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ISOLATION AND IDENTIFICATION OF ONE TACHYKININ- AND THREE KININ-RELATED PEPTIDES IN THE CENTRAL NERVOUS SYSTEM OF *PENAEUS VANNAMEI*

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ABSTRACT

One tachykinin-related and three kinin-related peptides were purified from the central nervous system of the white shrimp, *Penaeus vannamei*. Five thousand nervous systems were dissected and extracted in a methanolic solution followed by prepurification on Megabond Elute cartridges. In order to obtain pure peptides, the 50% CH_3CN/0.1% TFA Megabond eluant was further processed using four to five HPLC column systems. After each chromatographic separation, the fractions were monitored by the simple and fast *Leucophaea maderae* hindgut muscle preparation bioassay. The pure peptides were submitted to Edman degradation based automated microsequencing. MALDI-TOF mass spectrometry and chemical synthesis confirmed the sequences. Ala-Pro-Ser-Gly-Phe-Leu-Gly-Met-Arg-NH_2 (933Da) corresponds to a myotropin denominated Pev-tachykinin, belonging to the tachykinin family with members in all vertebrates as well as in the arthropod, annelid, and mollusc invertebrate phyla. A specific antiserum was developed against Pev-tachykinin which labels 4 neurosecretory cells in the brain of *P. vannamei*. Ala-Ser-Phe-Ser-Pro-Trp-Gly-NH_2 (750 Da), Asp-Phe-Ser-Ala-Trp-Ala-NH_2 (693.5 Da) and Pro-Ala-Phe-Ser-Pro-Trp-Gly-NH_2 (760 Da) were, respectively, designated as Pev-kinin 1, Pev-kinin 2, Pev-kinin 3. Pev-kinin 1 and 3 are the first kinin peptides found in crustaceans. Meanwhile, Pev-kinin 2 forms a new subfamily of the kinin peptide family, as it ends in a Trp-Ala-NH_2 instead of a kinin-typical Trp-Gly-NH_2 carboxyterminus.

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INTRODUCTION

Studies regarding crustacean neuroactive substances are scarce and fragmentary, especially in comparison to the current knowledge on insect neuropeptides and physiology. Crustacean neuropeptide research has mainly been focused on the study of the neurohemal organs in the eyestalk and more specifically on a peptide family group known as the CHH family, present in the X-organ-sinus gland complex of several species, as well as on other pigmentation hormones (Keller, 1992).

Until recently, not much attention had been conferred to neurohormonal centres other than the X-organ-sinus gland complex. In the search for bioactive compounds during the last decade, several studies have focused on other parts of the nervous system. The pericardial organs (neurohemal organs from the pericardial cavity (Keller, 1992)) and brain have been studied using an immunocytochemical approach, involving the localization of arthropod, mammalian, and even molluscan peptides. As a result, positive immunostaining has been found with antisera directed against Substance P, proctolin, FMRFamides, enkephalins, tachykinin-related peptides (locustatachykinin, leucokinins) and biogenic amines (serotonine, histamine, dopamine) (Fingerman et al., 1985; Callaway et al., 1987; Goldberg et al., 1988; Sandeman et al., 1990; Schmidt & Ache, 1994; Blitz et al., 1995; Langworthy et al., 1997; Schmidt, 1997), clearly demonstrating the presence of known bioactive substances.

Only a few bioactive substances such as cardioactive peptides and myotropins (peptides that influence visceral muscle contractility (in vitro), i.e., proctolin FMRFamide peptides (Mercier et al., 1993), tachykinin-related peptides (Christie et al., 1997), crustacean cardioactive peptide (CCAP), and orkinin (Mercier et al., 1992), have been identified. Myotropins have recently been shown to be of extreme importance in the neuro-endocrine system of insects. Not only do myotropins affect muscle contractility, but most of them have been found to play a dominant role in other physiological processes, such as diuresis, pheromone biosynthesis, juvenile hormone biosynthesis, pupariation, diapause, melanization, homeostasis and metabolism, and release of other hormones (Veelaert et al., 1997). It is, however, due to their myotropic effect that the isolation of most of these insect peptides has been possible.

Therefore, we undertook the purification of myotropic peptides from the central nervous system of the white shrimp, *Penaeus vannamei* Boone, 1931 using the same strategy as for the insect myotropic peptides (i.e., monitoring the fractions in the *Leucophaea maderae* (Fabricius, 1781) hindgut bioassay). We now report the isolation and characterization of four new neuropeptides from *P. vannamei* and provide evidence for a novel kinin subfamily.

MATERIALS AND METHODS

Animals

Only reproductive females (45-70 g) of *Penaeus vannamei* (white shrimp) were used; they were captured by fishermen using long-line nets, off the coast of the Ecuadorian provinces of Guayas and Manabí. Subsequently, the shrimp were transported to the CENAIM research centre, where they were kept at the indoor maturation and reproduction facilities. The females were reared until used, as previously described by Naessens et al. (1997).

Purification

Five thousand central nervous systems (CNS = brain, stomatogastric and thoracic ganglion, ventral nerve cord) of reproductive females of *P. vannamei* were dissected. Previous to dissection, females were kept in ice cold seawater for 30 min. Dissected CNS were placed in ice cold methanolic solution (methanol/water/acetic acid (v/v/v: 90/9/1)), homogenized, and the resulting mixture was centrifuged at 8,000 rpm for 10 min., recovering the supernatant. The pellet was resuspended once more in methanolic solution and the procedure repeated. The supernatants were pooled and rotavaped in order to evaporate methanol. The resulting solution was washed in two steps, one with ethyl acetate and another with hexane. The supernatants were dried and transported to Belgium for further purification. The extract was resuspended in 0.1% trifluoracetic acid (TFA), subsequently loaded on Megabond Elute cartridges and eluted with 50% CH_3CN/0.1% TFA. The elute was dried and further purified by means of HPLC. Columns and operating conditions for HPLC on a Gilson HPLC system (Gilson Medical Electronics, Villiers le Bel, France) 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% CH_3CN 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, 100Å, 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 Diphenyl column (4.6 × 250 mm; 30 nm, 5 μm, The Separations Group, Hesperia, CA, U.S.A.); 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-Sec-S-2000 size exclusion column (300 × 7.8 mm, Phenomenex, Torrance, CA, U.S.A.) run in normal phase; solvent A, 95% CH_3CN/0.01% TFA; solvent B, 50% CH_3CN/0.01% TFA;

conditions: see fig. 1, 0.2 AUFS; (v) Suplex PKB-100 (4.6 × 250 mm, 5 mm, Supelco Inc., PA, U.S.A.); solvents A and B same as in (i), conditions: see fig. 1; 1.5 ml/min.; 0.1 AUFS. Fractions were collected every 2 min. for (i) and (ii). Peaks were collected manually for (iii), (iv), and (v).

Structure determination

Following the last purification column, a peptide sample (one tenth of the final volume) was loaded on a precycled Biobren Plus-coated glass filter. N-terminal amino acid sequence analysis was carried out on a Perkin Elmer/Applied Biosystems Procise 492 microsequencer running in pulsed liquid mode. Mass analysis was performed on a Micromass Tofspec matrix assisted laser desorption time of flight mass spectrometer (MALDI-TOF MS) as described previously (Veelaert et al., 1997). The proposed primary structures were synthesized using Fmoc polyamide chemistry (J. W. Drijfhout, University Hospital, Leiden, The Netherlands). The synthetic peptides were used for verification of the sequence and antibody development.

Bioassay

After each chromatographic separation, the fractions were monitored by a simple and fast bioassay, the *Leucophaea maderae* hindgut muscle preparation bioassay (Holman et al., 1991).

Production of primary antiserum

A polyclonal antiserum was raised by immunizing rabbits with synthetic Ala-Pro-Ser-Gly-Phe-Leu-Gly-Met-Arg-NH_2 (Pev-tachykinin) coupled to thyroglobulin using EDC (1-ethyl-3-dimethylaminopropyl carbodiimide hydrochloride) as a cross-linker, via its terminal group. In this way, antisera would mainly be directed against the carboxyterminal portion of the peptide molecule. The bound peptide was dissolved in 1 ml of a Tris saline buffer pH 7.4, emulsified with an equal amount of Freunds adjuvant, and injected subcutaneously into New Zealand White rabbits. A a second and third boost was given, respectively, 3 and 6 weeks after the initial immunization. A total of 1 mg coupled Pev-tachykinin was injected into each of the two rabbits for initial immunization and three boosts.

The dot immunobinding assay (DIA) procedure used to characterize the antiserum is described in Nieto et al. (1998). Synthetic Pev-tachykinin was dotted in a concentration range from 1 pg up to 1 μg/dot. CCAP, proctolin, and locustatachykinin 1 were used as antigen controls. As an additional control for the immunoassays, the primary antiserum was incubated with synthetic Pev-tachykinin (10 μg/ml antiserum) before being used in DIA.

Immunocytochemical localization

Brains and thoracic ganglia were dissected in sterilized sea water, fixed in Bouin-Hollande's 10% sublimate solution for 24 hrs, dehydrated through ethanol and xylene series, and embedded in Paraplast. The immunocytochemical procedure followed is described by Paemen et al. (1992).

RESULTS AND DISCUSSION

Here we report the isolation and characterization of 4 neuropeptides from the central nervous system of the white shrimp, *Penaeus vannamei*. The amino acid sequences were established by a combination of automated Edman degradation, MALDI-TOF mass spectrometry and/or co-elution experiments in reverse phase HPLC with synthetic peptides. Fig. 1 presents the chromatograms for the first and last purification column corresponding to Pev-tachykinin, Pev-kinin 1, Pev-kinin 2, and Pev-kinin 3.

The primary structure of the first shrimp peptide purified is Ala-Pró-Ser-Gly-Phe-Leu-Gly-Met-Arg-NH_2 (933 Da) (table I), and was denominated Pev-tachykinin since it belongs to the tachykinin superfamily with members in verte-

TABLE I

Comparison of the amino acid sequences of tachykinins from vertebrate and invertebrate sources

CRUSTACEANS	
Penaeus vannamei tachykinin	**Ala-Pro-Ser-Gly-Phe-Leu-Gly-Met-Arg-NH₂**
Cancer borealis TRP 1	**Ala-Pro-Ser-Gly-Phe-Leu-Gly-Met-Arg-NH₂**
INSECTS	
Locustatachykinin 1	Gly-**Pro-Ser-Gly-Phe**-Tyr-**Gly**-Val-**Arg**-NH₂
Callitachykinin 1	**Ala-Pro**-Thr-Ala-**Phe**-Tyr-**Gly**-Val-**Arg**-NH₂
Culetachykinin 1	**Ala-Pro-Ser-Gly-Phe**-Met-**Gly-Met-Arg**-NH₂
Leucophea maderae TRP 1	**Ala-Pro-Ser-Gly-Phe-Leu-Gly**-Val-**Arg**-NH₂
MOLLUSKS	
Anodontatachykinin	pGlu-Tyr-**Gly-Phe**-His-Ala-Val-**Arg**-NH₂
ANNELIDS	
Urechistachykinin	Leu-Arg-Gln-Ser-Gln-**Phe**-Val-**Gly**-Ser-**Arg**-NH₂
VERTEBRATES	
Substance P (mammalian)	Arg-Pro-Lys-**Pro**-Gln-Gln-**Phe**-Phe-**Gly**-Leu-Met-NH₂
Hylambain (amphibian)	Asp-Pro-Pro-Asp-**Pro**-Asp-Arg-**Phe**-Tyr-**Gly-Met**-Met-NH₂
Scyliohinin (dogfish)	Ala-Lys-Phe-Asp-Lys-**Phe**-Tyr-**Gly**-Leu-Met-NH₂

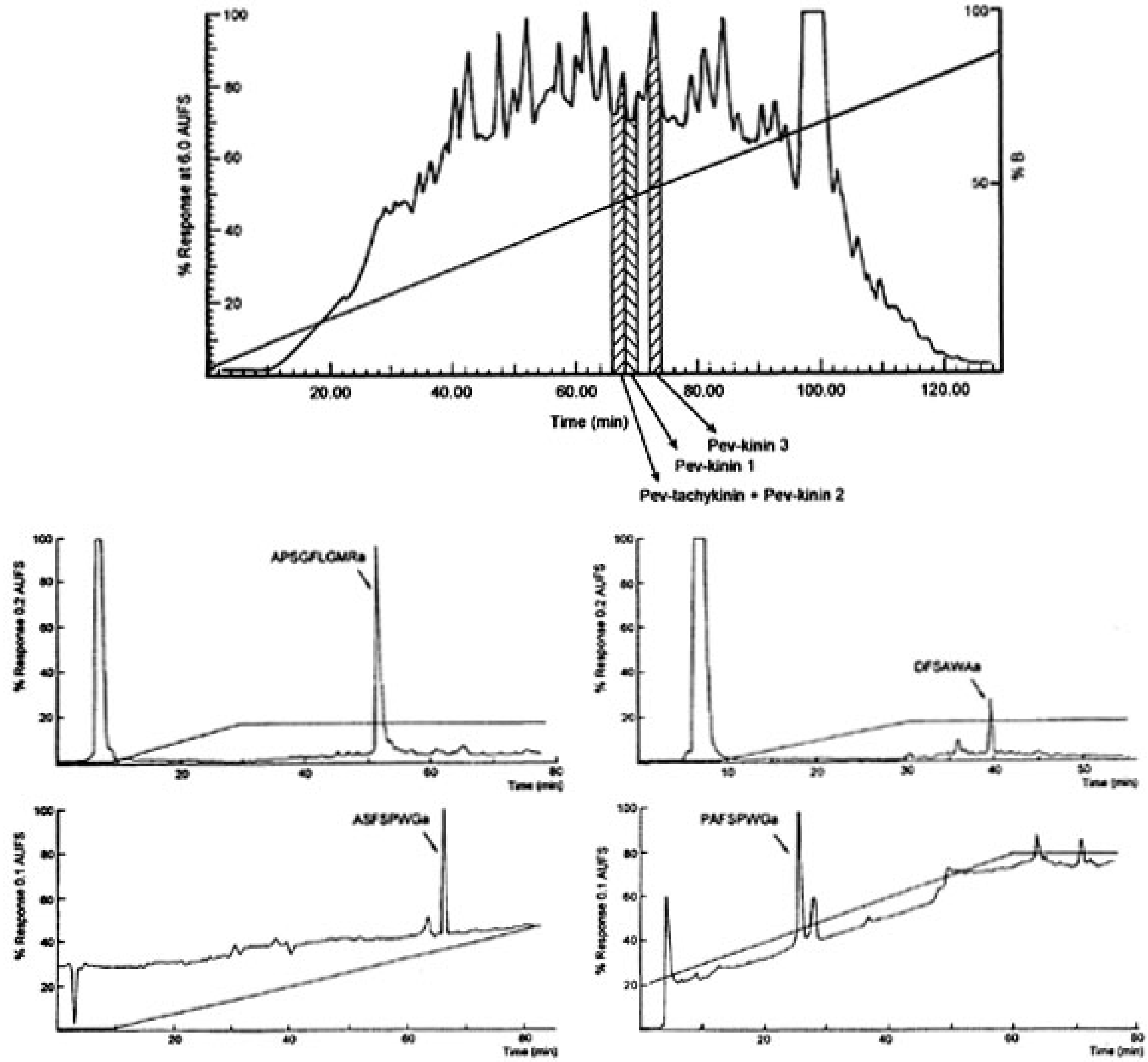

Fig. 1. HPLC chromatograms of the first (Bondapak column) and last purification step of Pev-tachykinin and Pev-kinin 1, 2, 3 (bioactive peak is indicated with arrow).

brate as well as invertebrate classes. Pev-tachykinin is identical to the CabTRP-Ia, the tachykinin-related peptide recently identified in the crab *Cancer borealis* Stimpson, 1859 (cf. Christie et al., 1997). The shrimp tachykinin sequence presented here, being exactly the same as the crab tachykinin, indicates that the tachykinin primary structure seems to be perfectly conserved in crustaceans, as is the case for other crustacean neuropeptides such as CCAP and RPCH (red pigment concentrating hormone (Keller, 1992)).

Characterization of the Pev-tachykinin antiserum by DIA revealed that the developed antiserum used in a dilution of 1/4000 recognized Ala-Pro-Ser-Gly-Phe-Leu-Gly-Met-Arg-NH_2 with a detection limit of 10 pg/dot (fig. 2a). Locustatachykinin was also recognized in a 100-fold higher concentration. There was no cross-reaction with the other peptides tested. In immunocytochemical stud-

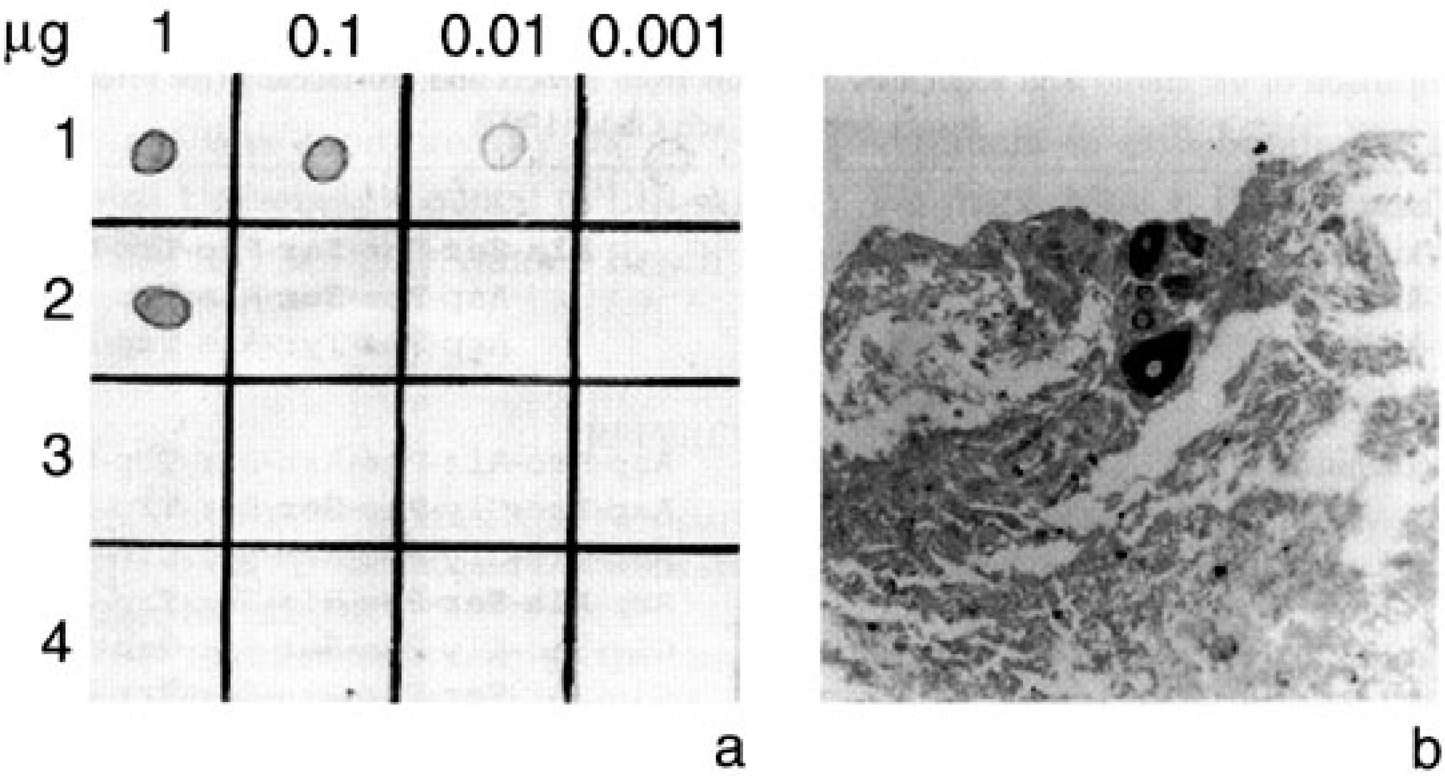

Fig. 2. a, Dot Immuno Assay with the *Penaeus* tachykinin antiserum (row 1, *Peneaus* tachykinin; row 2, locustatachykinin 1; row 3, proctolin; row 4, crustacean cardioactive peptide); b, neurosecretory cells with their processes in the protocerebrum of the *Penaeus vannamei* Boone, 1931 brain contain Pev-tachykinin-like immunoreactivity.

ies, 4 neurosecretory brain cells with their processes were intensively labeled with the antiserum against Pev-tachykinin (fig. 2b). Tachykinin immunoreactivity has also been reported in the crab *Cancer borealis*, where two neurons of the stomatogastric ganglion presented tachykinin-like immunoreactivity, when using Lom-TK antibody (Christie et al., 1993).

The primary structure of the other three identified peptides are novel for crustaceans (table II). Ala-Ser-Phe-Ser-Pro-Trp-Gly-NH_2 (750 Da) and Pro-Ala-Phe-Ser-Pro-Trp-Gly-NH_2 (760 Da) were designated as Pev-kinin 1 and Pev-kinin 3 (*Penaeus vannamei* kinin), since they include the carboxyterminal sequence FSPWGamide, which places them as members of the kinin peptide family, formerly with members only in insects. Kinins have been identified in a number of insects (for review see Gäde, 1997; Meola et al., 1998). All members of the insect kinin peptide family conform to the sequence Phe-(Asn, Ser, His, Phe or Tyr)-(Pro, Ser, or Ala)-Trp-Gly-amide. Therefore, Pev-kinin 1 and 3 are the first members of this family to be isolated from a crustacean. Initial immunological studies have shown that the leucokinin-IV antiserum (gift from J. Veenstra, University of Bordeaux, France) recognizes both Pev-kinins in DIA. On cross-sections of the brain of *P. vannamei*, numerous nerve fibers were labeled using this antiserum, but no cells were visualized as yet. In the future, the whole ventral nerve cord will be screened for kinin-like immunoreactivity. In contrast, Blitz et al. (1995) did not find any leucokinin-like immunoreactivity in the stomatogastric ganglion of *Cancer borealis*.

TABLE II

Comparison of the amino acid sequences of kinins from insects and crustaceans (for references of insect peptides, see Gäde, 1997)

CRUSTACEANS	
Pev-kinin 1	**Ala-Ser-Phe-Ser-Pro-Trp-Gly-NH2**
Pev-kinin 2	Asp-**Phe-Ser**-Ala-**Trp**-Ala-NH2
Pev-kinin 2	Asp-**Phe**-Tyr-Ala-**Trp**-Ala-NH2
INSECTS	
Leucokinins 1-8	Asp-Pro-Ala-**Phe**-Asn-Ser-**Trp-Gly**-NH2
	Asp-Pro-Gly-**Phe-Ser**-Ser-**Trp-Gly**-NH2
	Asp-Gln-Gly-**Phe**-Asn-Ser-**Trp-Gly**-NH2
	Asp-**Ala-Ser-Phe**-His-Ser-**Trp-Gly**-NH2
	Gly-Ser-Gly-**Phe-Ser**-Ser-**Trp-Gly**-NH2
	pGlu-Ser-**Ser-Phe**-His-Ser-**Trp-Gly**-NH2
	Asp-Pro-Ala-**Phe-Ser**-Ser-**Trp-Gly**-NH2
	Gly-**Ala-Ser-Phe**-Tyr-Ser-**Trp-Gly**-NH2
Locustakinin	Ala-**Phe-Ser**-Ser-**Trp-Gly**-NH2
Achetakinins 1-5	Ser- Gly-**Ala**-Asp-**Phe**-Tyr-**Pro-Trp-Gly**-NH2
	Ala-Tyr-**Phe-Ser-Pro-Trp-Gly**-NH2
	Ala-Leu-Pro-**Phe-Ser**-Ser-**Trp-Gly**-NH2
	Asn-Phe-Lys-**Phe**-Asn-**Pro-Trp-Gly**-NH2
	Ala-**Phe**-His-Ser-**Trp-Gly**-NH2
Culekin 1-3	Asn-Pro-**Phe**-His-Ser-**Trp-Gly**-NH2
	Ala-Asn- Ala-Asn-Val-**Phe**-Tyr-**Pro-Trp-Gly**-NH2
	Trp-Lys-Tyr-Val- Ser-Lys-Gln-**Phe**-Phe-Ser-**Trp-Gly**-NH2
Aedes leuckinin 1-3	Asn-Ser-Lys-Tyr-Val-Ser- Lys-Gln-Lys-**Phe**-Tyr-Ser-**Trp-Gly**-NH2
	Asn-Pro-**Phe**-His-Ala-**Trp-Gly**-NH2
	Asn-Asn- Pro-Asn-Val-**Phe**-Tyr-Pro-**Trp-Gly**-NH2
Heliokinin 1-3	Tyr-**Phe-Ser-Pro-Trp-Gly**-NH2
	Val-Arg-**Phe-Ser-Pro-Trp-Gly**-NH2
	Lys-Val-Lys-**Phe-Ser**-Ala-**Trp-Gly-NH2**

The primary structure of the fourth isolated shrimp neuropeptide is Asp-Phe-Ser-Ala-Trp-Ala-NH_2 (693.5 Da). The presence of an Ala at the C-terminal in the position that normally has a Gly is unusual, but the rest of the sequence clearly shows that it can be considered as a kinin. Therefore, it forms a new subfamily of the kinin peptide family and was designated Pev-kinin 2.

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, thoracic ganglia, and the ventral nerve cord) are important sources of neuropeptides. The identification of the three kinins provides evidence that the kinin peptide family, hitherto confined to insects, has a widespread occurrence in Arthropoda.

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