Isolation and Characterization of Two New Peptides, Mastoparan C and Crabrolin, from the Venom of the European Hornet, Vespa crabo*

Antonio Argiolas and John J. Pisano
From the Section on Physiological Chemistry, Laboratory of Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

Two peptides rich in hydrophobic amino acids have been isolated from venom sacs of the European hornet, Vespa crabo. One peptide (P-2) is structurally and functionally related to the tetradecapeptide mastoparan and has been named mastoparan C, Leu-Asn-Leu-Lys-Ala-Leu-Leu-Val-Val-Leu-Lys-Ile-Leu-NH₂. The other (P-1) is a tridecapeptide with a new sequence: Phe-Leu-Pro-Leu-Ile-Leu-Arg-Lys-Ile-Thr-Ala-Leu-NH₂ which we have named crabrolin. The peptide releases histamine from rat peritoneal mast cells with a threshold of ~2.5 μg/ml (~8 μm). Crabrolin also facilitates the action of purified phospholipase A₂ from different sources, but it is not quite as active as mastoparan. It is clearly less active than mastoparan in lysing erythrocytes, and it does not release amylase from dispersed guinea pig pancreatic acini. Given its unique sequence, the principal effect of crabrolin may be neither mast cell degranulation nor phospholipase facilitation, but a yet undiscovered action.

Venoms of bees, wasps, yellow jackets, and hornets contain a variety of biologically active peptides. Perhaps best known are the bee venom peptides melittin and apamin and the mast cell degranulating peptide (MCD peptide) (1) known also as peptide 401 (2). Different and more ubiquitous peptides, kinins (3-6), and mastoparans (7-10) are found in wasp, yellow jacket, and hornet venoms.

Kinin-like peptides are of interest because two kinins, bradykinin and lysyl-bradykinin, occur in man. They are produced by plasma and glandular kallikreins, respectively, and increase vascular permeability. They are suspected mediators of the pain and inflammation associated with certain pathologic conditions such as arthritis (11).

The mastoparans are an interesting new class of peptides discovered in wasp venom in a screening test for mast cell degranulating agents. We recently reported that the mastoparans are also potent stimulants of purified phospholipase A₂ from different sources (12). This broad spectrum of activity is not shared by melittin which only stimulates bee venom lipases of both venom and victims.

We now report the isolation and characterization of two new peptides mastoparan C and crabrolin from Vespa crabo venom sacs. Introduced from Europe about 140 years ago, V. crabo occurs in most of the United States east of the Mississippi. Weighing about four times more than the honey bee, Apis mellifera, this rather formidable hornet preys on other insects and is not a nuisance to man.

MATERIALS AND METHODS

The following were purchased: venom sacs of the European hornet, V. crabo (Vespa Laboratories, Spring Mills, PA); phospholipase A₂ from bee venom, rattle-snake (Crotalus adamanteus) venom, and porcine pancreas, and collagenase Type III (fraction A) (Sigma); trypsin, treated with 1-L-1-tosylamido 2-phenylethyl chloromethyl ketone and chymotrypsin (Millipore Corp.; carboxypeptidase Y, Pro- nase, thermolysin, and subtilisin (Boehringer Mannheim); masto- paran (Peninsula Laboratories); and 1-stearoyl-2-[1-14C]arachidonyl- sn-glycero-3-phosphocholine (55 mCi/mmol) (Amersham Corp.). All the reagents and chemicals for sequencing were Sequanal grade from Pierce Chemical Co. Other reagents were of the highest available purity.

**Extraction**

One hundred V. crabo venom sacs (stored at -10 °C for several months) were suspended in 2 ml of aqueous 80% methanol containing 0.1% (v/v) 2-mercaptoethanol. The mixture was homogenized in a glass Potter-Elvehjem tissue grinder fitted with a Teflon pestle. The pellet obtained by centrifugation was similarly extracted two more times, and the extracts were combined. Extract not used immediately was stored up to several weeks at -10 °C.

Aliquots were taken for direct bioassay or further purified by high-performance liquid chromatography. Methanol extract equivalent to 0.1-1.0 sac was evaporated at room temperature with a N₂ stream and the residue dissolved in water or appropriate buffer for bioassay. Extract equivalent to 98 sacs was evaporated, and the residue was dissolved in 1 ml of 0.1% trifluoroacetic acid. Peptides in this solution were purified in two steps: 1) initial fractionation on a Waters Sep-Pak C₈ cartridge (13); and 2) final purification on a Waters Bondapak C₈ column (0.39 × 30 cm).

The 1-ml trifluoroacetic acid solution was passed five times through a Sep-Pak cartridge previously washed with 5 ml of methanol and 10 ml of 0.1% trifluoroacetic acid. Retained material was eluted batchwise by the sequential additions of 3 ml of 0.1% trifluoroacetic acid and 2 ml of 20, 40, 60, 80, and 100% aqueous methanol, all containing 0.1% trifluoroacetic acid.

Active Sep-Pak fractions were evaporated to dryness in a Speed Vac (Savant) instrument and dissolved in 0.1% trifluoroacetic acid. Aliquots (<50 μl) equivalent to 0.2 sac were fractionated at room temperature on a μBondapak C₈ column (0.39 × 30 cm) in a Varian 5000 high-performance liquid chromatograph equipped with a variable wavelength UV detector. Gradients were prepared with two solvents. Solvent A consisted of 0.05% trifluoroacetic acid and 6.025% triethylamine in water (pH 2.7), and B was the same reagents in acetonitrile/water (80:20).

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.
Bioassays

Amylase Release from Dispersed Guinea Pig Pancreatic Acini—Dispersed guinea pig pancreatic acini were prepared by digestion of guinea pig pancreas with purified collagenase (14). Amylase released in the incubation medium was measured spectrophotometrically (15) using the Phadebas reagent (Pharmacia Fine Chemicals, Piscataway, NJ). Total amylase was measured by lysing the acini with 0.1% sodium dodecyl sulfate.

Histamine Release from Rat Peritoneal Mast Cells—Mast cells were harvested and purified as previously described (16). Cells, about 100,000 (purity 92 ± 5%), were incubated for 5 min at 37 °C without shaking in a final volume of 200 μl. After centrifugation at 1,000 × g, histamine in the supernatant was determined spectrophotometrically (17). Total histamine present was determined by sonication after disruption of the cells by sonication.

Phospholipase A2 Assay—Phospholipase A2 was assayed essentially as previously described (12) using egg yolk lecithin liposomes labeled with 1-stearyl-2-[1-14C]arachidonyl-sn-glycero-3-phosphocholine. Released [1-14C]arachidonic acid was extracted from the reaction mixture by addition of Dole's solvent (18) and isolated by means of small silica gel columns.

Hemolysis of Erythrocytes—Hemolysis of guinea pig erythrocytes was performed as previously reported (19). The red cells were diluted to 5–5.5 × 10^7/ml, and 10 μl aliquots were added to 2 ml of Na phosphate-buffered saline (pH 7.2) with or without peptides. After 20 min at 37 °C, the cells were centrifuged at 1500 × g, and the supernatant absorbance was measured at 415 nm. Total hemoglobin was determined by suspending the same aliquot of cells in 2 ml of distilled water.

Amino Acid and Sequence Analysis—Samples were hydrolyzed in evacuated, sealed tubes with 6 N HCl for 20 h at 110 °C, and amino acids were determined with a Beckman 6300 amino acid analyzer which incorporates refinements of the Spackman, Stein, and Moore procedure (20). Amino acid sequences were determined manually by dansyl (21)- and DABITC-Edman degradation methods (22). Dansyl amino acids and DABTH derivatives were identified by thin-layer chromatography using 5 × 5-cm polyamide sheets (Schleicher & Schuell).

RESULTS

Purification—Crude extracts equivalent to one venom sac caused the complete release of amylase from dispersed guinea pig pancreatic acini and the maximal release of histamine from rat peritoneal mast cells. Both activities were unaffected by boiling at neutral pH, but both were destroyed by incubation with Pronase, subtilisin, thermolysin, or trypsin, suggesting the peptide nature of the active agent(s). It was partially purified with a Sep-Pak cartridge where the activity was not eluted until the methanol concentration was raised to 80%. An aliquot of this fraction equivalent to 0.2 sac was dissolved in 50 μl of solvent A and injected on a μBondapak C18 column. Two major UV-absorbing peaks, P1 and P2, were observed (Fig. 1). Two-ml fractions were collected, evaporated to dryness, and the residue dissolved in appropriate buffer or water and assayed. Both P1 and P2 stimulated histamine release, but only P2 stimulated amylase release. Activity of both peaks was destroyed by trypsin. Sufficient quantities of P1 and P2 for the determination of their sequences and biological properties were obtained by repeated injections of 0.2 sac equivalents from the Sep-Pak step. From the amino acid analysis, we calculated that we isolated from one sac 12 nmol (18 pg) of P1 and 57 nmol (86 μg) of P2.

Sequence Analysis—Both P1 and P2 are rich in hydrophobic amino acids, especially leucine. Of the 13 residues in P1, 10 are hydrophobic, and of the 14 residues in P2, 10 also are hydrophobic (Table 1). Digestion of the peptides by trypsin

1 The abbreviations used are: dansyl, 5-dimethylaminonaphthalene-1-sulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; DABITC, 4-dimethylaminobenzene-4'-isothiocyanate; DABTH, 4-dimethylaminobenzene-4'-thiodyanotriion.

2 Crabrolin was synthesized by Peninsula Labs under contract.
same tryptic peptides (Table III).

Activity of P1 and P2—The peptides were compared in three tests: histamine release from rat peritoneal mast cells, hemolysis of guinea pig erythrocytes, and stimulation of purified phospholipase A₂ from various sources. P1 was about as potent as P2 in stimulating the release of histamine (Fig. 3), but P2 was about four times as potent a hemolytic agent (Fig. 4). In comparative tests with phospholipases from bee venom, rattlesnake venom, and porcine pancreas, P2 again was more potent than P1. The difference was greatest with the pancreatic enzyme (Table IV) and least with that from bee venom (Table IV and Fig. 5). Significant responses were seen in the various tests with 2.5 μg of active peptide/ml (~1.7 μM) and maximal responses with about 20–50 μg.

The structural and functional similarity of P2 to mastoparan (Table V) has led us to name the peptide crabrolin which indicates only its source.

**DISCUSSION**

Mastoparan C and crabrolin are the most abundant peptides observed in the HPLC chromatograms of *V. crabro* venom sac extracts. We isolated 57 nmol/sac of mastoparan C and estimate that a sac contains 80 nmol or 0.12 mg (Mᵣ = 1506). This is about 50% of the dry weight since the contents of one sac weigh 0.26 mg (24). Only one other peptide, melittin, is found in such a high concentration (1). Some other vespid species contain appreciable levels of mastoparans, although not as high as in *V. crabro*. *V. mandarinia* (10) and *V. tropica*³ contain 20 nmol/sac of mastoparan M and mastoparan T, but mastoparan, mastoparan X, and polistes mastoparan concentrations are probably much less than 20 nmol/sac (10).

We have previously reported that the mastoparans share many of the properties of melittin yet show certain qualitative and quantitative differences. The mastoparans have the same amino acid residues) and melittin (26 residues) by different mechanisms (25). While these peptides are structurally unrelated, all are amphiphilic, a property which pro-

³ T. Nakajima has informed us that his laboratory has isolated from *V. tropica* a tridecapeptide which shows 31% homology with crabrolin, personal communication.
New Peptides in Hornet Venom

The arrow (→) indicates residues determined by the dansyl procedure. Dansyl amino acids were identified by two-dimensional thin-layer chromatography on polyamide plates (5 × 5 cm). Solvent 1 = n-heptane, n-butyl alcohol, and formic acid (10:10:1). Solvent 2 = 0.15 M NH₄OH. The arrow (→) indicates the steps of DABITC-Edman degradation performed on 2–3 nmol of the peptides. DABTH derivatives were identified by two-dimensional thin-layer chromatography on polyamide plates (5 × 5 cm). Solvent 1 is water, CH₃COOH (10:5). Solvent 2 is toluene, n-hexane, and CH₃COOH (10:5:2.5). The vertical arrow indicates thermolysin cleavage of LeuNH₂. The amino acid amide was determined as its dansylated derivative (23). Dansyl-LeuNH₂ was also determined after 4, 1, and 2 Edman degradation steps of P1-3, P2-1, and P2-2, respectively.

Sequence of P1

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Fragment</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1-1</td>
<td>Phe-Leu-Pro-Leu-Ile-Leu-Arg-Lys-Ile-Val-Thr-Ala-LeuNH₂</td>
<td>10.6</td>
</tr>
<tr>
<td>P1-2</td>
<td>Ile-Leu-Arg</td>
<td>12.6</td>
</tr>
<tr>
<td>P1-3</td>
<td>Ile-Val-Thr-Ala-LeuNH₂</td>
<td>12.6</td>
</tr>
<tr>
<td>P1-4</td>
<td>Lys-Ile-Val-Thr-Ala-LeuNH₂</td>
<td>13.5</td>
</tr>
<tr>
<td>P1-5</td>
<td>Phe-Leu-Pro-Leu-Ile-Leu-Arg</td>
<td>13.5</td>
</tr>
</tbody>
</table>

Sequence of P2

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Fragment</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2-1</td>
<td>Leu-Asn-Leu-Lys-Ala-Leu-Leu-Ala-Val-Ala-Lys-Lys-Ile-LeuNH₂</td>
<td>12.6</td>
</tr>
<tr>
<td>P2-2</td>
<td>Ile-LeuNH₂</td>
<td>13.5</td>
</tr>
<tr>
<td>P2-3</td>
<td>Lys-Ile-LeuNH₂</td>
<td>13.5</td>
</tr>
<tr>
<td>P2-4</td>
<td>Leu-Asn-Leu-Lys</td>
<td>13.5</td>
</tr>
<tr>
<td>P2-5</td>
<td>Ala-Leu-Leu-Ala-Val-Ala-Lys</td>
<td>13.5</td>
</tr>
</tbody>
</table>

Table II

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Source of phospholipase A₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Bee venom</td>
</tr>
<tr>
<td>P2</td>
<td>Rattlesnake venom</td>
</tr>
<tr>
<td>P3</td>
<td>Porcine phospholipase</td>
</tr>
</tbody>
</table>

Table III

<table>
<thead>
<tr>
<th>Test</th>
<th>Natural</th>
<th>Synthetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine release ED₅₀ (μg/ml)</td>
<td>11.8 ± 0.6</td>
<td>12.0 ± 0.5</td>
</tr>
<tr>
<td>HPLC retention time (min): System A</td>
<td>7.9 ± 0.4</td>
<td>8.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>System B</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.5</td>
</tr>
</tbody>
</table>

Table IV

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Source of phospholipase A₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Bee venom</td>
</tr>
<tr>
<td>P2</td>
<td>Rattlesnake venom</td>
</tr>
<tr>
<td>P3</td>
<td>Porcine phospholipase</td>
</tr>
</tbody>
</table>

Perhaps of no less interest, the peptides also bind to another membrane component, calmodulin (26, 27), with the highest affinity ever observed for a peptide. The dissociation constant is ~0.3 nM (26). Calmodulin-sensitive phosphodiesterase is inhibited (27) by mastoparan (IC₅₀ 0.02 μM) and melittin (IC₅₀ 0.1 μM).

Mastoparans may be the most ubiquitous peptides in hymenopteran venoms, having been reported in Vespa lewisii, Vespa xanthoptera, Polistes jadwiga, and V. mandarinia. We have also observed mastoparan-like peptides in yellow jacket (Vespula) and bumble bee (Megabombus) venom sac extracts. Even more widely distributed in venoms is phospholipase A₂, making it highly likely that enzyme and peptide coexist in venom. Perhaps a biological role of mastoparans is to facilitate the phospholipase A₂ of both venom and victims, thereby promoting the generation of arachidonic acid, the precursor of prostaglandins and leukotrienes which are mediators of adverse reactions associated with immediate hypersensitivity. The high-affinity binding of mastoparan to calmodulin has led others to speculate that a role of the peptides is inhibition of calmodulin-mediated reactions (26, 27).

Crabrolin is also a major venom peptide. We isolated 12 nmol/sac and estimated that a sac contains 17 nmol (~25 μg). In addition to its prominence, the peptide is of interest because it releases histamine from rat peritoneal mast cells, stimulates phospholipases from different sources, and lyse erythrocytes. However, in none of the tests is it more potent than mastoparan C, and it does not release amylase from guinea pig dispersed pancreatic acini. Mastoparan C probably causes release by lysing the acinar cells.

* A. Argiolas and J. J. Pisano, unpublished data.
New Peptides in Hornet Venom

After completing this work, we found that Miroshnikov et al. (28) reported the structure of two new tetradecapeptides HR-I and HR-II isolated from the venom of the hornet, Vespa orientalis. From 250 mg of V. orientalis venom, 75 μg of HR-I and 45 μg HR-II were isolated. These concentrations are much less than mastoparan C and crabroin which they closely resemble. HR-I is Ile-Asn-Leu-Lys-Ala-Leu-Ala-Leu-Ala-Leu-Lys-Lys-Ile-LeuNH₂ and HR-II is Phe-Leu-Pro-Leu-Ile-Leu-Gly-Lys-Leu-Val-Lys-Leu-ValNH₂, and HR-II is Phe-Leu-Pro-Leu-Ile-Leu-Gly-Lys-Leu-Val-Lys-Leu-ValNH₂. Both V. orientalis peptides release histamine from rat peritoneal mast cells (MCD-peptide from bee venom was 100 times more potent) and lyse erythrocytes. While the potencies of mastoparan C and crabroin for releasing histamine are similar to the V. orientalis peptides, HR-II was four times more potent than HR-I in lysing erythrocytes. This was unexpected because crabroin, which it resembles (69% homology), is one-fourth as potent as mastoparan C in lysing guinea pig erythrocytes.

The occurrence of these peptides in V. orientalis supports our earlier remark on the widespread distribution of mastoparans in hymenopterae. It will be interesting to determine if HR-II/crabroin-like peptides have a similar distribution. Given the amino acid sequence of crabroin, it may also have a unique biological activity which is awaiting discovery.

Acknowledgments—We thank Diana Parker for her skilful assistance in the amino acid analyses. We also thank Dr. Allen Benton and Miles Guralnick of Vespa Laboratories for helpful discussions concerning insect characteristics and venom collection.

REFERENCES
New Peptides in Hornet Venom