Identification of One Tachykinin- and Two Kinin-Related Peptides in the Brain of the White Shrimp, *Penaeus vannamei*

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This paper reports the purification of three myotropic neuropeptides from the white shrimp *Penaeus vannamei*. The central nervous systems of 3500 shrimps were extracted in an acidified solvent, after which four to five HPLC column systems were used to obtain pure peptides. A cockroach hindgut muscle contraction bioassay was used to monitor all collected fractions. The pure peptides were submitted to Edman degradation based automated microsequencing. Mass spectrometry and chemical synthesis confirmed the sequences. Ala-Pro-Ser-Gly-Phe-Leu-Gly-Met-Arg-NH₂ (Pev-tachykinin, 934.1 Da) belongs to the tachykinin family with identified members in all vertebrate classes and some invertebrate classes: arthropods, annelids and molluscs. A very specific Pev-tachykinin antisera was developed, which labels 4 neurosecretory cells in the brain. Ala-Ser-Phe-Ser-Pro-Trp-Gly-NH₂ (Pev-kinin 1, 749.8 Da) and Asp-Phe-Ser-Ala-Trp-Ala-NH₂ (Pev-kinin 2, 694.7 Da) are the first crustacean kinins. Pev-kinin 2 is the first kinin with a Trp-Ala-NH₂ instead of a kinin-typical Trp-Gly-NH₂ carboxyterminus. © 1998 Academic Press

Although immunocytochemical and biochemical studies have indicated the presence of many neuroactive substances in the nervous system of crustaceans [1, 2], in comparison with insects, structural information on crustacean neuropeptides is fragmentary. The following peptides have been identified in several crustacean species: red pigment concentrating hormone and pigment dispersing hormone [3-5], a cardioactive peptide (CCAP) [6], proctolin [7], orokinin [8], a family of hyperglycemic hormones and molt-inhibiting hormones [9,10], a few peptides of the RFamide superfamily in Procambus clarkii [11] and two tachykinin-related peptides in Cancer borealis [12].

Peptides that influence visceral muscle contractility in *vivo* (designated as myotropins) have recently been shown to be of extreme importance in the neuroendocrine system of insects. Not only do they affect muscle contractility; most of them have been found to play a predominant role in other physiological processes, such as diuresis, pheromone biosynthesis, juvenile hormone biosynthesis, pupariation, diapause, melanization, homeostasis and metabolism, release of other hormones [13-15]. It is, however, due to their myotropic effect that the isolation of most of these insect peptide was possible.

Therefore, we undertook the purification of myotropic peptides from the central nervous system of the white shrimp, *P. vannamei*, using the same strategy as for the insect myotropic peptides (i.e. monitoring the fractions in the *Leucophaea maderae* hindgut bioassay). We now report the isolation and characterization of three new neuropeptides from *P. vannamei* and provide evidence for a novel kinin-type peptide.

**MATERIALS AND METHODS**

**Animals.** Mature specimens of the white shrimp, *Penaeus vannamei* were reared at the Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM, Ecuador) as described previously (16).

**Purification.** Three thousand central nervous systems (CNS) of the white shrimp, *P. vannamei*, were dissected. Batches of 500 CNS were extracted in a methanol/water/acetic acid (v/v/v, 90/9/1) solu-
The supernatants were dried and dissolved in 0.1% trifluoroacetic acid (TFA). Subsequently they were loaded on MegabondElute cartridges and eluted with 50% CH₃CN/0.1% TFA. The eluate was dried and transported to Belgium for further purification by means of HPLC. Columns and operating conditions for HPLC on a Gislon (Gislon Medical Electronics, Villiers-le-Bel, France) HPLC system with variable detector (set at 214 nm) were: (i) a preparative Bondapak C18 column (25 × 100 mm; 30 nm, 15 μm, Waters Associates, Milford, MA); solvent A, 0.1% TFA in water; solvent B, 50% CH₃CN in 0.1% aqueous TFA; conditions: 100% A for 10 min followed by a linear gradient to 100% B in 150 min; flow rate, 6 ml/min; detector range, 2.0 absorption units full scale (AUFS); (ii) a semi-preparative Pep-S C2-C18 column (5 μm, 100 A, Pharmacia Sweden); solvent A and B, same as in (i); conditions, a linear gradient from 0% to 25% B in 80 min, then to 50% B in 40 min; flow rate, 1.5 ml/min; detector range, 1.0 AUFS; (iii) an analytical Vydac Biphenyl column (4.6 mm × 250 mm, 30 nm, 5 μm, The Separations Group, Hesperia, CA, USA); solvent A and B, same as in (i); conditions, 15% B for 4 min followed by a linear gradient to 40% B in 60 min; flow rate, 1.5 ml/min; detector range, 0.5 AUFS; (iv) a Biosep-SCX-S-2000 size exclusion column (300 × 7.8 mm, Phenomenex, Torrance, CA, USA) run in normal phase; solvent A, 95% CH₃CN/0.01% TFA; solvent B, 50% CH₃CN/0.01% TFA; conditions, 100% A for 10 min followed by a linear gradient to 20% B in 20 min, then isocratic for 50 min; flow rate, 1.0 ml/min; detector range, 0.2 AUFS; (v) Supelco PKB-100, 4.6 × 250 mm, 5 μm, Supelco Inc. PA); solvents A and B same as in (i).

FIG. 1. HPLC chromatograms of the final purification step of Pev-tachykinin and Pev-kinin 1 and 2 (bioactive peak is indicated with arrow). Pev-tachykinin and Pev-kinin were pure after Biosep-SCX-S-2000 chromatography, whereas Pev-kinin 2 was further purified on a Supelco-PKB-100 column.
TABLE 1
Amino Acid Sequence, Molecular Mass, and Elution Characteristics of the Peptides Investigated

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Bondapak C18</th>
<th>C2-C18</th>
<th>Diphenyl</th>
<th>Biosep-Scotty</th>
<th>Suplex PKB-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>APSGLGMRA</td>
<td>68–70 (46%)</td>
<td>62–64 (20%)</td>
<td>31–32 (28%)</td>
<td>39 (25%)</td>
<td>41 (25%)</td>
</tr>
<tr>
<td>ASFSFWGa</td>
<td>68–70 (46%)</td>
<td>62–64 (20%)</td>
<td>39–41 (30%)</td>
<td>41 (25%)</td>
<td>41 (25%)</td>
</tr>
<tr>
<td>DFSWAAa</td>
<td>66–68 (45%)</td>
<td>52–54 (18%)</td>
<td>48–50 (20%)</td>
<td>40 (25%)</td>
<td>68 (40%)</td>
</tr>
</tbody>
</table>

Average mass (Da)

- Calculated: 934.11, 935.5, 935.9
- Observed natural (M + H)<sup>+</sup>: 749.8, 750.6, 751
- Observed synthetic (M + H)<sup>+</sup>: 694.7, 695, 695

Note. The average molecular mass was calculated with the addition of a hydrogen and an amide group for the N and C termini respectively.

RESULTS AND DISCUSSION

We report here the isolation of 3 neuropeptides from the central nervous system of the white shrimp, P. vannamei (Fig. 1). Table 1 shows their retention times in the four HPLC steps that were used. The amino acid sequences of the pure peptides (Fig. 1) were established by a combination of automated Edman degradation, MALDI-TOF or electrospray tandem mass spectrometry and/or co-elution experiments in reverse phase HPLC with synthetic peptides.

The primary structure of the first shrimp peptide is Ala-Pro-Ser-Gly-Phe-Leu-Gly-Met-Arg-NH<sub>2</sub> (934 Da) and is identical to the tachykinin-related peptide recently identified in the crab Cancer borealis [12]. It belongs to the tachykinin superfamily with members in vertebrate as well as invertebrate classes. Since we identified the first invertebrate brain tachykinin in Locusta migratoria [20], various tachykinins were identified in other insects, molluscs and worms [13, 21–26]. Only very recently, the first tachykinin in crustaceans was identified in the crab, Cancer borealis [12]. The shrimp tachykinin sequence presented here, being identical to crab tachykinin, indicates that the tachykinin primary structure is perfectly conserved in crustaceans, as is the case for other crustacean neuropeptides such as CCAP and RPCH. Amongst the insect tachykinins, Culetachykinin 1 (from Culex salinarius) and Leucophaea maderae tachykinin related peptide 1 display the strongest sequence similarities towards Penaeus tachykinin, as they differ only by a single amino acid (Fig. 2).

Characterisation by DIA revealed that the developed antiserum used in a dilution of 1/4000, recognizes Pev-tachykinin 1. The detection limit of the assay was 100 pg/dot. All other neuropeptides tested at a concentration up to 1 μg/dot did not interfere in the assay, except for the locustatachynkins 1-4, which were detected at a concentration of 10 ng/dot and higher. For an optimal immunoreaction on tissue sections a final dilution of 1/4000 appeared to be appropriate. No staining was observed when anti-Pev-tachykinin serum was re-
Crustaceans
*Peneaus tachykinin*

Cancer borealis tachykinin related peptide 1
Ala-Pro-Ser-Gly-Phe-Leu-Gly-Met-Arg-NH₂

Molluscs
Anodoctatachytachykinin

Annelids
Urechistachytachykinin 1

Insects
Locustatachytachykinin 1
Callitachytachykinin 1
Culetachytachykinin 1

Leucophaea maderae Tachykinin Related Peptide 1
Leu-Arg-Gln-Ser-Gln-Phe-Val-Gly-Ser-Arg-NH₂

Vertebrates

Vertebrates

substance P (mammalian)
Arg-Pro-Lys-Pro-Gln-Phe-Phe-Gly-Leu-Met-NH₂

kytambatin (amphibian)
Asp-Pro-Pro-Asp-Pro-Asp-Arg-Phe-Tyr-Gly-Met-Met-NH₂

FIG. 2. Comparison of the primary structures of *Peneaus* tachykinin with some vertebrate and invertebrate tachykinins [12, 13, 21-25]. Bold amino acids are shared by Pev-tachykinin and other tachykinins.

placed either by serum previously inactivated with synthetic Pev-tachykinin or with control serum. Likewise, no staining occurred when the secondary antibody, PAP-antibody or DAB respectively were omitted from the immunohistochemical procedure. In the brain, 4 neurosecretory cells were intensively labeled with the antiserum (Fig. 3).

The primary structures of the other two identified peptides are novel. The amino acid sequence of the second peptide is Ala-Ser-Phe-Ser-Pro-Trp-Gly-NH₂ (749.8 Da). It is designated as Pev-kinin (*Peneaus vannamei* kinin) since it includes the carboxyterminal sequence FSPWSGamide, which places it as a member of the kinin peptide family, hitherto having only members in insects. Since the discovery of the leucokins by Holman in 1986 [18], kinins have been identified in a number of insects, including *Acheta domesticus*, *Locusta migratoria*, *Culex salinarius*, *Aedes aegypti*, *Helio*
covera zea and Manduca sexta [27, review:28]. All members of the insect kinin peptide family conform to the sequence Phe-X₁xx-X₂xx-Trp-Gly-amide, X₁xx being Asn, Ser, His, Phe or Tyr, and X₂xx being Pro, Ser, or Ala. Therefore, Pev-kinin is the first member of this family to be isolated from a crustacean. Amongst the insect kinins, Achetakinin 2 and 3 and Heliokinin 1 display the strongest sequence similarities towards Pev-kinin 1 (Fig. 4).

The primary structures of the third isolated shrimp neuropeptide is Asp-Phe-Ser-Ala-Trp-Ala-NH₂ (694.7 Da). The presence of an Ala at the C-terminal in the position that normally has a Gly is unusual but the remainder of the sequence clearly shows that it can be considered as a kinin. Therefore it is the first kinin with an Ala-amide carboxyterminal.

In insects, kinins are involved in the regulation of diuresis as they increase fluid secretion in Malpighian tubules [29]. Table 2 shows that both Penaeus kinins are active at 1 µM on cricket tubules, providing further evidence that Pev-kinin 2 is a member of the kinin peptide family. In addition, we conclude that the C-terminal Gly can be substituted by Ala without substantial loss of biological activity. Whether the kinins are involved in diuresis in crustaceans is not known as yet and will be investigated in the future.

Pev-tachykinin and Pev-kinin 1 were approximately 1000 times more potent than Pev-kinin 2 in the cockroach hindgut bioassay, with threshold concentrations of 0.2, 0.5 and 500 nM respectively. Since alive penaeid shrimp were unavailable in Belgium, the synthetic peptides were tested for potential myotropic effects on the hindgut of the crustacean Astacus leptodactylus. Pev-kinin 2 was active at 0.5 µM whereas both Pev-kinin 1 and Pev-tachykinin were inactive.

In conclusion, our results indicate that in addition to the X-organ-sinus gland complex, other parts of the crustacean central nervous system, i.e. the brain, the thoracic ganglia and the ventral nerve cord are important sources of neuropeptides. Furthermore this study indicates that the Leucophaeae hindgut proves useful as a detection system for crustacean neuropeptides. The identification of the two kinins provides evidence that the kinin peptide family, hitherto confined to insects, has a widespread occurrence in arthropoda.

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