Identification of three novel peptides isolated from the venom of the neotropical social wasp *Polistes major major*

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Abstract: Three novel peptides designated as PMM1, PMM2, and PMM3 were isolated and characterized from the venom of the social wasp *Polistes major major*, one of the most common wasps in the Dominican Republic. By Edman degradation, and MALDI-TOF and ESI-QTOF mass spectrometry, the primary sequences of these peptides were established as follows: PMM1, H-Lys-Arg-Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg-OH (1357.77 Da); PMM2, H-Ile-Asn-Trp-Lys-Lys-Ile-Ala-Ser-Ile-Gly-Lys-Glu-Val-Leu-Lys-Ala-Leu-NH$_2$ (1909.19 Da); and PMM3, H-Phe-Leu-Ser-Ala-Leu-Leu-Gly-Met-Leu-Lys-Asn-Leu-NH$_2$ (1317.78 Da). The suggested sequences were confirmed by MS analysis of peptide fragments obtained by enzymatic digestion. The peptide PMM1 is a lysyl-arginyl-Thr[6]-bradykinine that belongs to the wasp kinins group. The sequence of the PMM2 peptide is unique; it resembles somewhat the tetradecapeptide amides of the mastoparan group; however, the chain is extended by three additional amino acid residues. The sequence of PMM3 dodecapeptide is homologous to the peptides of the wasp chemotactic group. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: mastoparans; wasp kinins; chemotactic peptides; *Polistes major major*; mass spectrometry

INTRODUCTION

The venom of the family Vespidae is a complex mixture of low molecular weight compounds, high molecular weight proteins, and small to medium size peptides. Many of these compounds exhibit a variety of physiological effects [1,2]. Human reactions to wasp stings vary widely. However, wasp venom is generally known to cause inflammation, severe and prolonged pain, local tissue damage, and in rare cases even death [3]. The social wasps are generally known to use their venom for self defense against predators [3], although on some occasions stinging of the prey has been observed [4]. The solitary wasps on the other hand use their venom to paralyze insects or spiders before depositing their eggs on the body of the pray [5,6]. Chemical identification and biological characterization of wasp venom peptides have attracted attention of researchers for decades. Results from such investigations enable us to comprehend better the pharmacological symptoms caused by wasp stings. Hitherto characterized oligomeric constituents of vespid venom are α-helical cationic peptides that bear amphipathic properties. Such properties permit wasp peptides to interact specifically with the anionic components of the cell or bacterial membranes and trigger a cascade of biological effects. These responses include: mast cell degranulation, lysis of the cell membranes, increase of the chemotaxis of neutrophils, activation of leucocytes in the inflammation site, as well as some *in vitro* antimicrobial effects [7,8]. The neurotoxic kinins, another group of cationic peptides found in vespid venom, play a major role in the paralysis of the prey [9,10]. The most important, and extensively studied families of cationic peptides of wasp venom are represented by mastoparans [7], chemotactic peptides [7], and wasp kinins [9,10], categorized according to their characteristic structure and biological activity.

The *Polistes major major* (Palisot de Beauvois) is one of the most abundant and largest wasp species on the Hispaniola island. In the Dominican Republic this species is known locally as Avispa de caballo (horse's wasp). The females build nests under the roofs, rims, and window frames of houses, or under palm tree leaves, to raise larvae. Up to now, no peptides have been identified from the venom of this wasp. In this work we report the sequence determination of three novel peptides from the venom of *Polistes major major*.

MATERIAL AND METHODS

Material

TLCK treated α-chymotrypsin and TPCK treated trypsin were purchased from Sigma (St Louis, MO), and aminopeptidase M, as a suspension in ammonium sulfate solution, was obtained

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from Roche (Indianapolis, IN). The H-Ile-Asn-Trp-Lys-Lys-Ile-Ala-Ser-Ile-Gly-Lys-Glu-Val-Leu-Lys-Ala-Leu-NH$_2$ (PMM2 [Leu[17]]) and H-Ile-Asn-Trp-Lys-Lys-Ile-Ala-Ser-Ile-Gly-Lys-Glu-Val-Leu-Lys-Ala-Ile-NH$_2$ (PMM2[Ile[17]] were synthesized manually in polypropylene syringes with a bottom Teflon filter using the protocol of N$^\alpha$-Fmoc chemistry [11] on Rink Amide MBHA resin (Novabiochem, San Diego, CA). TLC was carried out on Kieselgel 60 F$_{254}$ silica gel plates (Darmstadt, Germany) using a chloroform/methanol/17% NH$_4$OH (12 : 6 : 1) solvent system. Compounds were visualized under UV light and then stained with ninhydrin. α-Cyano-4-hydroxycinnamic acid, the matrix for MALDI spectrometry, was obtained from Aldrich Chemical Co. (St Loius, MO). All other reagents and solvents were of the highest purity provided by commercial sources.

Sample Preparation and Peptide Purification

Wasp specimens were collected from the ecological reserve areas of the Punta Cana Resort & Club, Punta Cana, on the eastern tip of the Dominican Republic, and frozen at −15 °C for several hours. The venom reservoirs, typically from five individuals, were removed by dissection and their contents were extracted with 25 µl of a mixture of acetonitrile–water (1 : 1) containing 0.1% TFA. The extract was centrifuged, and the supernatant was removed and its constituents were isolated by RP-HPLC. The chromatography was carried out on a Shimadzu instrument equipped with two LC-10AD pumps, an SCL-10A control unit, and a diode-array detector (SPD-M10AV). A Vydac C-18 column (250 × 4.6 mm; 5 µm) was eluted at a flow rate of 1 ml/min, using a linear gradient of solvents, starting from 5% of acetonitrile in water containing 0.1% TFA, and increasing to 70% acetonitrile (v/v) as the mobile phase within a period of 60 min. The elution was monitored by absorption at 222, 254 and 280 nm, and the UV spectra of selected peaks were evaluated by the Shimadzu CLASS-VP Chromatography Data system. The effluent fractions corresponding to chromatographic peaks were manually collected in Eppendorf tubes, and the solvent was evaporated by a Speed-vac centrifuge. Three peptides collected in this way, which we designated as PMM1, PMM2 and PMM3, were subjected to mass spectrometry, Edman degradation and enzymatic digestion.

Mass Spectrometry

Mass spectra of peptides were acquired on a Micromass Tof Spec 2E MALDI-TOF mass spectrometer, equipped with a UV nitrogen laser, operated in reflectron mode. The matrix, was prepared at a concentration of 10 mg/ml in 1 : 1 acetonitrile–water containing 0.1% TFA. Each sample (1 µl) mixed with the matrix (1 µl) was applied onto a spot on a MALDI plate and allowed to dry at room temperature. When working with peptide fragments smaller than 600 Da, (resulting from protease digestion), the spectra were recorded using a Micromass Q-Tof API-US mass spectrometer.

Enzymatic Digestions

The PMM1, PMM2 and PMM3 peptides, isolated by preparative RP-HPLC as dry residues after Sped-vac evaporation, were dissolved in 50 mM ammonium bicarbonate (10–50 µl) and digested overnight at 35 °C with trypsin, or α-chymotrypsin

(0.1–1.0 µg in 1 µl of water). After a certain time interval (1, 5, 15, 60, 120, and 360 min), 1 µl of the mixture was removed and mixed with the same volume of matrix, and applied onto a MALDI plate for mass spectrometric analysis. The peptides were subjected also to ladder sequencing [12] by adding aminopeptidase M, (1 µl) solution as provided by the supplier. In this case, 1 µl of the digestion mixture was removed at 1, 2, 5 and 10 min time intervals and mixed with a stop solution of 50% acetonitrile containing 1% TFA (1 µl), followed by the matrix solution (2 µl) before applying the mixture (1 µl) onto a MALDI plate.

Moreover, PMM2 isolated by preparative HPLC was digested by immobilized trypsin. PMM2 dissolved in ammonium bicarbonate buffer (20 µl, 50 mM, pH ∼ 7.8) was aspirated into a NuTip Trypsin (Bovine) tip (Glygen Corp., MD, USA) and kept for 5 min. The digestion mixture was then dispensed into an Eppendorf tube for further analysis by nanospray ESI-MS on the Q-Tof API-US instrument. Samples were infused at a flow rate of 0.3 µl/min, as 50 : 50 acetonitrile : water solutions containing 0.1% formic acid.

Peptide Sequencing by Edman Degradation

The peptides were loaded onto a Biobrene-coated and pre-cycled glass fiber filter disc and sequenced using model cLC-Procise sequencer/PTH Analyzer (Applied Biosystems, Foster City, California) using manufacturer's pulse-liquid Edman degradation chemistry cycles.

RESULTS

Purification

HPLC analyses of the venom extracts obtained from five *Polistes major major* reservoirs gave very similar qualitative and quantitative profiles (Figure 1) independent of the date or the location of collection of the wasp samples. The effluent corresponding to more prominent peaks within the first 10 min of the chromatogram were collected and analyzed by thin layer chromatography, which revealed the presence of several amines and amino acids when the spots were visualized by ninhydrin reagent. Moreover, ESI-MS analysis of these fractions did not show the presence of any oligopeptides. On the other hand, when fractions that eluted after 20 min were examined by MALDI-TOF MS, some of the samples clearly showed the presence of medium-size peptides. Peptides corresponding to the peaks A, B and C of the HPLC profile showed monoisotopic mass spectral peaks for their proton adducts ([M + H]$^+$) at m/z 1359.1, 1909.9 and 1319.1, respectively. The determination of the amino acid sequence of these three peptides was the main focus of our study. A direct analysis of *Polistes major major* venom extract by MALDI-TOF MS demonstrated the presence of these peptides in the venom in an unmodified form (Figure 2).

Sequence Determination of the PMM1 Peptide

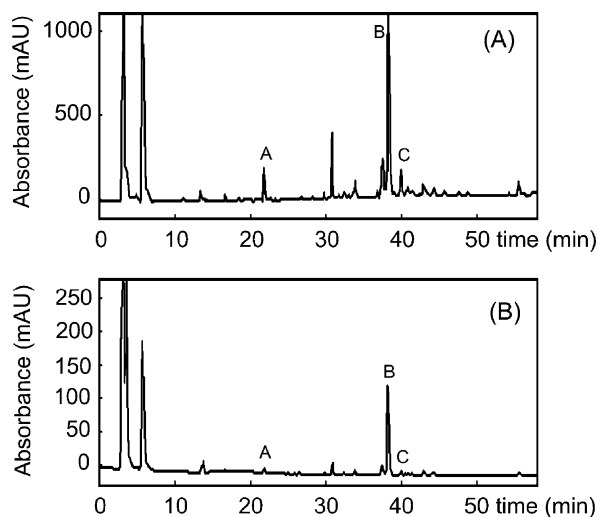The short retention time of peak A, (Figure 1), the low intensity of UV absorptions at 220 and

Figure 1 Reversed-phase HPLC profiles recorded from *Polistes major major* venom extracts. The HPLC was carried out on a C-18 Vydac (250 × 4.6 mm, 5 µm) column using a linear solvent gradient of 5% acetonitrile in 0.1% TFA-water to 70% acetonitrile in 0.1% TFA-water during a period of 60 min at flow rate 1 ml/min. UV absorption was monitored simultaneously at 222 nm (A) and 254 nm (B).
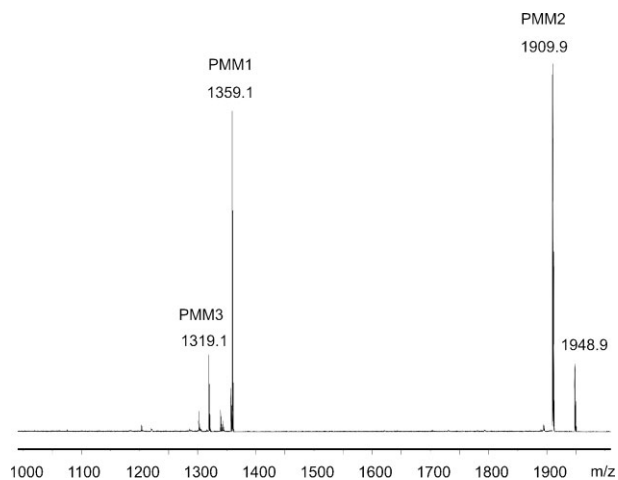

Figure 2 MALDI-TOF MS recorded from a sample of a venom extract from the wasp *Polistes major major*.

254 nm, and the absence of any detectable light absorption at 280 nm, (not shown) indicated the

hydrophilic nature of this peptide, and the absence of Tyr or Trp residues in its structure. These first clues indicated that PMM1 peptide might be a wasp kinin. Since all kinins contain Phe and Arg residues within the peptide chain, we envisaged that an α-chymotrypsin or a trypsin digestion, in combination with MALDI-TOF MS analysis, would provide information about the sequence of PMM1.
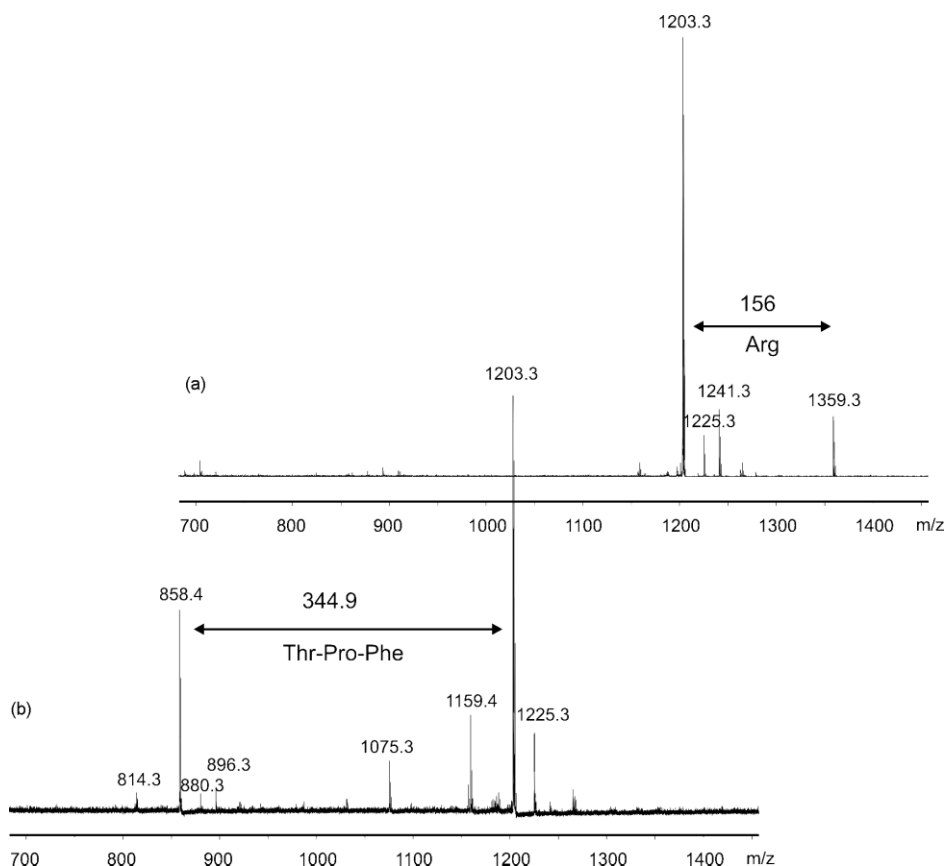

Figure 3 MALDI-TOF mass spectra obtained from the digestion mixture of PMM1 peptide (H-Lys-Arg-Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg-OH) and α-chymotrypsin after 15 min (A), and 6 h (B).
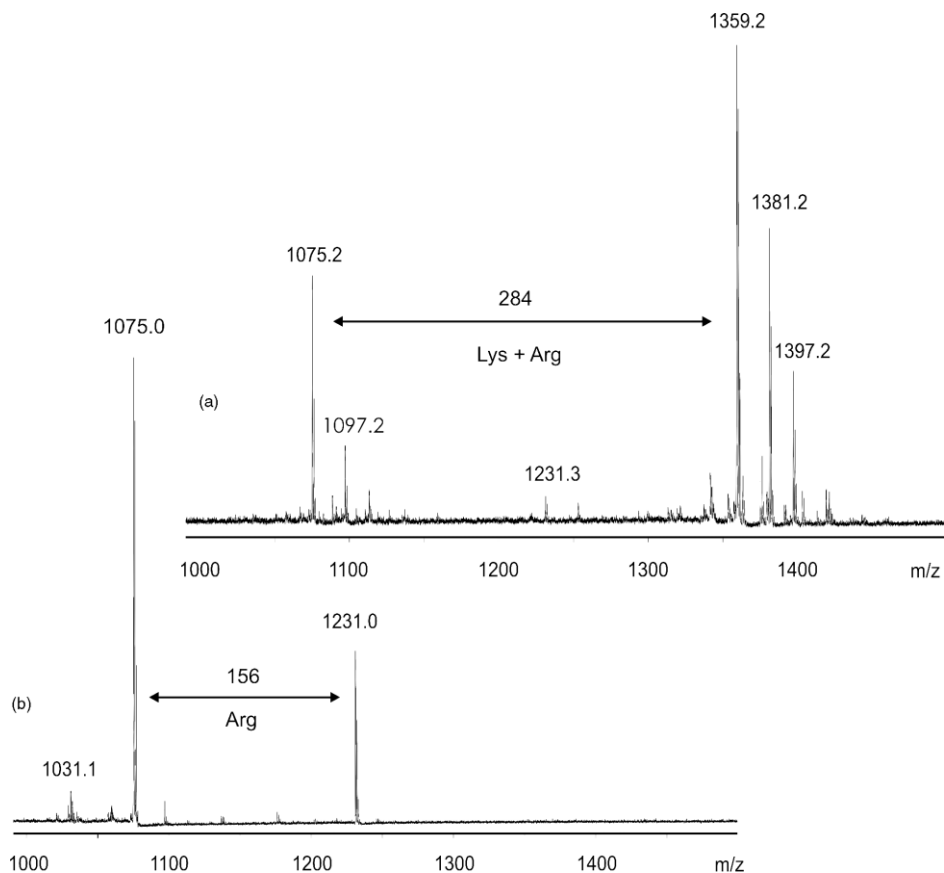
Figure 4 MALDI-TOF mass spectra obtained from the digestion mixture of PMM1 peptide (H-Lys-Arg-Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg-OH) and trypsin after 1 min (A), and 2 h (B).

Usually, wasp kinin homologues encompassing the bradykinin sequence are elongated by several amino acid residues either at the *N*- or the *C*-terminal end. The bradykinin-like part is either identical to bradykinin (H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) itself, or differs from it by a substitution of the proline residue at positions 3 by a hydroxyproline (Hyp), or the serine residue at position 6 by a threonine. A typical set of results obtained from the α-chymotrypsin digestion of the PMM1 peptide is shown in Figure 3. The MALDI spectrum recorded from the peptide digest after 15 min still showed the presence of the original peptide as an MS peak at m/z 1359.3. However, the most intense mass spectral peak at m/z 1203.3 indicated a removal of a 156 Da residue, which was attributed to a removal of an Arg residue from the *C*-terminus by cleaving of the Phe-Arg peptide bond. These results clearly indicated that the PMM1 peptide is a kinin extended at the *N*-terminal end. The mass spectral analysis of the digest mixture after 6 h revealed the presence of two major peaks at m/z 858.4 and 1203.3. The mass separation between these two peaks (344.9 Da) corresponded to the Thr-Pro-Phe part of the Thr[6]-bradykinin sequence (hydrolysis of Phe-Thr peptide bond afforded by α-chymotrypsin). On the other hand, the MALDI MS analysis of the tryptic digest of PMM1 at

two different time intervals (Figure 4) revealed a mass spectral peak at m/z 1075.2, which represented Thr[6]-bradykinin. The spectrum obtained after 2 h showed an intense peak at m/z 1231.0 for the removal of a Lys moiety. Taken into consideration the fact that trypsin usually does not hydrolyze the Arg-Pro peptide bond, the 128.2 Da mass difference between the original peptide (at 1359.2) and the fragment at m/z 1231.0 corresponds to a Lys residue, and the 156 Da mass difference between the m/z 1231.0 and 1075.0 corresponds to an Arg residue, it was evident that the sequence of the PMM1 peptide is H-Lys-Arg-Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg-OH and the m/z 1231.0 fragment corresponds to H-Arg-Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg-OH (1229.68 Da).

Sequence Determination of the PMM2 Peptide

The UV spectrum corresponding to the most intense peak of the HPLC profile (Peak B, Figure 1) confirmed that this peptide contains Trp within its sequence. The presence of the *N*-terminal H-Ile-Asn-Trp- sequence is a common structural feature found in many hemolytic mastoparans [7]. The MALDI spectrum of the PMM2 peptide showed a peak at m/z 1909.9 for the $[M + H]^+$ However, the molecular weight of PPM2 obtained
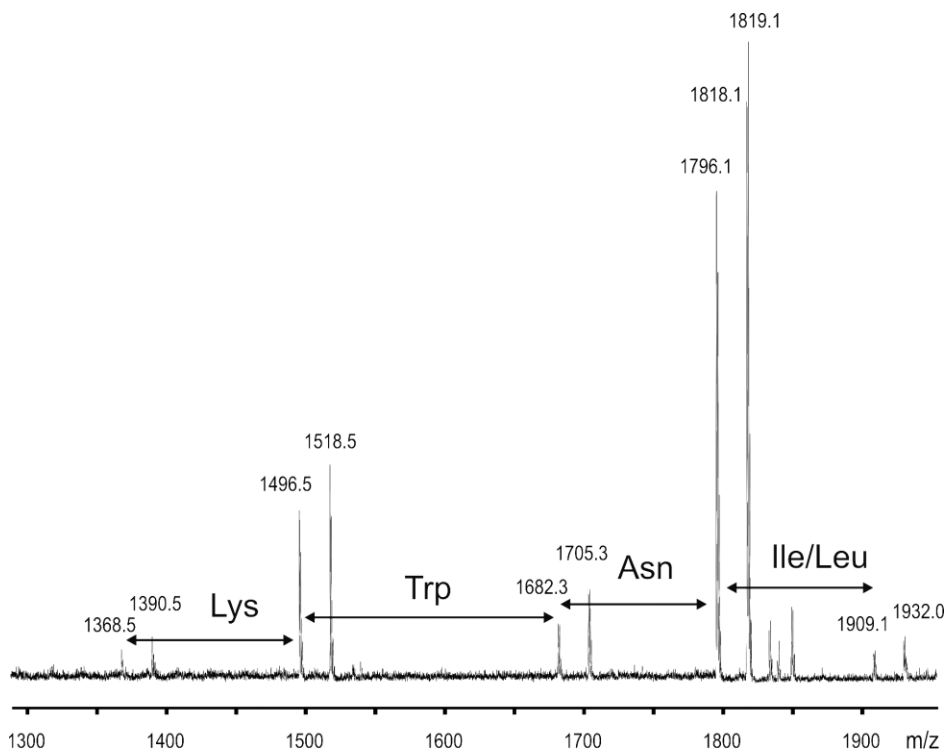
Figure 5 MALDI-TOF MS obtained from the digestion mixture of PMM2 peptide and aminopetidase M after 1 min of incubation. Peak pairs represent $[M + H]^+$ and it corresponding sodium adduct $[M + Na]^+$. (Sodium ions in the buffer salts of the commercial enzyme caused excessive abundance of sodium adducts).

in this way was in fact significantly larger than masses reported in the literature for mastoparans. The partial amino acid sequence of the *N*-terminal end of PMM2 was determined to be Ile/Leu-Asn-Trp-Lys/Gln- by an aminopeptidase-M digestion experiment (Figure 5). Although this indicated that PMM2 might be a mastoparan, it was not a tetradecapeptide. When the sample was sequenced by Edman degradation using 19 cycles, it yielded the following *N*-terminal sequence: Ile-Asn-Trp-Lys-Lys-Ile-Ala-Ser-Ile-Gly-Lys-Glu-Val-Leu-Lys. Since the mass of the peptide was determined by ESI-QTOF MS to be 1909.16 (monoisotopic), it was evident that two amino acid residues were still missing from the *C* terminus. Assuming a *C*-terminus is an amide, the mass difference between the Edman degradation suggested sequence, and the mass of the peptide was 184 Da. This result indicated that the two *C*-terminal residues that were not revealed by Edman degradation were either [Ala + Ile/Leu] or [Pro + Ser]. The $[M + H]^+$ ion derived from PMM2 was then subjected to for CID MS/MS. The resulting tandem mass spectrum not only confirmed the sequence determined by Edman degradation, but also identified the missing two amino acids from the *C*-terminus to be Ala-Ile/Leu-NH$_2$. Additionally, the peptide was hydrolyzed with endopeptidases and the digest was analyzed by MALDI-MS. The MALDI mass spectrum proved the

presence of H-Ile-Asn-Trp-Lys-Lys-Ile-Ala-Ser-Ile-Gly-Lys-Glu-Val-Leu-OH (1597.96 Da) and H-Lys-Lys-Ile-Ala-Ser-Ile-Gly-Lys-Glu-Val-Leu-OH (1184.75 Da) in the α-chymotryptic digest, and H-Lys-Ile-Ala-Ser-Ile-Gly-Lys-Glu-Val-Leu-Lys-OH (1184.75 Da) and H-Ile-Ala-Ser-Ile-Gly-Lys-Glu-Val-Leu-Lys-OH (1056.65 Da) in the tryptic digest. Moreover, when PMM2 was subjected to digestion by immobilized trypsin, and the resulting reaction mixture was analyzed by ESI-MS, mass spectral peaks were observed at m/z 488.31, 529.34, 560.32, 588.37, and 620.9. The mass separation of isotopic peaks reveled that the m/z 529.34 and 620.9 peaks represented double charged ion, whereas all others were from singly charged species. The peaks observed could be correlated to T_4, T_{3-4}, T_1, T_3, and T_{3-5} fragments expected from trypsin digestion, respectively (Figure 6). Finally, the distinction between Ile and Leu at the *C*-terminus was unambiguously achieved by chemical synthesis of both the compounds. An HPLC comparison of the natural PMM2 peptide with the two synthetic peptides PMM2[Ile[17]] (Rt 38.05 min) and PMM2[Leu[17]] (Rt 38.80 min) demonstrated that the natural PMM2 peptide co-eluted with the synthetic PMM2[Leu[17]].

Sequence Determination of the PMM3 Peptide

The peptide corresponding to the minor Peak C that eluted after PMM2 was sequenced using 14 Edman

H-Ile-Asn-Trp-Lys-Lys-Ile-Ala-Ser-Ile-Gly-Lys-Glu-Val-Leu-Lys-Ala-Leu-NH₂

T₁ (m/z 560.32)

T₂

T₃ (m/z 588.37)

T₄ (m/z 488.31)

$\overset{++}{T}_{3-4}$ (m/z 529.34)
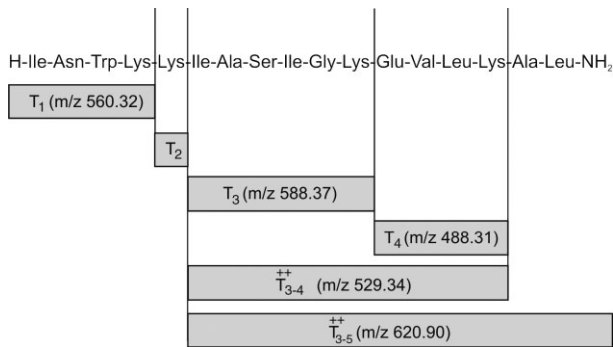
$\overset{++}{T}_{3-5}$ (m/z 620.90)

Figure 6 Fragments expected from trypsin digestion of PMM2.

degradation cycles, which suggested the *N*-terminal sequence   Phe-Leu-Ser-Ala-Leu-Leu-Gly-Met-Leu-Lys-Asn-Leu. The monoisotopic mass determined by MALDI-TOF MS for the $[M+H]^+$ was 1318.94 Da. Assuming the *C*-terminus to be an amide, the monoisotopic mass of the neutral peptide was determined as 1317.78. The presence of a H-Phe-Leu-Ser-Ala-Leu-Leu-Gly-Met-Leu-Lys-OH peptide fragment (monoisotopic mass 1091.64) in the tryptic digest of PMM3, as the dominant peak in the MALDI-TOF spectrum, supported the proposed sequence. A comparison of the primary sequence of the PMM3 peptide to those of other peptides known from social wasp venom revealed some similarities with those known for chemotactic peptides, which contain many hydrophobic amino acid residues and a single Lys residue in the central part of the sequence or at the position 10. Some of these peptides have characteristically Phe-Leu-Pro tripeptide signature at the *N*-terminus.

DISCUSSION

Recently, two new antimicrobial heptadecapeptide amides, named Dominulin A (H-Ile-Asn-Trp-Lys-Lys-Ile-Ala-Glu-Val-Gly-Gly-Lys-Ile-Leu-Ser-Ser-Leu-NH₂) and Dominulin B (H-Ile-Asn-Trp-Lys-Lys-Ile-Ala-Glu-Ile-Gly-Lys-Gln-Val-Leu-Ser-Ala-Leu-NH₂), have been found in the venom and on the cuticle of the social paper wasp *Polistes dominulus* (collected in Italy). The authors [13] speculate that these two antimicrobial peptides may play an important role in the protection of the wasp colony against infections since they are applied to the surface of the whole nest. PMM2 heptadecapeptide, which possesses the same sequence of the first seven residues from the *N*-terminal of the Dominulins A and B, and differs only in three residues (positions 8, 12 and 15) of the entire sequence of Dominulin B, is currently being tested for antimicrobial and mast cell degranulation activity.

REFERENCES

1. Habermann E. Bee and wasp venoms. *Science* 1972; **177**: 314–322.
2. Hoffman DR. Hymenoptera venom proteins. *Adv. Exp. Med. Biol.* 1996; **391**: 169–186.
3. Nakajima T. Pharmacological biochemistry of vespid venoms. In *Venoms of the Hymenoptera: Biochemical, Pharmacological and Behavioural Aspects*, Piek T (ed.). Academic Press: London, 1986; 309–327.
4. Olson EJ. Parachartergus fraternus (Gribodo) (Hymenoptera: Vespidae: Polistinae) uses venom when taking caterpillar pray. *Psyche* 1996; **103**: 85–93.
5. Steiner A. Stinging behaviour of solitary wasps. In *Venoms of the Hymenoptera: Biochemical, Pharmacological and Behavioural Aspects*, Piek T (ed.). Academic Press: London, 1986; 63–160.
6. Hisada M, Satake H, Masuda K, Aoyama M, Murata K, Shinada T, Iwashita T, Ohfune Y, Nakajima T. Molecular components and toxicity of the venom of the solitary wasp, *Anoplius samariensis*. *Biochem. Biophys. Res. Commun.* 2005; **330**: 1048–1054.
7. Nakajima T, Uzu S, Wakamatsu K, Saito K, Miyazawa T, Yasuhara T, Tsukamato Y, Fujino M. Amphiphilic peptides in wasp venom. *Biopolymers* 1986; **25**: 115–121.
8. Mendes MA, Souza BM, Marquez MR, Palma MS. Structural and biological characterization of two novel peptides from the venom of the neotropical social wasp *Agelaia pallipes pallipes*. *Toxicon* 2004; **44**: 67–74.
9. Piek T. Neurotoxic kinins from wasp and ant venoms. *Toxicon* 1991; **29**: 139–149.
10. Konno K, Palma MS, Hitara IY, Juliano MA, Juliano L, Yasuhara T. Identification of bradykinins in solitary wasp venoms. *Toxicon* 2002; **40**: 309–312.
11. Fields GB, Noble RL. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acid. *Int. J. Pept. Protein Res.* 1990; **35**: 161–214.
12. Doucette A, Li L. Investigation of the applicability of a sequential digestion protocol using trypsin and leucine aminopeptidase M for protein identification by matrix-assisted laser desorption/ionization mass spectrometry. *Eur. J. Mass. Spectrom.* 2001; **7**: 157–170.
13. Turillazi S, Mastrobuoni G, Dani FR, Moneti G, Pieraccini G, la Marca G, Perito B, Lambardi D, Cavallini V, Dapporto L. Dominulin A and B: two new antimicrobial peptides identified on the cuticle and in the venom of the social paper wasp *Polistes dominulus* using MALDI-TOF, MALDI-TOF/TOF, and ESI-Ion trap. *J. Am. Soc. Mass Spectrom.* 2006; **17**: 376–383.