar mass range of 1400 to 7000 Da and together comprise up to 70% of weight of freeze-dried Hymenoptera venom (57).

In a previous work by Palma (58), the author described how important peptides are in several physiological processes, working as neurotransmitters, hormones, toxins, antibiotics, and defensins since venom peptides act on a wide variety of membrane protein receptors and may interact directly with phospholipids of the plasma/organelle membranes or with cytosolic proteins to regulate their activities.

Several peptides have been described from wasp venoms, including mastoparans, protonectins, chemotactic peptides, kini related peptides (wasp kinins or bradykinin homologue), sylverin and cabrolin (59). Whereas chemotactic peptides recruit macrophages and polymorphonuclear leukocytes near the site of stinging, mastoparans and protonectins were proven to act as mast cell degranulating peptides responsible for histamine release (12, 60). Mastoparans present an amphipathic  $\alpha$ -helical conformation that permits the creation of porin structures in membranes and the release of histamine. Besides, these peptides are assumed to trigger histamine release via G-protein coupled receptor cascades; additionally, regarding the venom proteome, mastoparans are thought to regulate some venom enzymes (12, 60, 61). It is known that some mastoparans bind to G-protein coupled receptors (GPCRs) that are involved in the activation of different types of basophils, chemotaxis of

leukocytes, and neurotoxicity (62, 63). Several mastoparan and protonectin homologues have been sequenced from different wasp species using Edman degradation and tandem mass spectrometry (MS/MS) (6, 59). Studies have shown that the use of two or more peptides from wasp venoms can potentiate biological activities, for example, the mixture of two (protonectin 1-6 and protonectin) can amplify the activities of mast cell degranulation, LDH releasing from mast cells, and antibiosis (17).

The literature shows that mastoparans, wasp kinins, antimicrobial peptides and myotoxic peptides were identified among the peptides found in social wasp venoms (64-70). Furthermore, it was also reported that some wasp venom peptides provoke myonecrosis and apoptosis, probably involving caspases signalling, which is corroborated by mitochondrial damage and cytokine activation (71).

Regarding bee venom, it is composed of several peptides including melittin, apamin, adolapin, mast-cell degranulating peptide and secapin that were previously characterized and analyzed (16). A novel melittin isoform from Africanized *Apis mellifera* venom was found to be less hemolytic and to present a less organized secondary structure (72). In this scenario, the use of matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and nano-electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometry permitted the identification of several peptide sequences including melittin and different breakdown products. Matysiak *et al.* (73) discovered a new peptide [HTGAVLAGV + amidated (C-term)] in the bee venom, but its function is yet unknown.

Several Hymenoptera venom peptides were evaluated by injecting them into the hind paw of mice (74). The results showed that mellitin (*Apis mellifera*), polybia-MP-I, N-2-polybia-MP-I (*Polybia paulista*), protonectarina-MP-NH2 and protonectarina-MP-OH (*Protonectarina sylveirae*) produced hyperalgesic and edematogenic effects. These results indicate that these peptides could contribute to better understand about the inflammation process and pain induced by *A. mellifera*, *P. paulista*, and *P. sylveirae* venoms (74).

Although social wasps, bee and ants are better known as venom producers, the vast majority of Hymenoptera are solitary wasps. Whereas the

social Hymenoptera use their venoms for hunting and defending themselves and their colonies, the solitary wasps use them only to paralyze their prey (75). Therefore, neurotoxic compounds and bioactive substances such as eumenine mastoparan-AF, anoplins, pompilidotoxins and, mainly, bradykinin-related peptides present in the venoms of solitary wasps are responsible for causing long-term and non-lethal paralysis of the prey (9, 53).

Several neurotoxic components and antimicrobial peptides have been isolated from solitary wasp venoms. The discovery of venom gland/sac-specific genes has encouraged further studies on biologically active components in the venom of *Orancistrocerus drewseni* (53). Moreover, philanthotoxins found in the venom of *Philanthus triangulum* inhibit neuromuscular transmission by blocking postsynaptic glutamate (76).

The venoms of *Megascolia flavifrons*, *Anoplius samariensis*, *Batozonellus maculiformis*, and *Cyphononyx dorsaris* were proven to contain several kinins that block nicotinic acetylcholine receptors (8, 77-80). Furthermore, antimicrobial peptides and mastoparans were found in *Anterhynchium flavomarginatum*, *A. samariensis*, and *Eumenes rubronotatus* (8, 77-83).

Concerning ant venoms, Myr 1 from *Myrmecia pilosa* was identified as a major allergenic product followed by Myr 2, and they may dissociate or be cleaved into minor fragments, such as pilosulin-1 and -2. Pilosulins, in their turn, were shown to have antimicrobial peptides, hemolysins and histamine release peptides (84).

Other researchers identified and described: poneratoxins that are neuropeptides obtained from the venom of *Paraponera clavata* ant, ponerocins that have hemolytic effects, insecticidal and antimicrobial activities, and ectatomins that are neurotoxins isolated from *Ectatomina turbeculatum* venom (85-87).

In addition to inflammatory responses, the pilosulins of the venom of jack jumper ant (*Myrmecia pilosula*) were proven to be immunological molecules. New variants of pilosulins were identified in serum samples from patients with a history of jack jumper ant sting allergy. One of these peptides was designated as the minor allergen Myr p 3 according to the International Union of Immunological Societies nomenclature. Although peptidomes are mainly

characterized via high performance liquid chromatography (HPLC) and mass spectrometry, a gel-based protocol for studying the peptidome of the jack jumper ant was also revealed. Using SDS-PAGE, new disulfide-linked dimmers were identified by comparing non-reduced and reduced/alkylated venom (12, 88).

## LOW MOLECULAR MASS COMPOUNDS

Besides proteins and peptides, Hymenoptera venoms present biologically active amines, which are non-peptide components with a variety of pharmaceutical properties (89). Among the low mass compounds are histamine, serotonin, lysine, octopamine, GABA, spermidine, spermine, arginine, acetylcholine and several other molecules (90-92). These toxins are neurotransmitters, agonists and/or antagonists of ion channels (93). Such compounds are used by solitary wasps to modulate neurotransmission in insect prey permitting them to paralysis, since they use their venoms only for active defense (94-97).

Recently, a novel low mass compound was isolated from the venom of the social wasp *Polybia paulista* and identified as polybioside. Its structure was assigned as 3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl-3-(1H-imidazol-4-yl) propanimidate by magnetic resonance and MS protocols. The application of this low mass compound in rat brain, followed by the detection of c-Fos protein expression in some brain regions indicated that the compound is neuroactive in a number of brain areas, causing convulsions in rats, even when peripherally applied (17).

## STATE OF THE ART IN HYMENOPTERA VENOM BIOPROSPECTING

The bioprospecting of novel molecules in the pharmaceutical and biomedical areas employs the proteomics analysis strategy. But what is proteomics? Proteomics is a term that refers to all the proteins expressed by a genome; it involves the identification of proteins in the body and the determination of their role in physiological and pathophysiological functions. Proteomic studies represent only a subset of all possible gene products, since the genetic machine of organisms produces different molecules depending on the genes that will be activated or inhibited at a certain point of

time (98). Thus, post-translational modifications or degradation processes that affect protein structure, localization and function are targets of numerous studies in this area, particularly proteolytic degradation products of proteomes that contain disease-specific information (98).

The development of proteomic strategies offered new drug candidates for clinical assays, including the identification of novel biomarkers for disease detection and evaluation of drug adverse effects. Besides the standardization of proteomic methods, the accessibility of proteomic data in public databases has aided research of molecules derived from known genomes, such as Hymenoptera venoms.

The implementation of proteomic approach helped in the discovery of new drugs by using the sensitivity and high-throughput process of modern mass spectrometers. Over the last decade, mass spectrometry has achieved a high status in bioprospecting research since it identifies and characterizes several proteins that would previously have been overlooked. Some types of mass spectrometry equipments are responsible for large-scale investigations and detection of complex structures like post-translational modifications. Other methods such as two-dimensional gel electrophoresis, differential gel electrophoresis (DIGE), in gel protein digestion and bioinformatics tools can be useful in proteome analysis since they can identify more specifically drug candidates.

Hymenopteran venoms have been intensively studied and they are subjected to modern venom profiling studies. Originally, crude venom extracts were separated by liquid chromatography and proteins or peptides of interest were further biochemically characterized using bioassays, Edman degradation chemistry, spectroscopy and eventually X-ray crystallography (20). Although these techniques showed to be useful in the initial characterization of venom compounds, they are single compound oriented, time-consuming and little sensitive. Such characteristics contrast sharply with novel profiling techniques like mass spectrometry (12).

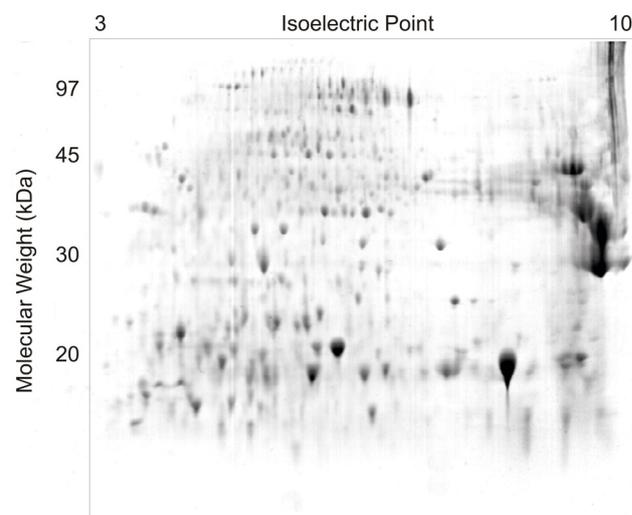
The main applications of MS in proteomics are: identification of protein expression, elucidation of protein-protein interactions and confirmation of post-translation modifications. For any MS experiment, it is necessary to select mass spectrometry equipment depending

on the molecule to be analyzed and the fragmentation analysis method (99). But, what constitutes a mass spectrometry equipment? Mass spectrometers consist of an ion source that transforms the molecules into gas-phase ions, a mass analyzer that separates ionized molecules according to  $m/z$  ratio, and a detector that records the number of ions at each  $m/z$  value. The development of soft ionization techniques – including electrospray ionization and matrix-assisted laser desorption/ionization – capable of ionizing peptides or proteins revolutionized molecules analysis (100, 101).

In the same way, a mass spectrum shows the mass-to-charge ratio ( $m/z$ ) of gas-phase ions representing a chemical analysis. The mass spectrum of a sample is a pattern representing the distribution of ions by mass in a sample. However, some molecules cannot be analyzed by mass spectrometry. Depending on their biochemical characteristics, a molecule will not ionize and it will be necessary that the researcher chooses other ionization methods.

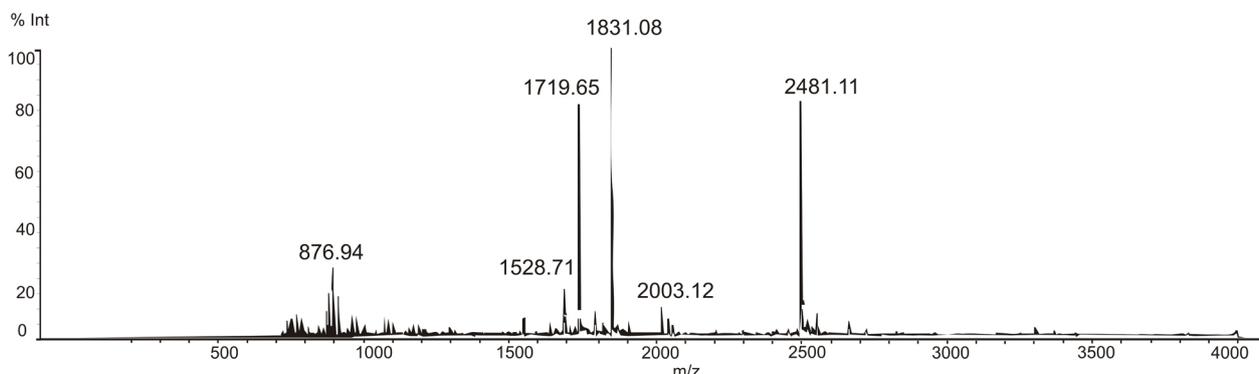
Many complete genomes are becoming publicly available with the recent high throughput analytical process and therefore stimulating large-scale protein experiments. In a classical bottom-up proteomic approach, proteins are obtained by several chemical preparations and separated by 2D-SDS-PAGE (Figure 2) (102, 103). Then, individual protein spot are excised from gel electrophoresis and proteolytically digested and identified by using peptide mass fingerprinting (PMF) (Figure 3) or/and tandem mass

spectrometry (MS/MS or MS2) with database searching algorithms as MASCOT, ProFound, ProteinProspector, SEQUEST, for example (104, 105). Multidimensional liquid chromatography combined with modern mass spectrometers is one of new approach in recent proteome researches named shotgun strategies (106). This approach is able to isolate the molecules by liquid chromatography and sequence them by mass spectrometry at the same time, e.g., this strategy is very useful when there is a small amount of sample or the researcher needs to identify minor compounds of the sample.



**Figure 2.** Two-dimensional SDS-PAGE of *Polybia paulista* wasp venom by Santos et al. (44). The proteins of this venom were separated based on two biochemical properties: charge and molecular mass.

Shimadzu Biotech Axima Performance : Reflectron Mode



**Figure 3.** Peptide mass fingerprint spectrum from Matrix Assisted Laser Desorption Ionization (MALDI-ToF) mass spectrometry (AXIMA Performance®, Shimadzu, Japan). Each ion is a peptide fragment from a protein digested with a proteolytic enzyme.

In addition to analytical equipments, the development of bioinformatics tools was very important to proteomic approach. Several protein profiles were elucidated when these automated database tools identified molecules by means of the genomic information. Besides, proteins can be identified from organisms which genomes were not sequenced. If the genomic information is not available, proteins still might be identified through cross-species protein identification. PMF or MS/MS allow identifying homolog proteins from strongly related species only differ by a few amino acids (12).

Recently, a novel mass spectrometry approach, called matrix-assisted laser desorption-ionization imaging mass spectrometry (MALDI Imaging), was employed in the proteomic strategy. This technology has been used to identify proteins in tissue specimens through direct analysis of histological sections and presents some analytical advantages because of its ability to provide information on the localization and relative abundance of multiple molecules in a sample. The powerful step for this type of analysis is the ability to map the spatial distribution of specific molecular species throughout a tissue section and sequencing the molecular targets at the same time (107). Usually, fresh or formalin-fixed tissue and paraffin-embedded samples can be analyzed by this analytical process. As the molecules must be ionized through a matrix in the MALDI strategy, this matrix can be deposited manually or by a chemical printer in specific points on the tissues.

MALDI imaging mass spectrometry has emerged as a powerful technique for analyzing the spatial arrangement of proteins, peptides, lipids, and small molecules in biological tissues. In a recent publication, a MALDI imaging experiment in rat tissue stung by *Apis mellifera* was studied (108). This strategy was able to record the distribution of several venom peptides and proteins in envenomed tissue. In the same experiment, they pointed out the appearance of rat peptides and proteins that were previously identified as markers for the inflammatory event evoked by bee venom. Thus, MALDI imaging can assist in the elucidation of many physiological processes in stinging accidents to promote a better understanding of the immune/allergic responses against Hymenoptera venom.

Animal venoms contain highly selective and efficient bioactive molecules that are clinically

challenging and scientifically fascinating, which have already led to the development of several new drugs. During billion of years, the nature accumulated and combined powerful libraries of hundreds of thousands of potentially active substances, resulting in useful molecules synthesized from several animal venoms. Bioprospecting activities have improved the identification, collection, cloning and synthesis of new drug candidates for commercial application. Usually, the targets of interest of bioprospecting are purified for further biochemical, enzymatic, functional and structural characterization for the investigation of their structure, functional activities and mechanism of action. Thus, several analytical equipments were developed to provide sensitive analysis with more resolution. Mass spectrometry has become an indispensable proteomic tool to identify the protein expression, protein-protein interactions and post-translational modifications (1).

## CONCLUDING REMARKS

The bioprospecting of animal venoms reached an important status in the development of drugs for specific treatments or diagnostic tests. The strategy of isolating a natural allergen from Hymenoptera venom and characterize it comprises a biochemical and immunological important task, since it provides the possibility of investigating such allergens and better understanding its structure/activity. Such knowledge will necessarily reveal the level of specificity of this allergen in the immunotherapy process, which will prevent incomplete treatment of a patient and side effects during treatment, including severe anaphylaxis. The understanding of major molecules from Hymenoptera venom is a very important step for future development of new extracts for immunotherapy.

With the new scenario of nanobiotechnology, several animal venom molecules have been revealed through some analytical techniques such as two-dimensional electrophoresis, high performance liquid chromatography and mass spectrometry. New separation techniques are constantly being created with increasing sensitivity and resolution and the development of modern mass spectrometry equipments has promoted an important insight into complex biological samples such as Hymenoptera venom.

These results were achieved because these new technologies used in the proteomics approaches have high sensitivity and resolution. Besides the improvement of bioinformatics tools for proteomics, databases and formation of human resources have permitted the discovery of numerous animal venom molecules and their biochemical and/or structural characterization.

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## CONFLICTS OF INTEREST

There is no conflict.

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