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Structure and biological activities of a new mastoparan isolated from the venom of the hornet *Vespa basalis*

Chewn-Lang HO and Ling-Ling HWANG
Institute of Biological Chemistry, Academia Sinica P.O. Box 23–106, Taipei, Taiwan 10798, Republic of China

By gel filtration on a Fractogel TSK HW 50 column followed by cation-exchange chromatography on CM-Trisacryl M, a tetradecapeptide amide, designated 'mastoparan B', was purified from the venom of the hornet *Vespa basalis*. Its amino acid sequence was determined as:

Leu-Lys-Leu-Lys-Ser-Ile-Val-Ser-Trp-Ala-Lys-Lys-Val-Leu-NH_2

and its molecular mass was measured to be 1611 Da by fast-atom-bombardment mass spectrometry. In addition to having a common structure of vespid mastoparans, the peptide shows a less hydrophobic sequence at positions 1, 2, 5, 8 and 9. The peptide caused liberation of histamine from rat peritoneal mast cells and induced oedema in the rat paw. However, the latter effect was inhibited by 'anti-serotonin' (anti-5-hydroxytryptamine) (cyproheptadine), but not by antihistamine (chlorpheniramine). The peptide also possesses a potent haemolytic activity which acts in synergy with the lethal protein of the venom, suggesting the possible involvement of mastoparan B in the lethal effect of *Vespa basalis* venom.

INTRODUCTION

The hornet *Vespa basalis* is one of the most dangerous species of wasps found in Taiwan. The insect is aggressive and its venom is highly toxic [1]. The venom causes local oedema, severe haemolysis and circulation failure in animals [1,2]. Our recent study on this venom has revealed that the venom fraction of high molecular mass contains a haemolytic lethal protein [1] which is responsible for the death of animals. In a preliminary bioassay, the major fraction containing small peptides was also found to possess mastocytotropic and haemolytic activities. Mastoparans, the mast-cell-degranulating peptides of the vespid venom, have been reported to be the major peptide in many species of the hornets [3]. Although several such peptides have been isolated and characterized as a histamine liberator of the mast cells [3,4], the contribution of the peptide to the toxic effects of the whole venom remains to be elucidated. Here we report the purification, from *Vespa basalis* venom, of a mastocytotropic peptide which has an amino acid sequence distinct from those of other vespid mastoparans. The peptide possesses a potent haemolytic activity which acts in synergy with the lethal protein of the venom.

MATERIALS AND METHODS

Hornet venom was collected by pressing the venom sacs dissected from worker insects. Isolation of the venom components was performed on a gel-filtration column (2.5 cm × 100 cm, Fractogel TSK HW 50; fine grade; Merck) eluted with ammonium acetate buffer (0.05 M, pH 5.5), followed by a cation-exchange column (1.0 cm × 20 cm; CM-Trisacryl M; IBF Biotechnics, Garenne, France) eluted with a linear gradient of ammonium acetate (0.05 M, pH 5.5, to 1.0 M, pH 6.8). Reversed-phase h.p.l.c. was performed on a Nucleosil 5 C_{18} column (4 mm × 250 mm) eluted with a linear gradient of acetonitrile (5–60%, v/v) in 6 mM-trifluoroacetic acid.

Amino acid analysis was carried out using a method that utilizes a hydrolysis conditions of 7 M-HCl, 10% (w/v) trifluoroacetic acid and 0.1% (v/v) phenol at 158 °C for 30 min [5] and subsequent reversed-phase h.p.l.c. analysis of dimethylaminoazobenzenesulphonyl amino acids [6]. Tryptophan was determined by the method of Simpson *et al.* [7] with an amino acid analyzer (Beckman model 6300). The amino acid sequence of the peptide was determined by automated Edman degradation with an Applied Biosystems 477 A gas-phase sequencer. The phenylthiohydantoin amino acid derivatives were separated using an on-line Applied Biosystems phenylthiohydantoin analyser. The molecular mass of the peptide was measured with a fast-atom-bombardment mass spectrometer (JEOL JMS-HX 110) as described in [8].

Hydrolysis of the peptide with carboxypeptidase A was carried out in 0.05 M-Tris buffer, pH 7.5, containing 1.0 M-NaCl at 25 °C for 30, 60 and 90 min. A synthetic decapeptide with a free α-carbonyl group at the *C*-terminus was used as the control. The released amino acid was allowed to react with dimethylaminoazobenzenesulphonyl chloride and analysed as mentioned above [6].

Peritoneal mast cells were isolated from rats (Wistar; 250–400 g) by the method of McClain *et al.* [9]. The isolated cells (~ 10^6/ml) were incubated with the peptide at 37 °C for 15 min. Histamine released and remaining in the cells (after lysis by heating at 70 °C in water) was determined fluorometrically by the method of Håkanson & Rönnberg [10].

Hind-paw oedema was induced by a single subplantar injection of the peptide (in 50 μl of saline) into the left hind-paws of Wistar rats. An equal volume of the saline was injected into the right ones as control. The volume of each paw was measured with a plethysmometer (Ugo Basile, model 7150). The degree of paw swelling was expressed as percentage of the initial volume. Antihistamine and 'anti-serotonin' were given subcutaneously 1 h before the peptide injection.

Direct haemolytic activity was assayed on washed red cells of the rat and guinea pig. The red cells (1%) were incubated with the peptide or toxin in Tris-buffered (0.01 M, pH 7.4) saline (0.15 M) at 37 °C for 60 min [11].

BSA, mastoparan (from the wasp *Paravespula lewissi*) and cyproheptadine were purchased from Sigma Chemical Co. Chlorpheniramine was from Schering.

RESULTS AND DISCUSSION

Isolation, amino acid sequence and molecular mass

By gel permeation on a Fractogel TSK HW 50 column, the hornet venom (500 mg) was first separated into 15 fractions (Fig.

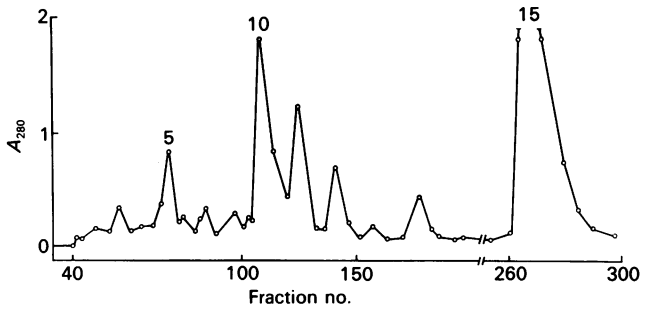

Fig. 1. Gel-filtration chromatography of the crude venom of *Vespa basalis*

Freeze-dried venom (500 mg) was applied to a Fractogel TSK HW 50 column (2.5 cm × 100 cm) and eluted with an ammonium acetate buffer (0.05 M, pH 5.5). Fraction 10 was collected for further purification.

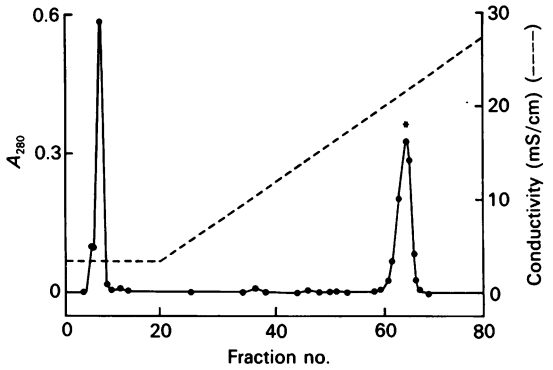

Fig. 2. Cation-exchange chromatography of fraction 10 obtained from a gel-filtration column

Fraction 10 (48 mg) was applied to a CM-Trisacryl M column (1.0 cm × 20 cm) and eluted with a linear gradient of ammonium acetate buffers (0.05 M, pH 5.5, to 1.0 M, pH 6.8). The last fraction (*) was collected for chemical and pharmacological characterization.

1). The major fraction (10), which contained small peptides, was further purified on a cation-exchange (CM-Trisacryl M) column eluted with a linear gradient of ammonium acetate buffer (0.05–1.0 M) (Fig. 2). The bioactive substance obtained from the last fraction (denoted by *) of this chromatographic procedure showed a symmetric peak upon passing through a reversed-phase h.p.l.c. column (Nucleosil 5 C_{18}). The yield of the substance was about 3.4% of the crude venom. Amino acid analysis revealed that it was a peptide with a high content of lysine and leucine (Table 1). The amino acid sequence of the peptide was determined as:

Leu-Lys-Leu-Lys-Ser-Ile-Val-Ser-Trp-Ala-Lys-Lys-Val-Leu

by automated Edman degradation. On the basis of its amino acid composition, the molecular mass of the peptide was estimated to be 1612 Da. However, using the fast-atom-bombardment mass spectrometer, its molecular mass was found to be 1611 Da rather than 1612 (Fig. 3). The peptide was resistant to the hydrolytic action of carboxypeptidase A, which catalyses the cleavage of peptides with the absolute requirement for a free α-carbonyl group in the *C*-terminal position [12]. This finding, together with the data concerning its molecular mass, suggests that the *C*-terminus of the peptide was amidated. The presence of lysine residues at the positions 4, 11 and 12 of the sequence is a structure common to most vespid mastoparans [3]. The tetradecapeptide isolated from the *Vespa basalis* venom, therefore, should be a homologue of vespid mastoparans. Using the nomenclature of Hirai *et al.* [13], we designated this peptide as 'mastoparan B'. However, distinctive differences in sequence were found at the positions 1, 2, 5, 8 and 9, where mastoparan B has less hydrophobic Leu^1-Lys^2, $Ser^{5,8}$ and Trp^9, instead of Ile^1-Asn^2, $Ala^{5,8}$ and Leu^9 (or other amino acids) found in most *Vespa* mastoparans [3,14,15]. A comparison of the sum of hydropathy indexes [16] at these positions showed that mastoparan B had a lowest value (most hydrophilic) among the known mastoparans, except *Polistes* mastoparan which does not possess the common Lys^{11}-Lys^{12} structure in its sequence [17]. According to the helical structure of mastoparan (from *Paravespula lewissi*), Ile^1, $Ala^{5,8}$ and $Lys^{4,11,12}$ were shown to be located on the hydrophilic side of the molecule, and the other amino acids on the hydrophobic side [3,18]. This conformation reveals the amphiphilic properties of the peptide. As compared with the sequence of this prototypic mastoparan (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2) [13], mastoparan B, which possesses several less hydrophobic amino acids at positions 1, 2, 5, 8 and 9, would result in a more hydrophilic surface on the molecule and might, thereby, lead to the change in its biological activities.

Biological activities of mastoparan B

Like other mastoparans, mastoparan B caused release of histamine from the isolated peritoneal mast cells of the rat in a dose-dependent manner. At concentrations of 3, 10 and 20 μM, the histamine released (expressed as a percentage of the cell content) was 2.2 ± 0.7, 25.9 ± 4.4 and 46.4 ± 7.0% (means ± S.E.M.,

Table 1. Amino acid composition, sequence and molecular mass of mastoparan B isolated from *Vespa basalis* venom

Parameter	Value						
Amino acid* composition	Lys	Ser	Ala	Val	Ile	Leu	Trp
Molar ratio	4.01	1.42	1.00	1.59	0.76	3.02	1.0
Nearest integer	4	2	1	2	1	3	1
Amino acid† sequence	1 Leu-Lys-Leu-Lys-Ser-Ile-Val-Ser-Trp-Ala-Lys-Lys-Val-Leu-NH_2		5		10		
Molecular mass‡	1611 (M+H = 1612)						

* Analysed by gas-phase hydrolysis and dimethylaminoazobenzenesulphonyl chloride derivatives [5,6], except tryptophan, which was determined by the method of Simpson *et al.* [7].

† Determined by automated Edman degradation.

‡ Measured by fast-atom-bombardment mass spectrometry [8].

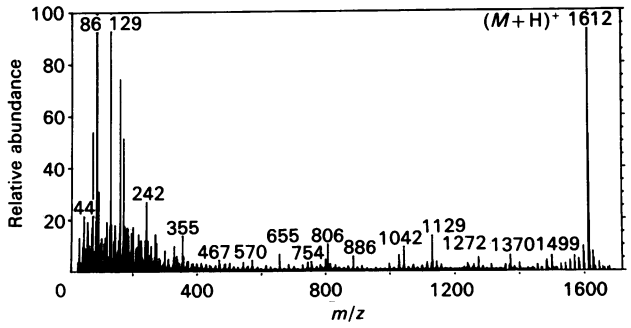

Fig. 3. Fast-atom-bombardment mass spectra of mastoparan B

The peptide (10 μg) was suspended in a thioglycerol matrix and bombarded with xenon atoms accelerated at 6.0 KeV.

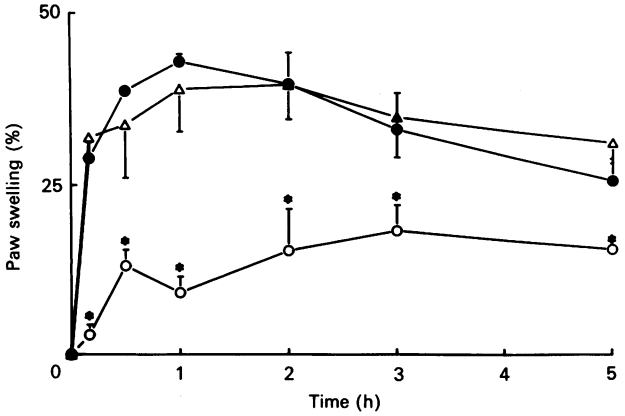

Fig. 4. Swelling of rat hind-paw induced by mastoparan B and the effects of 'anti-serotonin' and antihistamine

Mastoparan B (●) (80 μg/paw) was given by subplantar injection. Cyproheptadine (○) (5 mg/kg) and chlorpheniramine (△) (10 mg/kg) were given subcutaneously 1 h before the peptide injection. Each point represents the mean ± S.E.M. of three or four experiments. $*P < 0.05$ as compared with the effect of mastoparan B alone.

Table 2. Direct haemolytic activity of mastoparan B alone and in the presence of the lethal protein isolated from *Vespa basalis* venom

Washed red blood cells (RBC; 1%) were incubated with mastoparans or lethal protein in Tris-buffered (0.01 M, pH 7.4) saline at 37 °C for 60 min. $*P < 0.05$ as compared with that of mastoparan B alone at the corresponding concentration. Results are means ± S.E.M., with the numbers of experiments given in parentheses.

Mastoparan or toxin	Concn. (μM)	Direct haemolysis (%)	
		Guinea-pig RBC	Rat RBC
Mastoparan B (*V. basalis*)	10	8.7 ± 3.0 (9)	4.1 ± 0.6 (9)
	15	87.8 ± 3.0 (9)	11.3 ± 1.5 (9)
	20	100.0 ± 0.0 (9)	71.1 ± 9.7 (9)
Mastoparan (*P. lewissi*)	15	3.6 ± 2.0 (4)*	3.1 ± 0.8 (4)*
	20	5.5 ± 1.0 (4)*	6.4 ± 0.8 (8)*
Lethal protein (*V. basalis*)	0.015	1.7 ± 0.2 (6)	7.0 ± 2.1 (6)
Mastoparan B + lethal protein (0.015 μM)	10	81.3 ± 13.1 (6)*	54.5 ± 10.1 (6)*
	15	99.7 ± 0.1 (6)*	82.8 ± 4.4 (6)*
	20	–	91.3 ± 1.5 (6)

$n = 3$) respectively. Injection of the peptide into the rat paw caused swelling of that paw. The oedematous response could be observed within 10 min and reached its maximum in about 1–2 h after the injection (Fig. 4). This local effect appeared to be related to 5-hydroxytryptamine, but not histamine, liberated by the peptide, since 'anti-serotonin' (cyproheptadine, 5 mg/kg) effectively reduced the swelling, whereas a rather high dose of antihistamine (chlorpheniramine, 10 mg/kg) was unable to inhibit the effect (Fig. 4). Mastoparan B also showed a marked haemolytic action on the red cells of several species of animals, including the guinea pig and the rat (Table 2). Mastoparan from *Paravespula lewissi* was much weaker in this respect. However, the haemolytic activity of mastoparan B was still less potent as compared with the lethal protein of *Vespa basalis* venom (95% haemolysis at less than 1.0 μM) [1]. Addition of a non-lytic dose (0.015 μM) of the hornet lethal protein caused a remarkable increase in the haemolysis induced by mastoparan B (Table 2), indicating a synergistic effect of the two peptides on haemolytic action.

Vespa basalis venom is highly lethal, owing to its potent action on the cardiovascular system. The main factor responsible for the high toxicity is the lethal protein, which possesses phospholipase A_1 activity and is extremely haemolytic [1]. The finding that mastoparan B acts synergistically with lethal protein on haemolysis suggests that mastoparan B may play a supporting role in the lethal effect of the venom.

Recent studies on the structure–activity relationship of mastoparan have revealed the important role of the first three amino acid residues in the Ca^{2+} influx, affinity to liposomes or inhibition of calmodulin-sensitive phosphodiesterase [14]. In addition, a correlation between the histamine-releasing activity or binding to calmodulin and the hydrophobic moments of mastoparan has been reported [14,19]. Mastoparan B, with a different amino acid sequence and hydrophobicity, should be useful for studying the structure–activity relationship of mastoparans.

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