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RESEARCH REPORT

Identification and characterisation of novel wasp mastoparans and chemotactic peptides from the venom of social wasp *Polistes stigma* (Hymenoptera: Vespidae: Polistinae)

Rajaian Pushpabai Rajesh[1], R. Arun[1], M Masilamani Selvam[2], Carlton Ranjith Wilson Alphonse[1], M Rajasekar[1], Jayaseelan Benjamin Franklin[3*]

[1]Molecular & Nanomedicine Research Unit, Centre for Nanoscience and Nanotechnology, Sathyabama Institute of Science and Technology, Chennai 600119, India

[2]Department of Biotechnology, Sathyabama Institute of Science and Technology, Chennai 600119, India

[3]Marine Conservation, Bombay Natural History Society, Hornbill House, SBS Road, Mumbai 400001, Maharashtra, India

***Correspondence to:** Jayaseelan Benjamin Franklin, Email: b.franklin@bnhs.org, Tel: +91 80729 68170

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ABSTRACT

Polistes stigma is a common social wasp found in continental Southeast Asia. Despite its wide distribution and abundance, hitherto, there are no studies on small or medium molecular weight components of the venom. For the first time, this study has described the amino acid sequences and its post-translation modifications (PTM's) of four wasp-mastoparans (Ps 1524, Ps 1540, Ps 1556 and Ps 1630), three chemotactic peptides (Ps1417, Ps1434 and Ps1474) and one more (Ps1549) lysine rich peptide from the venom of *P. stigma*. There were 27 mass traces obtained from the crude natural venom, in which the complete amino acid sequences of 8 peptides were solved. Further, single disulphide bonded peptides uncommon in wasp venoms were identified. The mastoparan peptides were rich in hydrophobic residues. In addition, the peptides Ps1549, Ps1630, Ps1434 and Ps1417 were found to have unusual PTM's of C-terminal amidation. This preliminary study comprehends the untapped compounds present in wasp venom that are equally valuable to widely studied venoms of snakes, spiders and cone snails.

KEYWORDS: *Polistes stigma*, social wasp, venom, mastoparans, chemotactic peptides, peptide sequencing, mass spectrometry

INTRODUCTION

The social wasp genus *Polistes* consists of 86 species and 34 sub-species distributed from Africa to Australia. The genus *Polistes* has been classified into four groups namely *P. adustus, P. stigma, P. sagittarius,* and *Stenopolistes* group (Carpenter, 1996a; Santos et al*,* 2015). The *P. stigma* group are species with an apically spotted forewing (Carpenter, 1996b). Originally, this group contained two species, namely *P. stigma* and *P. latinis* (Das and Gupta, 1989). In continental Southeast Asia, hitherto, only one species *P. stigma* has been recorded in this group. The predatory wasp species *P. stigma* normally subdue their prey by stinging it. Generally, wasp venom contains amines (histamine, tyramine, serotonin and catecholamines), peptides and proteins, including many hydrolases (Morgan and Wilson, 1999). This mixture of biologically active substances can exert toxic effects, contributing to certain clinical signs and symptoms of envenomation (Postma, 2009). However, *Polistes* venom is a complex secretion reported to have several functions in addition to its antimicrobial properties. The venom also contains both alarm and sexual pheromones.

The chemical composition of *Polistes* venom is different from other members of social Hymenoptera. It consists of low, medium (*i.e.,* kinins and mastoparans) and high molecular weight (*i.e.,* proteins) components (Turillazzi, 2006). The low molecular weight components are active amines (*i.e.,* serotonin, histamine, dopamine and thyromine) (Vogel, 2018), which are reported to be the pain producing components in the venom; however, no pharmacological properties of these components are known so far (Hisada et al*,* 2005). The medium molecular weight components are the peptidic fractions that have short amino acid (AA) chain peptides (900Da to 3000Da). These include Kinins with 9 to 18AA residues containing bradykinin–like sequences, and Mastoparans that contain 7 to 10 hydrophobic amino acid residues and possess 2 to 4 lysine residues in the primary sequence. Both kinins and mastoparans are lesser known venom peptide components of the genus *Polistes*. However, *Polistes* were the very first genus in which the primary structure of a kinin and an octadecapeptide named Polisteskinin 3 was described from the venom of *P. fuscatus, P. exclamans* and *P. annularis* (Turillazzi, 2006). Further, Polisteskinin R was reported from the venom of a Japanese species *Polybiapaulista*, and two forms of kinins with 11 AA residues from *P. jawigae* (Dos Anjos et al*,* 2016). In addition, six vasoactive peptides were isolated and sequenced from the venom of *P. gallicus*, of which four of them resembled the structural analogues of bradykinins (Mukhamedov and Akhunov, 1989; Pantera et al*,* 2003). Kinins are reported to be the main pain producers in the venom against vertebrates and help effectively in defence during colony threats. They are also effective against conspecifics or congenerics in the case of aggressive interactions from usurpers or social parasites (Nagy et al*,* 2007).

Mastoparans are typically made of 14 amino acids chain largely represented by hydrophobic residues such as leucine, isoleucine, valine, and alanine (Mahadevappa et al, 2017). This compound induces haemolysis in animal cells (Schmidt et al, 1983) and can activate venom enzymes (Mendes and Palma, 2006). *Polistes* mastoparan was first described in *P. jadwigae* from Japan ("A New Mast Cell Degranulating Peptide 'Mastoparan' in the Venom of Vespula lewisii"). Additionally, two heptadecapeptides (mastoparans) were isolated from the venom of *P. dominulus*. Turillazzi et al (2006) and his group reported two mastoparans namely Dominulin A and Dominulin B as a strong antimicrobial agent against Gram+ and Gram- bacteria found in the venom of female members of *Polistes dominulus* (Turillazzi et al*,* 2006). They also observed different mass spectral profiles of several compounds ranging from 900 to 3000Da in the venom of three European (*P. dominulus, P. gallicus and P. mimphus*) and one North American (*P. exclamans*) species.

Finally, the *Polistes* venom consists of high molecular weight components composed of various proteins reported to have enzymatic activity. These are the most studied fractions in Aculeates reported to be powerful antigen and component that cause allergic reactions in humans and animals (Golden, 2005). Vespid chemotactic peptide (VCP) a major constituent of wasp venom, show similarities with the antimicrobial peptides (AMPs) from Ranid frog, *Amolops loloensis.* These peptides posses antimicrobial property and induce the cellular chemotactic response (Yu et al*,* 2007).

In this study, we have explored the lesser or unknown medium molecular weight components (wasp-mastoparans and chemotactic peptides) from the venom of *Polistes stigma.* Despite the abundance of *P. stigma* in Southeast Asia, as far our literature knowledge there is no study on mastoparans and chemotactic peptides of this species. Mass spectrometry-guided venom peptide profiling is a powerful tool to investigate novel substances from venomous animals in a highly sensitive manner. We have applied this peptide profiling approach to successfully explore and characterize the amino acid sequences of four mastoparans and three chemotactic peptides from the venom of *P. stigma*. These results may play an important role to further explore and reveal the biological and functional aspects of these peptides and to promote research on wasp venoms towards drug development.

MATERIALS AND METHODS

Wasp and venom collection

Five healthy worker wasps from different nests were collected from Tambaram, Chennai district, Tamilnadu, India and the venom was milked directly into a sterile 1.5ml eppendorf vial. Immediately, the venom was preserved and diluted by adding fine droplets of 0.5ml of 50% (v/v) HPLC grade acetonitrile (ACN) on the sides of the vial. This original stock solution was stored in -20^{o}C and used for further analyses.

Mass Spectrometry

The protocol for mass spectrometry follows methods of our everyday task that we use to analyse marine animal venoms, mostly the predatory cone snail venoms (Rajesh, 2015; Franklin and Rajesh, 2015; Franklin et al*,* 2017). Nevertheless, we have slightly modified the procedure so to acquire results in a highly sensitive manner and add an extremely low mass range of components from wasp venom.

Materials

Tris (2-carboxyethyl) phosphine (TCEP) was procured from Thermo Fisher Scientific, Inc., United States of America (USA). N-ethylmaleimide (NEM) was purchased from Sigma Aldrich, United States of America. Analytical grade acetonitrile (ACN), Methanol and Tri fluoro acetic acid (TFA),α-Cyano-4-hydroxycinnamic acid (**CCA)**were obtained from Merck Ltd., India.

Electrospray ionisation (ESI)

ESI mass spectra were recorded on a Bruker Daltonics Esquire 3000 Plus Ion-Trap Mass Spectrometer attached to an Agilent 1100 series HPLC system. The samples were infused into the mass spectrometer through a HPLC column (Agilent Zorbax analytical C18 column, 150 × 4.6mm, 5µm, 90Å pore size) and eluted using a binary gradient of water (TFA 0.1% v/v): acetonitrile (TFA 0.1% v/v) at a flow rate of 0.2 (ml/min). Data were acquired from 100 to 3000m/z in positive ion mode.

Mass determination and chemical modifications

Molecular mass analysis was performed on a *matrix-assisted laser desorption/ionization* Ultra flex time of flight-mass spectrometer (MALDI-TOF-MS) (Bruker Daltonics, Bremen, Germany) using a positive-ionization mode in

a 90ns time delay, and a 25kV accelerating voltage. The system utilizes a 50Hz pulsed nitrogen laser, emitting at 337nm. The ion source and the flight tube were kept at a pressure of about 7×10^{7} mbar by turbo molecular pumps. To identify the number of disulfide-rich peptides and to establish the number of disulfides, reduction and alkylation experiments were carried out by adding 20µl of *TCEP* (final concentration 20mM) into the crude venom extract. The mixture was incubated at 37°C for 1hr. To this reaction mixture, NEM was added to a final concentration of 40mM and again incubated for 45min at room temperature. CCA was used as matrix in MALDI-TOF-MS experiments. The results were analysed and data was acquired by LC-ESI-MS and MALDI-TOF.

Amino acid sequencing

Auto MS(n) experiments (CID (Collision-Induced Dissociation) and ETD (Electron-Transfer Dissociation) fragmentation) were performed for the reduced and alkylated peptides. The chemically modified peptides were chromatographically separated based on their polarity using a reverse phase C_{18} column. Peptides eluting from the column was fragmented using nitrogen gas (CID fragmentation) and by collision of these peptides with thermal excited electrons in a methane atmosphere (ETD fragmentation). The daughter ions derived from CID fragmentation were manually analyzed to determine amino acid sequences (Supplementary Table **2**). Multiple sequence alignment was done separately for each peptide to identify similar peptides from wasp venom or from other animal venoms using NCBI blast. NCBI blastpsuite (protein-protein BLAST) was used to search the closest matching peptide sequencing to calculate the % of sequence identity for each individual sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins).

RESULTS

Twenty-seven different venom components (excluding Na and K adducts) between the mass range of 100 to 3000 were identified from the venom of *P. stigma.* The complete fingerprint of MALDI-TOF-MS spectrum is given as (Supplementary Figure **1**) of which, eight of them were isolated and sequenced successfully. Three peptides, namely Ps1417, Ps1434 and Ps1474 were classified as chemotactic peptides and four peptides that contain 8 (Ps1524), 11 (Ps1540), 10 (Ps1556) and 8 (Ps1630) number of hydrophobic amino acid residues and possesses 2, 2, 3 and 2 lysine residues, respectively in the primary sequences and were determined as Mastoparans (Supplementary Table **1**). Additionally, three peptides, 2824.6, 2858.6 (Supplementary Figure **2**) and 2695.6 (Supplementary Figure **3**) with single disulphide bonds that are usually rare in wasp venom was identified with an increase of 252Da from its original mass through global reduction alkylation experiments.

Examination of 'b' and 'y' ions allowed us to generate the amino acid sequences of Ps1417 (Supplementary Figure **4**), Ps1434 (Supplementary Figure **5**) and Ps1474 (Supplementary Figure **6**). The peptide Ps1417 was obtained from the Na adduct parent mass, in which the glycine residue (10^{th}position) was attached with the Na ion (adduct). Amidation at C-terminal end of Ps1417 and Ps1434 was calculated by a difference of 1 Da in 'y' series ions (Supplementary Figure **4** and **5)**. Peptides Ps1417 and Ps1434 showed 91% identity and Ps1474 showed 89% identity towards Ropalidian wasp chemotactic peptides (Koike et al*,* 1991). These peptides are involved in Mast cell degranulation and induce chemotaxis of neutrophils belonging to the MCD family and Crabrolin subfamily. Further, the peptide Ps1474 showed 62% identity towards ant toxin U1-poneritoxin-Da2a from *Dinoponera australis* (Johnson et al*,* 2010). Ps1417 and Ps1434 has 8 hydrophobic amino acids and 61% of defined hydrophobic ratio. Similarly, the peptide Ps1474 had 9 hydrophobic amino acids and 64% of defined hydrophobic ratio. Hitherto, chemo-tactic peptides isolated from wasp venom possess several lysine residues with amidated C-terminus (Koike et al*,* 1991; Mukhamedov and Akhunov, 1989) and peptides mostly start with Phenylalanine residue (Lee et al, 2016). Nevertheless, in this study, sequence homology of Ps1434, Ps1474 and Ps1417 suggests chemo-tactic functions that are closely similar to venom peptides of hunting wasps with peptide codes Ves-CP-T and Ves-CP-M. In addition, the peptides Ps1417 and Ps1434 have unusual PTM's of C-terminal amidation as seen in the chemotactic peptides from Ropalidian wasp.

The amino acid sequence of Ps1549 was derived from the fragmented spectrum of doubly charged parent ion with m/z 775.5$[M+2H]^{+2}$ (Supplementary Figure **8**) and the sequence was derived as RKPJKRKJKKKK-NH_2. The sequence of Ps 1549 possesses 7 number of acetylation sites (Supplementary Figure **8**) that indicated the presence of 7 lysine residues in the sequence. The peptide was positively charged with a total net charge of +9 due to 9 (K: 7, R: 2) positively charged aminoacids.

The amino acid sequences of other four peptides namely Ps1524, Ps1540, Ps1556 and Ps1630 (Supplementary Figure **9, 10, 11 and 12**) were determined as mastoparans. The peptide Ps1524 amino acid sequence was confirmed as IDWAKIGKIAJJAJ (Supplementary Figure **9**). Acetylation of this peptide indicated the presence of 3 amino groups that clarified the L/Q ambiguity in 5^{th} and 8^{th} position of the amino acid sequence with 128 Da (Supplementary Figure **10**). The peptide Ps1524 showed the doubly m/z 816.9$[M+2H]^{+2}$ and triply 544.8$[M+3H]^{+3}$ charged state of the peptide suggesting the presence of the 2 charged Lysine residues (Supplementary Figure **11**). Ps1524 sequence showed 60% identity with pd-mastroparan PDD-B of *Polistes dorsalis* (Cerovský et al*,* 2008) with the presence of 10 hydrophobic aminoacids and 71% of defined hydrophobic ratio.

The peptides Ps1540 (Supplementary Figure **12)** and Ps1556 (Supplementary Figure **13**) showed a similar amino acid chain except that the latter possessed a Lysine residue in the 2^{nd} position. ESI-MS spectrum of Ps1540 (Supplementary Figure **14**) revealed a doubly charged 771.47 $[M+2H]^{+2}$ and a triply charged 514.3$[M+3H]^{+3}$ that indicated the presence of 2 charged residues and confirmed the presence of 2 charged amino acid, Lysine. Similarly, the ESI-MS spectrum of Ps1556 (Supplementary Figure **15**) has shown a doubly charged 779.47 $[M+2H]^{+2}$, a triply charged 519.8$[M+3H]^{+3}$ and a quadruply charged 391.0$[M+4H]^{+4}$ indicating the presence of 3 charged residues that confirmed the presence of three charged aminoacid, Lysine. Multiple sequence alignment with mastoparans of *Polybiapaulista* (Venom protein 13a) (de Souza et al, 2009), *Protopolybiaexigua*

(Mastoparan-1) (Mendes et al, 2005) and *Agelaiapallipes* (Agelaia-mastoparan) (Mendes et al*,* 2004) showed a 35.7% identity with a conserved region of aminoacids KLGKXXXXXL. Ps1540 11 (I: 1, L: 6, F: 1, A: 3) hydrophobic aminoacids and 73% of defined hydrophobic ratio, similarly Ps1556 has 10 (I: 1, L: 5, F: 1, A: 3) hydrophobic aminoacids and 66 % of defined hydrophobic ratio.

De novo sequencing of doubly charged parent ions 816.91$[M+2H]^{+2}$ and triply charged parent ion 544.87 $[M+3H]^{+3}$ derived as sequence INWSNPAEMAKKML-NH_2 (Supplementary Figure **16**). Acetylated Ps1630 has shown an increase in 126 Da delta mass indicating the presence of 3 sites for acetylation (Supplementary Figure **17**). The multiple of 3, 42 Da (126 Da) increase was observed that confirmed the amino group at the amino terminus and Lysine residues at $11^{th}$and 12^{th} position. Carboxy terminal amidation was confirmed by inspection of the 'y' series ions which were less than 1 Da from the original mass (Supplementary Figure **16**). ESI-MS spectrum of Ps1630 (Supplementary Figure **18**), have shown a doubly 816.9$[M+2H]^{+2}$, a triply 544.8$[M+3H]^{+3}$and a quadruply 408.7$[M+4H]^{+4}$charged ions that suggested the presence of at least 3 charged amino acids (2 Lysine and 1 Glutamic acid) in the amino acid sequence of Ps1630. Ps1630 showed 64.28% identity towards a mastoparan X peptide of bee venom (Nakajima et al*,* 1986) and Mastoparan-like peptide 12a of *Vespa magnifica* (Yu et al*,* 2007) belonging to the mastoparan family and has 7 (I: 1, L: 1, M: 2, A: 2, W: 1) hydrophobic aminoacid with 50% of defined hydrophobic ratio.

DISCUSSION

Wasp venom contains numerous bioactive substances that are of importance to the animal for hunting and defending against intruders, but have also attracted attention of biomedical researchers due to their potential physiological, pharmacological and therapeutic applications (Moreno and Giralt, 2015; Chen et al*,* 2018) and represent an untapped source of template molecules for the design of novel drug candidates. Neverthless, perhaps due to limitation in sample or venom collection, only very few previous studies have been conducted to identify and characterize the constituents of the wasp venoms, especially *Polistes stigma*. But, their venoms are rich sources of novel bioactive substances. Wasp venom is more variable in composition among species. A significant characteristic feature in the peptide composition of wasp venom is the predominance of mastoparan and bradykinins (Moreno and Giralt, 2015). Mastoparan is a membrane-active amphipathic peptide with 14 amino acid residues. It is rich in hydrophobic and basic residues that form amphipathic helical structures, the latter with the capacity to form pores in membranes. Mastoparan induces a effective mitochondrial permeability alteration that affects cell viability (Pfeiffer et al*,* 1995). Mastoparan show diverse biological effects and the key biological activities have been described such as antimicrobial activity, increased histamine release from mast cells, haemolytic activity (Cabrera et al*,* 2011) induction of a potent mitochondrial permeability transition, and tumor cell cytotoxicity (Pfeiffer et al*,* 1995). Chemotactic peptides show its biological activity by increasing neutrophils and T helper cells chemotaxis (Hancock and Brown, 1995; Wu and Hancock, 1999). Although wasp venom has attracted much less attention than bee venom, extensive research over recent decades has shown the pharmacological properties. In addition, the availability of highly sensitive mass spectrometry techniques allows for a more efficient proteomic/peptidomic analysis of a limited amount of wasp venoms.

In summary, this study identified and characterised eight novel wasp venom peptides from a lesser-known species, *Polistes stigma.* Four mastoparans and three chemotactic peptides were sequenced using highly sensitive mass spectrometry techniques. Further, work is ongoing in our laboratory to identify the possible bioactivities of these peptides and the corresponding molecular mechanisms. One peptide, Ps1549 was found rich in lysine residue (7 no.) and three peptides (Ps 1524, Ps 1556 and Ps 1630) were rich in hydrophobic residues (8 to 10 no.). Interestingly, single disulphide bonded peptides were found in *P. stigma*, which is a rare occurrence in wasp venom. Unusual post-translation modifications were observed in peptides Ps1549, Ps1630, Ps1417 and Ps1434. The presence of several lysine residues in most of the sequences suggested the chemotactic functions of the peptides. Even though mastoparans are toxic to humans and other animals, they may serve as potential antimicrobial agents (Mendes and Palma, 2006). Wasp mastoparan has been used as a transduction peptide to transfer drug molecules through cell membrane (Fuchs et al*,* 2005). Broad studies on allergens of various species may also contribute to the development of more reliable and effective venom immunotherapy for allergic people (Haskel and Paul, 2007). Unlike other venomous animals like snakes, spiders or cone snails, wasp venom is less explored for medical or pharmacological purposes. This preliminary study on *P. stigma* allow other researchers to explore novel compounds for further biomedical research. Although a wide variety of compounds are currently available, the future therapeutic applications of wasp venom components are further complicated by strong competition with millions of potential new molecules several venomous creatures.

CONFLICT OF INTEREST

None declared

LIST OF ABBREVIATIONS

MALDI-TOF: Matrix Assisted Laser Desorption/ Ionization Time-of-Flight
ESI-MS: Electro spray ionization-mass spectrometry
TFA: Trifluoroacetic acid
TCEP: Tris (2-carboxyethyl) phosphine
NEM: N-Ethylmaleimide
CCA: α-Cyano-4-hydroxycinnamic acid
ACN: Analytical grade acetonitrile
PTM: Post Translational Modification

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