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Mass spectrometric characterization of two novel inflammatory peptides from the venom of the social wasp *Polybia paulista*

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The social wasp *P. paulista* is relatively common in southeast Brazil causing many medically important stinging incidents. The seriousness of these incidents is dependent on the amount of venom inoculated by the wasps into the victims, and the characteristic envenomation symptoms are strongly dependent on the types of peptides present in the venom. In order to identify some of these naturally occurring peptides available in very low amounts, an analytical protocol was developed that uses a combination of reversed-phase and normal-phase high-performance liquid chromatography (HPLC) of wasp venom for peptide purification, with matrix-assisted laser desorption/ionization time-of-flight post-source decay mass spectrometry (MALDI-Tof-PSD-MS) and low-energy collision-induced dissociation (CID) in a quadrupole time-of-flight tandem mass spectrometry (QTof-MS/MS) instrument for peptide sequencing at the sub-picomole level. The distinction between Leu and Ile was achieved both by observing *d*-type fragment ions obtained under CID conditions and by comparison of retention times of the natural peptides and their synthetic counterparts (with different combinations of I and/or L at N- and C-terminal positions). To distinguish the isobaric residues K and Q, acetylation of peptides was followed by Q-Tof-MS analysis. The primary sequences obtained were INWLKLGKMVIDAL-NH$_2$ (MW 1611.98 Da) and IDWLKLGKMVMDVL-NH$_2$ (MW 1658.98 Da). Micro-scale bioassay protocols characterized both peptides as presenting potent hemolytic action, mast cell degranulation, and chemotaxis of polymorphonucleated leukocyte (PMNL) cells. The primary sequences and the bioassay results suggest that these toxins constitute members of a new sub-class of mastoparan toxins, directly involved in the occurrence of inflammatory processes after wasp stinging. Copyright © 2004 John Wiley & Sons, Ltd.

The venoms of social Hymenoptera, such as hornets, paper wasps and honeybees, are used both for self-defense and the defense of larvae from predators and intruders in the nest. The stingings caused by these insects produce severe pain, inflammation, local tissue damage and occasionally death in large vertebrates, including man.[1] Hymenoptera venoms are complex mixtures of biochemically and pharmacologically active components such as biogenic amines, peptides and proteins.[2] The composition of neotropical vespid venoms has been subjected to few investigations, since the production of venoms by social wasps is very low and most large wasp species usually present a population of only some tens of individuals; thus, there is a limited availability of venoms as raw materials.

It has been shown that Vespinae venoms contain many different protein components, such as phospholipases A and B, hyaluronidases, acid phosphatases, proteases and nucleases. In addition, these venoms may contain several peptides presenting biological activities related to mast cell degranulation, chemotaxis of polymorphonucleated leukocytes (PMNL), cytolysis and smooth muscle contraction.[3]

The identification of peptides that occur in each venom is important for characterization of the type of pharmacological symptoms caused by each stinging incident; this knowledge will help physicians to interpret the anamnesis of the victims of Hymenoptera sting incidents. The identification of each peptide is performed initially through the examination of the primary sequences of these peptides and complemented by some biological assays.[3,4] The sequencing of peptides naturally occurring at low abundance is generally a difficult task, requiring the use of powerful analytical tools.[4,5]

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Tremendous progress in biomedical sciences is now possible, due, in part, to recent advances in bioanalytical methods, in particular biological mass spectrometry. In concert with separation techniques such as capillary electrophoresis and high-performance liquid chromatography, mass spectrometry allows characterization of a large array of small organic molecules, peptides, proteins, oligonucleotides and RNA fragments.[6] Concerning peptide sequencing, the use of low-energy collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) using a quadrupole instruments has emerged as a potent technique.[7,8]

Peptide sequencing has become one of the major fields of mass spectrometry, driven by the growing analytical demand. Sequencing peptides by mass spectrometry has some important advantages in certain fields of application, compared with classical sequencing methods such as Edman degradation: (1) the ability to perform sequence analysis of a peptide within a mixture because it can be mass-selected by the instrument; (2) fragmentation of a molecular ion is possible even in the presence of an N-terminal modification of the peptide, a considerable problem for the Edman degradation protocols; and (3) the sample amount necessary for analysis is usually less than 1 pmol to obtain a high quality mass spectrum, which provides the molecular weight (MW) of the sample as well as an indication of its purity.[9]

In order to isolate and characterize peptide toxins from wasp venoms through standard bioassay-guided fractionation, it is necessary to collect a large number of insects. The highly reduced concentrations of these peptides in crude venom of wasps would demand large amounts of biological material in order to purify a peptide and obtain the primary sequence through Edman degradation protocols. However, the collection of large numbers of insects is both extremely difficult and ecologically unacceptable. Therefore, micro-bioassays and micro-scale isolation, purification and chemical characterization of venom peptides are required. Thus, we have set up an analytical protocol to purify, sequence, and characterize the biological activities of low-abundance peptides from wasp venoms by using a combined strategy of reversed-phase high-performance liquid chromatography (RP-HPLC), mass spectrometry, manual solid-phase peptide synthesis, and micro-scale bioassays. This approach has permitted the isolation and determination of the primary sequences of two novel inflammatory peptides naturally occurring in the venom of the social wasp *Polybia paulista*. These toxins are both tetradecapeptides exhibiting a potent cytolysis, mast cell degranulation and chemotaxis of PMNL.

EXPERIMENTAL

Sample preparation

The wasps collected in Rio Claro-SP, southeast Brazil, were immediately frozen and stored at −20°C. The venom reservoirs were removed by wasp dissection with surgical micro-scissors and were then washed with 1:1 acetonitrile (MeCN, Aldrich)/water containing 0.1% (v/v) trifluoroacetic acid (TFA, Aldrich) to solubilize the peptides. The extract was then centrifuged at 10 000 *g* for 15 min at 4°C; the supernatant was collected, dried by centrifugation under reduced

pressure in a Speed-Vac system, and maintained at −20°C until use.

Peptide purification

The biological material from the dried extract was solubilized in 5% (v/v) MeCN at a concentration of 100 μg/mL, and chromatographed under RP-HPLC conditions on a Shiseido Nucleosil C-18 (ODS) column (250 × 4.6 mm, 5 μm), at a flow rate of 700 μL/min, using a gradient from 5–60% (v/v) MeCN (containing 0.1% TFA), at 30°C during 45 min. The elution was monitored at 215 nm with a UV-DAD detector (Shimadzu, model SPD-M10A) and all eluted peaks were manually collected in 2-mL plastic vials. A peak of interest was resubmitted to chromatography under normal-phase conditions using a Brownlee CN column (250 × 4.6 mm, 5 μm), under isocratic elution with 50% (v/v) MeCN containing 0.1% TFA at a flow rate of 600 μL/min, during 25 min at 30°C. The elution was monitored at 215 nm, and fractions were manually collected in 2-mL plastic vials.

ESI-MS analysis

All ESI mass spectrometric analyses were performed using a hybrid quadrupole/time-of-flight (Q-Tof™) mass spectrometer (Micromass, model QTOF 1). Samples were dissolved in 50% (v/v) MeCN containing 0.1% (v/v) formic acid, and analyzed by positive electrospray ionization (ESI+) using typical analytical conditions as described elsewhere,[8,9] which permitted operation of the instrument at a resolution of 6000 (based on FWHM).

Peptide sequencing by Q-Tof-MS/MS

All the tandem mass spectrometry experiments were performed using ESI+ using the ESI-Q-Tof™ mass spectrometer, with peptide solutions in water: MeCN (1:1) acidified with formic acid 0.1% (v/v); a detailed description of the analytical protocols for this analysis was described in a previous publication.[8,9] The doubly charged (diprotonated) ions were subjected to CID with argon as collision gas in the 60–70 eV collision energy range. Peptide sequences were determined manually from the MS/MS product ion mass spectra with the help of the PepSeq software (Micromass, UK).

The distinction between the isobaric I/L residues in the natural peptides was partially achieved by searching for the presence of *d*-type fragment ions obtained under low-energy CID conditions with argon gas. The *m/z* values of *d*-type ion fragments in the experimental spectra were compared with the *m/z* values of simulated spectra of model peptides with different I and/or L residue combinations at the ambiguous positions, generated with the software BioLynx/MassLynx (Micromass, UK).

MALDI-Tof-MS analysis

The peptide sample was dissolved in 5 μL matrix, a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% MeCN/0.9% TFA (v/v); 1 μL was applied onto the target slide using the dried-droplet method.[10] Mass spectra were acquired using an Ettan MALDI-TOF/MS (Amersham Biosciences); the instrument was set up with a reflectron, presenting an effective flight path of 2.4 m. The ions were accelerated using 20 kV and an extraction time delay of 100–200 ns. The laser

was a standard 337-nm nitrogen laser with a repetition rate of 0.8–8 shots/s. Ions were detected using double microchannel plates and a 500 MHz samples/s analogue/digital converter. Spectra were calibrated using internal reference peptides, such as adrenocorticotropic and angiotensin II.

PSD analysis

A simple switch to fragmentation analysis in the MALDI instrument allowed further investigation of unidentified peptides. Peaks were selected from complex spectra using the timed ion gate. The instrument has a reflectron based on Z^2 technology to create a quadratic field so that the ions created during post-source decay (PSD) are all focused at the detector; each pulse of the laser provides a PSD spectrum over the entire m/z range, without the need for data stitching. Since calibration uses data from a single spectrum covering the entire range, higher mass accuracy can be obtained. The spectra were acquired using an accelerating potential of 20 kV, adjusting the pulsed laser to 1 shot/s; 300 shots/spectrum were accumulated.

Peptide synthesis and purification

Peptides were prepared by step-wise manual solid-phase synthesis using N-9-fluorophenylmethoxycarbonyl (Fmoc) chemistry with Novasyn TGS resin (NovaBiochem). Side-chain protective groups included t-butyl for serine and t-butoxycarbonyl for lysine. Cleavage of the peptide–resin complexes was achieved by treatment with TFA/1,2-ethanedithiol/anisole/phenol/water (82.5:2.5:5:5:5 v/v), using 10 mL/g of complex at room temperature during 2 h. After filtering to remove the resin, diethyl ether at 4°C was added to the soluble material causing precipitation of the crude peptides, which were collected as a pellet after centrifugation at 1000 g, during 15 min at room temperature. The crude peptides were solubilized in water and chromatographed under RP-HPLC conditions using a semi-preparative column (Shiseido C18, 250 × 10 mm, 5 μm), under isocratic elution with 40% (v/v) MeCN in water containing 0.1% (v/v) TFA at a flow rate of 2 mL/min. The elution was monitored at 215 nm with a UV-DAD detector (Shimadzu, model SPD-M10A) and each fraction eluted was manually collected in 2-mL plastic vials. The homogeneity and sequences of the synthetic peptides were assessed by analytical HPLC and QTof-MS analysis.

Biological activities

Mast cell degranulation activity was determined by measuring the release of β-D-glucosaminidase, which co-localizes with histamine, as proposed by Hide et al.[11] Mast cells were obtained by peritoneal washing of adult Wistar rats with a solution containing 0.877 g NaCl (Merck), 0.028 g KCl (Merck), 0.043 g NaH$_2$PO$_4$ (Synth), 0.048 g KH$_2$PO$_4$ (Synth), 0.10 g glucose (Synth), 0.10 g BSA (Sigma), 90 μL CaCl$_2$ (Merck) 2 M solution, 50 μL Liquemine (heparin, Roche) in 100 mL water. Mast cells were incubated in the presence of peptides for 15 min at 37°C. After centrifugation, the supernatants were sampled for β-D-glucosaminidase assay. Briefly, 50-μL aliquots of the samples were incubated in 50 μL of the substrate (3 mg of p-nitrophenyl-N-acetyl-β-D-glucosaminidine (Sigma) dissolved in 10 mL of 0.2 M sodium citrate, pH

4.5 solution), for 6 h. The absorbance of colored product was assessed at 405 nm, and the values were expressed as the percentage of total β-D-glucosaminidase, which was determined from lysed mast cells in the presence of 0.1% (v/v) Triton X-100 (Aldrich).

Washed rat red blood cells (WRRBC) were used to evaluate the hemolytic activity of the peptides. WRRBC were prepared by washing 50 μL of Wistar rats red blood cell suspensions three times with physiological saline solution (NaCl 0.85% and CaCl$_2$ 10 mM), and then suspended in 25 mL of the same solution. Aliquots of WRRBC were then incubated at 37°C in the presence of each peptide for 120 min, with gentle mixing. Samples were then centrifuged, and the absorbances of the supernatants were measured at 540 nm. The absorbance measured from lysed WRRBC in the presence of 1% (v/v) Triton X-100 was considered as 100%.

Chemotaxis was assayed in a specific multi-chamber apparatus (Neuro Probe)[12] using polymorphonucleated leukocytes (PMNL) obtained from subcutaneous inflammatory induction in Wistar rats. The upper chambers were filled with 200 μL of a PMNL suspension ($\sim$2.7 × 10^5 cells/mL in 0.9% NaCl solution, i.e., physiological saline solution) and the lower chambers were filled with 400 μL of physiological saline solution containing the peptides (about 10 ng per assay). A polycarbonate membrane (10 μm pore diameter, Neuro Probe) was placed between the two chambers. The chemotaxis chamber was incubated at 37°C for 1 h. After incubation, cells in the lower chamber were counted using a Neübauer chamber.

RESULTS AND DISCUSSION

We have found two new peptides extracted from *Polybia paulista* wasp venom. The chromatogram obtained by RP-HPLC (Fig. 1(a)) reveals 13 peaks (designated 1 to 13). Fraction 13 showed pronounced hemolytic, chemotactic and mast cell degranulation activities. However, the MALDI-Tof-MS spectrum of this fraction showed a series of different molecular masses, with peptides of molecular mass 1612 and 1659 Da representing the major compounds (data not shown). The re-chromatography of peak 13 under normal-phase conditions resulted in two fractions, designated 13a and 13b (Fig. 1(b)). When these fractions were analyzed by QTof-MS, peptides with molecular masses 1612.07 and 1658.60 Da were identified (data not shown). We attempted to obtain the amino acid sequences of these peptides by Edman degradation chemistry, but the available amounts of each purified peptide were not sufficient to perform the sequencing. The second attempt to determine the amino acid sequences was through the use of MALDI-Tof/PSD-MS. This approach was only partially successful, since it was not possible to determine the complete amino acid sequences. However, we were able to determine the entire amino acid sequence using QTof-MS/MS.

PSD analysis of natural peptides

An enormous number of structurally different peptides is possible, especially considering the occasional occurrence of amino acids modified by post-translational processes, and any notion of developing a 'library' of all of them for
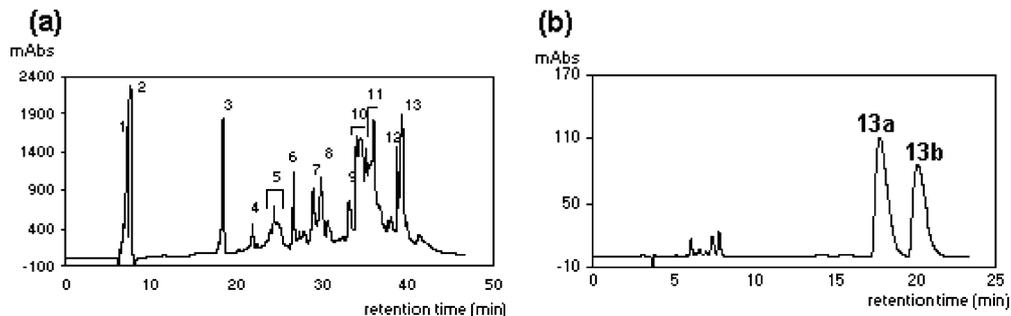
Figure 1. (A) Reversed-phase HPLC chromatogram of *Polybia paulista* venom with a C-18 column (10 × 250 mm, 5 μm) under gradient elution from 5–60% (v/v) MeCN, containing 0.1% TFA (v/v). (B) Normal-phase HPLC chromatogram of peak 13 with a CN column (4.6 × 250 mm, 5 μm) under isocratic elution with 50% (v/v) MeCN containing 0.1% TFA; (a) and (b) represent the purified peptides from fraction 13.

use in any matching-type identification must be immediately discarded.

MALDI-PSD analysis of both toxins was performed as an initial experimental approach in order to determine the primary sequences of the peptides corresponding to fractions 13a and 13b. In these PSD experiments high laser power was applied to increase the available energy, generating metastable ions of high internal energy which, in turn, promoted peptide fragmentation. The quadratic-field reflectron of the MALDI instrument used focuses all ions in time, irrespective of their energy, over the entire *m/z* range, providing a complete PSD spectrum for each laser shot. Figures 2 and 3 show the MALDI-Tof-PSD-MS spectra for the two natural peptides.

The assignment of amino acid sequence was performed by searching for consecutive peaks of mass differences corresponding to the mass of the residues of natural amino acids. As a result, it was possible to recognize a series of peaks representing the *b*-type ions in each spectrum. However, it was not possible to assign the complete amino acid sequence of either peptide. Figure 2 reveals a partial sequence for the peptide with [M+H]$^+$ of *m/z* 1613 as: K/Q-M-V-I/L-D-A-I/

L; Fig. 3 shows the partial sequence for the peptide with [M+H]$^+$ of *m/z* 1660 as: I/L-G-K/Q-M-V-M-D-V-I/L. In both cases, the sequencing method could not distinguish the isobaric residues I/L and K/Q. Thus, in order to obtain a reliable and complete sequence, it was necessary to use the QTof-MS instrument with capability to perform MS/MS analysis under CID conditions.

Characterization of peptides by MS/MS

Using 50% (v/v) MeCN containing 0.1% (v/v) formic acid, and a potential of 30 V at the sample cone, the molecular mass of each peptide was obtained by QTof-MS, resulting in molecular masses of 1612.07 and 1658.60 Da for fractions 13a and 13b, respectively (data not shown).

In order to establish the structural identities of these peptides, the CID spectra of the [M+2H]$^{2+}$ ions of *m/z* 807.07 and 830.30 were generated (Figs. 4 and 5, respectively). The low-mass region (*m/z* <200) of such MS/MS spectra often contains ions that are indicative of the presence of specific amino acid residues in the peptides. These immonium ions (H$_2$N=CHR)$^+$, where 'R' is a side-chain group, arise from the fragmentation of two internal bonds. The low-mass regions
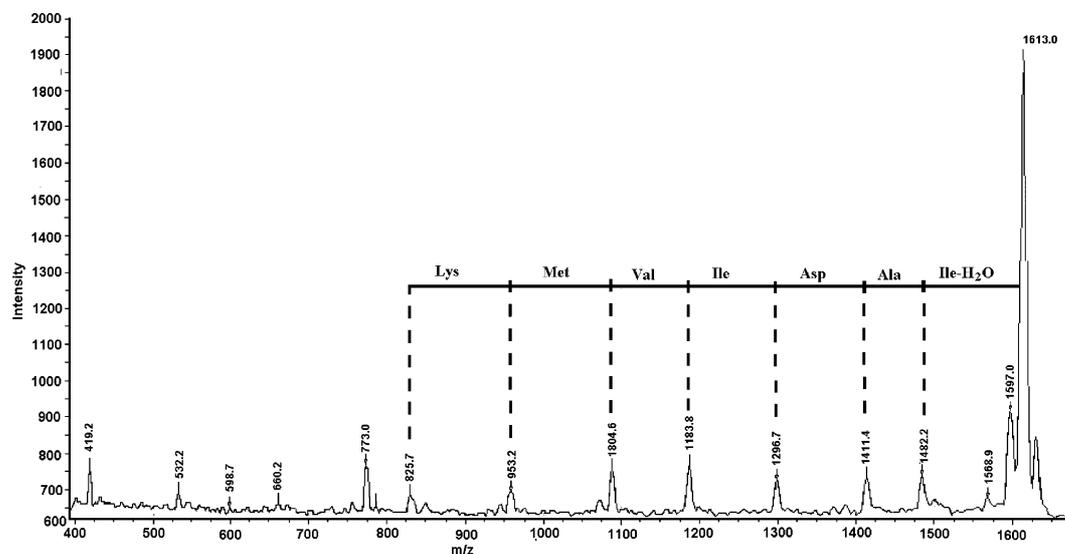

Figure 2. MALDI-PSD-MS spectrum of the peptide [M+H]$^+$ ion of *m/z* 1613 as the precursor ion.
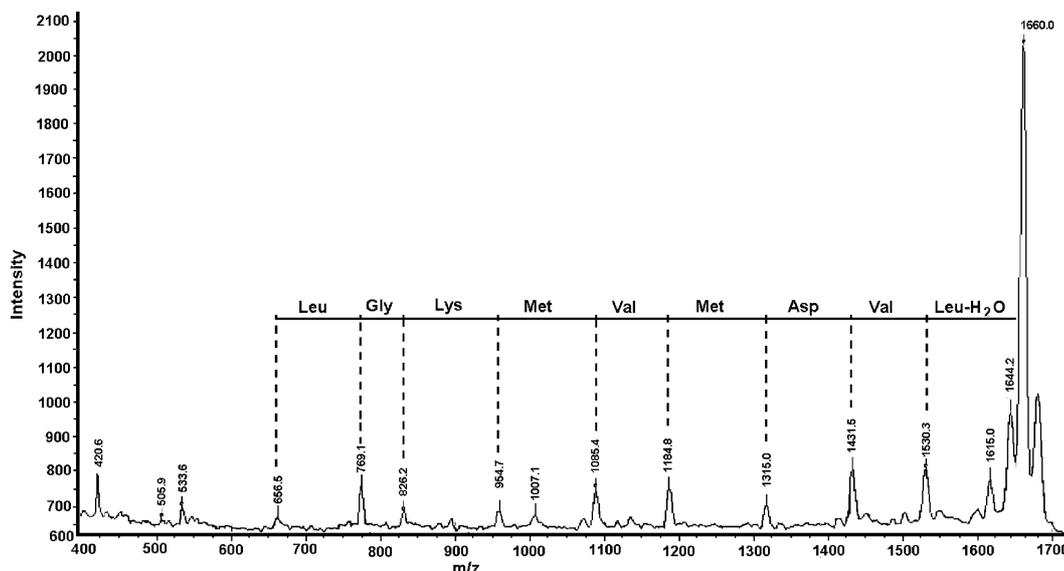
Figure 3. MALDI-PSD-MS spectrum of the peptide [M+H]$^+$ ion at *m/z* 1660 as the precursor ion.

of both Figs. 4 and 5 contain ions of *m/z* 72.09, 86.11, 87.10, 104.06 and 159.10, suggesting the presence of the amino acid residues V, I/L, N, M and W, respectively, in both peptides.

By searching for mass differences between consecutive peaks that correspond to the masses of (–NH–CHR–CO–) residues of natural amino acids, it is possible to recognize one or more series of ion fragments. In Figs. 4 and 5 these peaks were assigned as a series of *b*-type ion fragments for both peptides. As discussed below, the complete sequence identification of the peptides is corroborated by the presence of some *y*-type ions, and also by the presence of peaks corresponding to neutral losses (NH$_3$ or H$_2$O) from the main peaks.

The MS/MS spectrum of peptide **13a** (Fig. 4) shows two ions series, i.e., a few *y*-type ions with *b*-type ions as the major ones. The major peak with *m/z* 1613 was assigned to the molecular ion [M+H]$^+$. Loss of ammonia (17 Da) from the [M+H]$^+$ ion will result in the ion at *m/z* 1596. A series of *b*-ions can be observed in this spectrum: *m/z* 1482.96 (b_{13}), *m/z* 1411.92 (b_{12}), *m/z* 1296.92 (b_{11}), *m/z* 1183.81 (b_{10}), *m/z* 1084.80 (b_9), *m/z* 953.78 (b_8), 825.53 (b_7), 768.54 (b_6), *m/z* 655.45 (b_5), *m/z* 527.34 (b_4), *m/z* 414.24 (b_3) and *m/z* 228.16 (b_2). These permitted the assignment of the sequence of the peptide **13a** with a few ambiguities with respect to the isobaric residues (I/L and K/Q), as shown in Fig. 4: I/L –N- W- I/L- K/Q- I/L- G- K/Q- M- V- I/L- D- A- I/L.
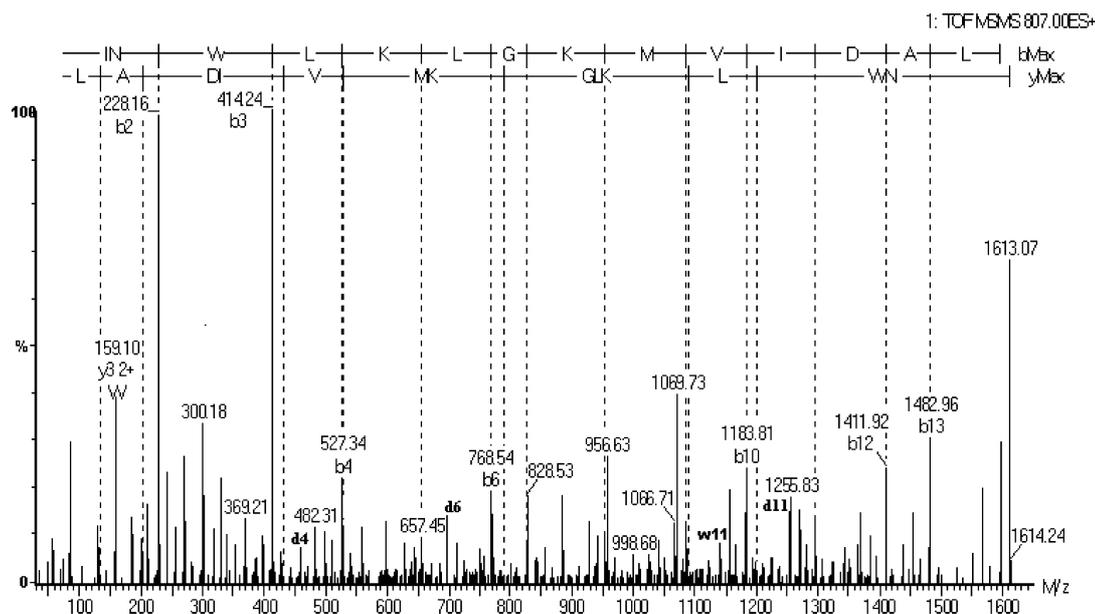

Figure 4. Tof-MS/MS spectrum of the [M+2H]$^{2+}$ ion (*m/z* 807) acquired in the 60–70 eV collision energy range. The mass differences of the consecutive b_n ions and their correspondence to the amino acid sequence are shown.

Figure 5. Tof-MS/MS spectrum of the $[M+2H]^{2+}$ ion (m/z 830) in the 60–70 eV collision energy range. The mass differences of the consecutive b_n ions and their correspondence to the amino acid sequence are shown.
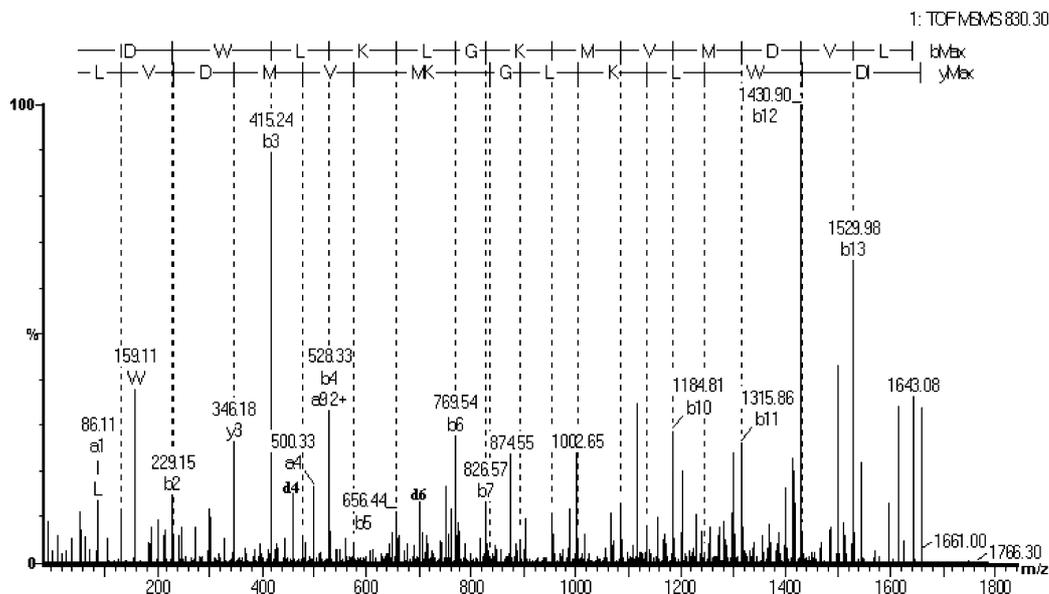
The MS/MS spectrum of peptide **13b** (Fig. 5) also shows two ion series, again with a few *y*-type ions and *b*-type ions as the major ones. The peak of m/z 1660.00 was assigned as the molecular ion $[M+H]^+$. Loss of ammonia (17 Da) from the $[M+H]^+$ ion results in the ion at m/z 1643.08. A long series of *b*-ions can be observed in this spectrum: m/z 1529.98 (b_{13}), m/z 1430.90 (b_{12}), m/z 1315.86 (b_{11}), m/z 1184.81 (b_{10}), m/z 1085.90 (b_9), m/z 954.87 (b_8), m/z 826.57 (b_7), m/z 769.54 (b_6), m/z 656.44 (b_5), m/z 528.33 (b_4), m/z 415.24 (b_3) and m/z 229.15 (b_2). Again this series permitted the assignment of the primary sequence of peptide **13b**, with a few ambiguities in relation to the isobaric residues (I/L and K/Q), as shown in Fig. 5: I/L- D-W- I/L- K/Q- I/L- G- K/Q- M- V- M- D- V- I/L.

These experiments allowed the determination of the amino acid sequences of both peptides as follows:

I/L N W I/L K/Q I/L G K/Q M V I/L D A I/L-NH$_2$ 1612.07 Da
I/L D W I/L K/Q I/L G K/Q M V M D V I/L-NH$_2$ 1658.60 Da

However, it must be emphasized that, by considering only the *b*- and *y*-types of fragment ions, some doubts still remain concerning the discrimination between the isobaric residue pairs I/L and K/Q.

In tandem mass spectrometry (MS/MS), the isomeric side chains of the residues of leucine and isoleucine may be distinguished from each other by inducing secondary fragmentation in the side chains together with backbone bond dissociation, to form *d*- and *w*-fragment ions.[9,12,14] The use of high cone voltage fragmentation and low-energy CID MS/MS can thus supply sufficient structural information to distinguish leucine from isoleucine, due to the differential formation of *d*-type ions for each amino acid residue.[7]

In order to solve these outstanding questions, we considered the presence of the *d*-type ion fragments in order to distinguish between L and I. As an example, the presence of a *d*-ion corresponding to the loss of the side chain yields a mass difference of 42 Da in the case of L, or of 14 Da in the case of

I.[14–16] When two *d*-ions can be formed due to the presence of two different substituents attached to the β-carbon, the loss of the larger substituent is conventionally indicated by an added subscript 'a' (d_a), while the loss of the smaller group is indicated by 'b' (d_b). Other side-chain fragment ions, like *w*-ions, are indicated the same manner.[7,13–16]

Simulated spectra were also created by using the software Protein/Peptide Editor in BioLynx/Mass Lynx (v 3.2) from Micromass, with different combinations of I and/or L at positions 4, 6, and 11 for peptide **13a**, and at positions 4 and 6 for peptide **13b**, to provide the theoretical m/z values for *d*-type ions corresponding to the Ile/Leu residues (Table 1). Careful inspection of the QTof-MS/MS spectrum for peptide **13a** (Figs. 6(a)–6(c)) reveals the presence of the ions d_4, d_6 and d_{11} (m/z 457.31, 698.47 and 1254.86, respectively), which fit the theoretical m/z values found for the simulated sequence presenting L_4, L_6 and I_{11} residues, respectively (Table 1). Similarly, the QTof-MS/MS spectrum for peptide **13b** (Figs. 7(a) and 7(b)) showed the presence of the ions d_4 and d_6 (m/z 457.82 and 699.96, respectively), corresponding to the theoretical m/z values expected for the sequence incorporating Leu residues at both positions 4 and 11 (Table 1). However, doubt still remains concerning the distinction between I and L at the N- and C-terminal positions of both peptides, since the fragmentation of side chains does not occur for residues at these positions.

In order to resolve these remaining sequence ambiguities, four possible sequences with different combinations of I and/or L at the terminal positions for both peptides were synthesized and purified (as described in the Experimental section). Retention times were obtained both for the synthetic peptides and for the natural ones (peptides **13a** and **13b**) using normal-phase chromatography on a CN column (250 × 4.6 mm, 5 μm) with isocratic elution with 50% (v/v) MeCN containing 0.1% TFA at a flow rate of 600 μL/min during 25 min at 30°C; the retention times are presented in

Table 1. List of theoretical and experimental *m/z* values for the *d*-type ions resulting from the side-chain fragmentations of the amino acid residues I and L at different positions in the primary sequences of the peptides **13a** and **13b**

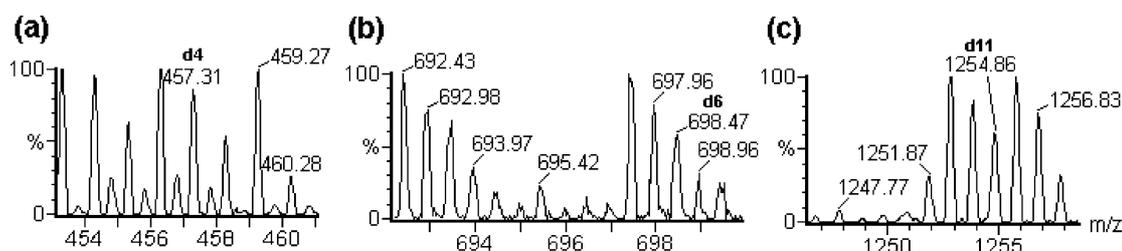| *d*-ion		Peptide **13a**			Peptide **13b**		
		Theoretical (*m/z*)			Theoretical (*m/z*)		
	Residue	Ile	Leu	Experimental (*m/z*)	Ile	Leu	Experimental (*m/z*)
d_4		570.3	457.3	457.3	473.3	458.1	457.8
d_6		712.5	698.2	698.4	713.4	699.7	699.9
d_{11}		1254.8	1226.7	1254.8			

Figure 6. Magnified representation of the Tof-MS/MS spectrum obtained under continuum mode, for the natural peptide component as the $[M+2H]^{2+}$ ion (*m/z* 807) from peak **13a**; acquired at 60–70 eV collision energy to permit the observation of the *d*-type ions originated from the I/L side-chain fragmentations. (a), (b) and (c) represent different sections of the MS/MS spectrum.
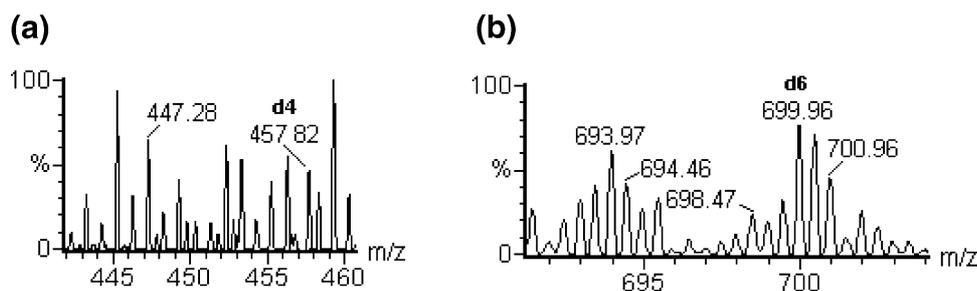
Figure 7. Magnified representation of the Tof-MS/MS mass spectrum obtained under continuum mode, for the natural peptide component as the $[M+2H]^{2+}$ ion (*m/z* 830) from peak **13b**; acquired at 60–70 eV collision energy to permit the observation of the *d*-type ions originated from the I/L side-chain fragmentations. (a) and (b) represent different sections of the MS/MS spectrum.

Table 2. Each different sequence corresponds to a different retention time, ranging from 17.85 to 22.07 min. Comparison of these results shows that the peptides **13a** and **13b** had the same retention times as the synthetic peptides with amino acid residues I_1 and L_{14} (at the N-and C-terminal positions, respectively). Consequently, it can be concluded at this point that the primary sequences of peptides **13a** and **13b** are:

Peptide **13a** I N W L K/Q L G K/Q M V I D A L-NH₂
Peptide **13b** I D W L K/Q L G K/Q M V M D V L-NH₂

The distinction between K and Q residues was clarified by acetylation of both peptides. The esterification occurs for free amino groups at α- and ε-positions; thus, the unblocked amino groups at the N-terminal residue and in the side chain of any K residue are acetylated. After acetylation, the $[M+H]^+$ ions of fractions **13a** and **13b** were determined by MALDI-Tof-MS as *m/z* 1738 and 1784, respectively (data not shown). Thus, both molecular masses were increased by 126 Da (corresponding to 42 Da × 3), indicating three sites of

Table 2. Retention times for both the natural (peptides **13a** and **13b**) and synthetic peptides with different combinations of the residues I and/or L at N- and C-terminal positions. Data refer to normal-phase chromatography using a CN column (4.6 × 250 mm, 5 µm), under isocratic elution with 50% (v/v) MeCN containing 0.1% TFA (v/v) and at a flow rate of 600 µL/min

Peptide	Sequence	Retention time (min)
13a	**I-N-W-L-K-L-G-K-M-V-I-D-A-L-NH₂**	**19.02**
	L-N-W-L-K-L-G-K-M-V-I-D-A-L-NH₂	20.60
	I-N-W-L-K-L-G-K-M-V-I-D-A-I-NH₂	17.85
	L-N-W-L-K-L-G-K-M-V-I-D-A-I-NH₂	20.01
	Peptide 13a	**18.96**
13b	**I-D-W-L-K-L-G-K-M-V-M-D-V-L-NH₂**	**21.48**
	I-D-W-L-K-L-G-K-M-V-M-D-V-I-NH₂	18.83
	L-D-W-L-K-L-G-K-M-V-M-D-V-L-NH₂	22.07
	L-D-W-L-K-L-G-K-M-V-M-D-V-I-NH₂	20.03
	Peptide 13b	**21.43**

acetylation. Since the α-amino group of the N-terminal residue was free in both peptides, the reactive sites were the terminal α-amino groups and two ε-amino groups of internal lysine residues for each peptide. Therefore, the putative K/Q positions were identified as lysine; thus, the complete sequences of peptides **13a** and **13b** were defined as:

I N W L K L G K M V I D A L-NH$_2$ 1612.07 Da
I D W L K L G K M V M D V L-NH$_2$ 1658.60 Da

The sequences described above are quite similar to those of mastoparan peptides from other wasp species; however, the presence of methionine residue(s) in the primary sequences indicates that these peptides are somewhat uncommon among the Vespid toxins.

Biological activities

Both peptides presented potent chemotactic activities, i.e., about 90% of rat PMNL cells were chemo-attracted in the presence of only 1.0×10^{-5} M of each peptide. The peptides from fractions **13a** and **13b** presented high hemolytic activity, causing the disruption of 100% of the WRRBC at concentrations of 6.2×10^{-5} M peptide. We also observed the degranulation of 57% of rat peritoneal mast cells at a concentration of 6.2×10^{-5} M of each peptide. The hemolytic activity and degranulation of rat peritoneal mast cells are characteristics of mastoparan peptides from wasp venoms.[14,16] However, cytolytic mastoparans generally present only a weak chemotactic activity for PMNL cells.[16] Thus, the present study identified a new sub-family of mastoparan peptides, occurring in the venom of neotropical wasps in very reduced amounts, only detectable by using mass spectrometry. These novel toxins are tetradecapeptides containing methionine residue(s) in their primary sequences, and apparently exhibiting both a potent cytolysis and chemotaxis of PMNL.

CONCLUSIONS

Most components of wasp venom are peptides, but nothing is known about the peptide composition in the venom of the wasp *P. paulista*. This wasp produces a very small amount of venom (a few micrograms per individual), making difficult the task of peptide identification. We set up an analytical protocol using a combination of reversed-phase and normal-phase HPLC of wasp venom for peptide purification. MALDI-Tof-PSD-MS and low-energy CID in a QTof-MS/MS instrument were applied for peptide sequencing at the sub-picomole level. The primary sequences obtained were:

I N W L K L G K M V I D A L-NH$_2$ (MW 1611.98 Da) and
I D W L K L G K M V M D V L-NH$_2$ (MW 1658.98 Da).

The biological activities were investigated using microscale bioassay protocols, which permitted the observation that both peptides caused very potent hemolysis, mast cell

degranulation and chemotaxis of PMNL cells. The primary sequences and the bioassay results suggest that these toxins appear to constitute members of a new sub-class of mastoparan peptides directly involved with the occurrence of inflammatory processes.

The analytical protocols described in this manuscript may be applied to the reliable identification of biologically active peptides occurring at reduced concentrations in the venoms from other aggressive wasp species from neotropical regions of the planet.

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