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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-NH_2 (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 antiserum was developed, which labels 4 neurosecretory cells in the brain. Ala-Ser-Phe-Ser-Pro-Trp-Gly-NH_2 (Pev-kinin 1, 749.8 Da) and Asp-Phe-Ser-Ala-Trp-Ala-NH_2 (Pev-kinin 2, 694.7 Da) are the first crustacean kinins. Pev-kinin 2 is the first kinin with a Trp-Ala-NH_2 instead of a kinin-typical Trp-Gly-NH_2 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], orcokinin [8], a family of hyperglycemic hormones and molt-inhibiting hormones [9,10], a few peptides of the RFamide superfamily in *Procambarus clarkii* [11] and two tachykinin-related peptides in *Cancer borealis* [12].

Peptides that influence visceral muscle contractility *in vitro* (designated as myotropins) have recently been shown to be of extreme importance in the neuro-endocrine 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-

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tion. The supernatants were dried and dissolved in 0.1% trifluoracetic acid (TFA). Subsequently they were loaded on MegabondElute cartridges and eluted with 50% CH_3CN/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 Gilson (Gilson 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% 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, 100 Å, 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 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-Sec-S-2000 size exclusion column (300 × 7.8 mm, Phenomenex, Torrance, CA, USA) run in normal phase; solvent A, 95% CH_3CN/0.01% TFA; solvent B, 50% CH_3CN/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) Suplex PKB-100, 4.6 × 250 mm, 5 μm, Supelco Inc. PA); solvents A and B same as in (i),

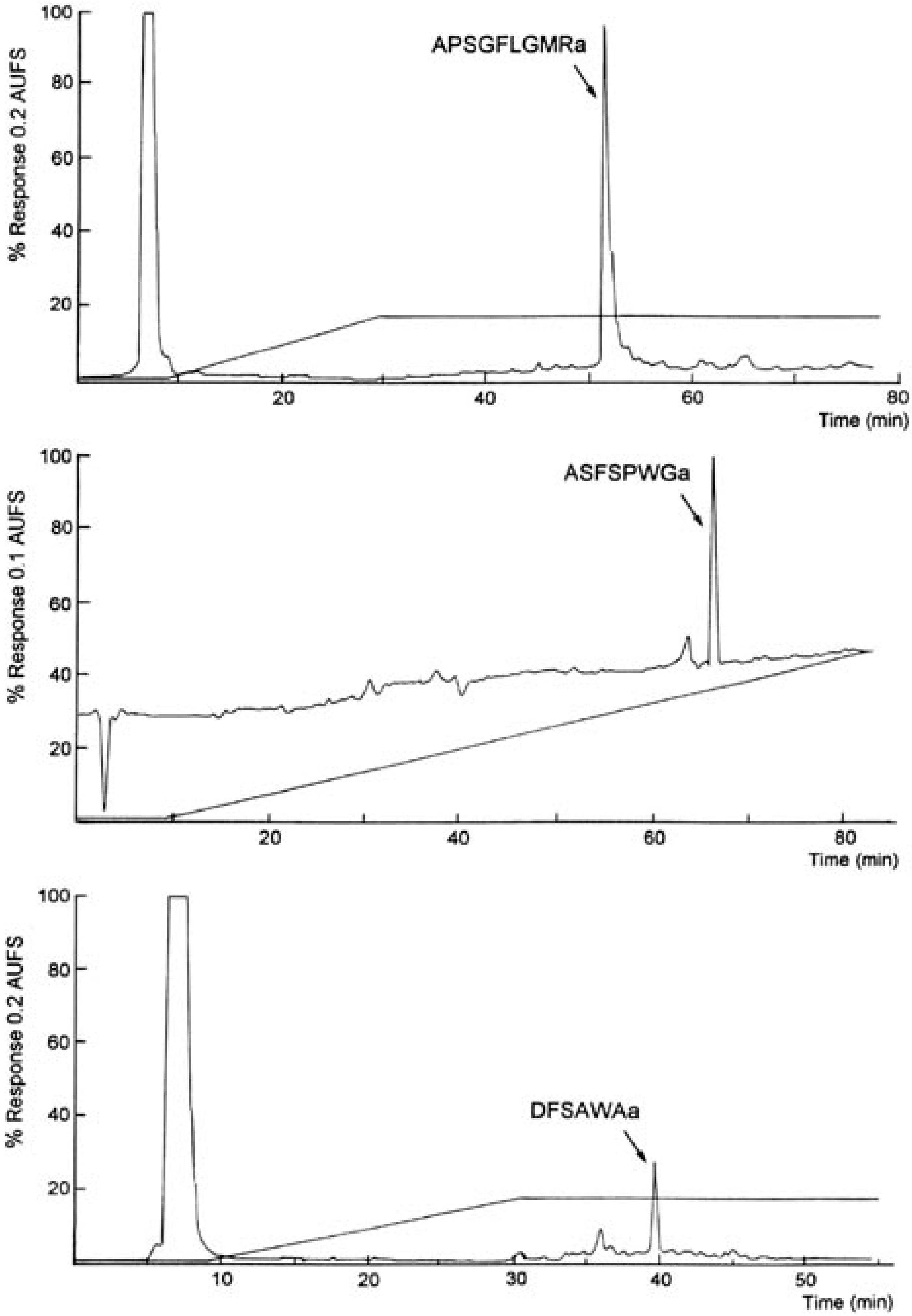

FIG. 1. HPLC chromatograms of the final purification step of Pev-tachykinin and Pev-kinin 1 and 2 (bioactive peak is indicated with arrow). Pev-tachykinin and Pev-kinin were pure after Biosep-Sec-S-2000 chromatography, whereas Pev-kinin 2 was further purified on a Suplex-PKB-100 column.

TABLE 1

Amino Acid Sequence, Molecular Mass, and Elution Characteristics of the Peptides Investigated

Peptide	Column material retention time (% solvent B)					Average mass (Da)		
	Bondapak C18	C2-C18	Diphenyl	Biosep-Sec-S-2000	Suplex PKB-100	Calculated	Observed natural $(M + H)^+$	Observed synthetic $(M + H)^+$
APSGFLGMRa	68–70 (46%)	62–64 (20%)	31–32 (26%)	39 (25%)		934.11	935.5	935.9
ASFSPWGa	68–70 (46%)	62–64 (20%)	39–41 (30%)	41 (25%)		749.8	750.6	751
DFSAWAa	66–68 (45%)	52–54 (18%)	48–50 (20%)	40 (25%)	68 (40%)	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.

100 % A for 10 min, then linear gradient to 50% B over 80 min; 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 normal phase chromatography, 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 the pulsed liquid mode. Mass analysis of Pev-tachykinin and Pev-kinin 1 was performed on a Micromass Tofspec matrix assisted laser desorption time of flight mass spectrometer (MALDI-TOF MS) as described previously [17]. The primary structure of Pev-kinin 2 was confirmed by electrospray tandem mass spectrometry of both the natural and synthetic peptide. The proposed primary structures were synthesized using Fmoc polyamide chemistry (J.W. Drijfhout, Universital Hospital, Leiden, The Netherlands). The synthetic peptides were used for verification of the sequence and/or antibody development.

Bioassay. After each chromatographic separation, the fractions were monitored by a simple and very fast bioassay, the *Leucophaea maderae* hindgut muscle preparation [18]. The potential diuretic effect of the *Penaeus* kinins was tested in a Malpighian tubule secretion assay [19].

Immunochemistry. A polyclonal antiserum was raised by immunizing rabbits with synthetic Ala-Pro-Ser-Gly-Phe-Leu-Gly-Met-Arg-NH_2 coupled to thyroglobulin using EDC (1-ethyl-3-(dimethylaminopropyl carbodiimide hydrochloride) as a cross-linker.

A dot immunobinding assay (DIA) according to Salzet [20] was used to characterize the antiserum. One μl of each of the following synthetic peptides, Pev-tachykinin, Pev-kinins 1 and 2, CCAP, proctolin and locustatachykinins 1-4 was spotted onto a nitrocellulose membrane (0.45 μm pore size) in a dilution series ranging from 1 pg up to 10 μg/dot. Membranes were baked (30 min, 110°C), blocked with skimmed milk in 50mM TBS (Tris buffered saline) to reduce background staining (1h gentle agitation; room temperature), and then incubated overnight at 4°C with the Pev-tachykinin antiserum (diluted 1:2000 in TBS). Membranes were washed several times and incubated with peroxidase-conjugated goat anti-rabbit IgG for 2 h. Following detection by enhanced chemiluminescence (Amersham Int. Rainham, Great Britain) immunoreactive spots were visualised by a short exposure to a blue-light sensitive autoradiography film. As a control, the primary antiserum was incubated with synthetic Pev-tachykinin (10 μg/ml antiserum) before use in DIA.

For immunocytochemistry, brains were dissected and immersed in Bouin Hollande's sublimate (10%) fixative and embedded in Paraplast. Alternating sections were cut at 4 μm and sections were processed using the peroxidase-antiperoxidase method. Specificity was controlled (1) by immunoabsorption of the tachykinin antiserum with synthetic Pev-tachykinin and (2) by the processing of a series of stainings in which the various steps compared to the regular staining sequence were omitted one by one.

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_2 (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 locustatachykinins 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

Penaeus tachykinin
Ala-Pro-Ser-Gly-Phe-Leu-Gly-Met-Arg-NH_2

Cancer borealis tachykinin related peptide 1
Ala-Pro-Ser-Gly-Phe-Leu-Gly-Met-Arg-NH_2

Molluscs

Anodontatachykinin
pGlu-Tyr-Gly-Phe-His-Ala-Val-Arg-NH_2

Annelids

Urechistachykinin 1
Leu-Arg-Gln-Ser-Gln-Phe-Val-Gly-Ser-Arg-NH_2

Insects

Locustatachykinin 1
Gly-Pro-Ser-Gly-Phe-Tyr-Gly-Val-Arg-NH_2

Callitachykinin 1
Ala-Pro-Thr-Ala-Phe-Tyr-Gly-Val-Arg-NH_2

Culetachykinin 1
Ala-Pro-Ser-Gly-Phe-Met-Gly-Met-Arg-NH_2

Leucophaea maderae Tachykinin Related Peptide 1
Ala-Pro-Ser-Gly-Phe-Leu-Gly-Val-Arg-NH_2

Vertebrates

substance P (mammalian)
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2

hylambatin (amphibian)
Asp-Pro-Pro-Asp-Pro-Asp-Arg-Phe-Tyr-Gly-Met-Met-NH

FIG. 2. Comparison of the primary structures of *Penaeus* 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-NH_2 (749.8 Da). It is designated as Pev-kinin (*Penaeus vannamei* kinin) since it includes the carboxyterminal sequence FSPWGamide, which places it as a member of the kinin peptide family, hitherto having only members in insects. Since the discovery of the leucokinins by Holman in 1986 [18], kinins have been identified in a number of insects, including *Acheta domesticus, Locusta migratoria, Culex salinarius, Aedes aegypti, Heli-*

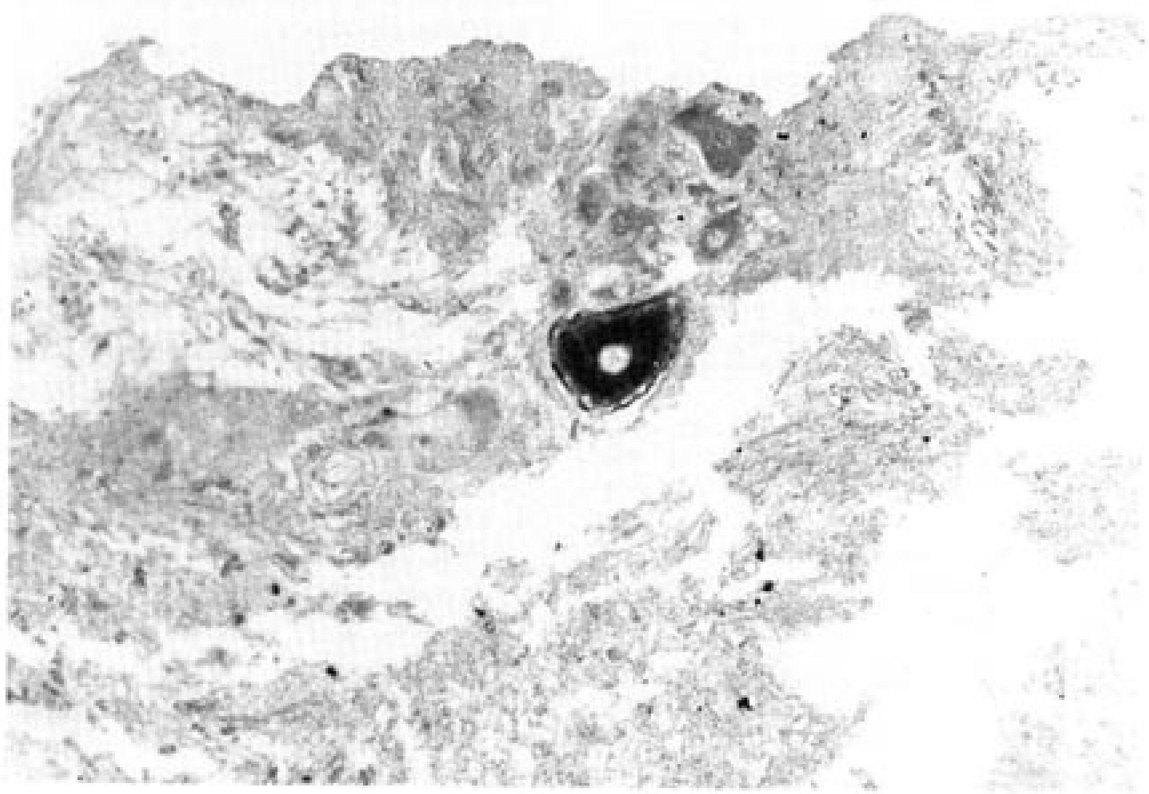

FIG. 3. Neurosecretory cells with their processes in the protocerebrum of the *Penaeus* brain contain Pev-tachykinin-like immunoreactivity.

coverpa zea and *Manduca sexta* [27, review:28]. All members of the insect kinin peptide family conform to the sequence Phe-X_1xx-X_2xx-Trp-Gly-amide, X_1xx being Asn, Ser, His, Phe or Tyr, and X_2xx 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-NH_2 (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 *Leucophaea* 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.

TABLE 2

Diuretic Effect of Pev-kinin 1 and 2

ASFSPWGa	183 ± 34 (n = 4)
DFSAWAa	101 ± 27 (n = 4)
Max (LKI 1 nM)	192 ± 20 (n = 4)
Controls	−16 ± 11 (n = 7)

Note. Results as change in rate of fluid secretion in pl/mm/min (± SE).

Crustaceans	
Pev-kinin 1	Ala-Ser-Phe-Ser-Pro-Trp-Gly-NH_2
Pev-kinin 2	Asp-Phe-Ser-Ala-Trp-Ala-NH_2
Insects	
Leucokinins 1-8	Asp-Pro-Ala-Phe-Asn-Ser-Trp-Gly-NH_2
	Asp-Pro-Gly-Phe-Ser-Ser-Trp-Gly-NH_2
	Asp-Gln-Gly-Phe-Asn-Ser-Trp-Gly-NH_2
	Asp-Ala-Ser-Phe-His-Ser-Trp-Gly-NH_2
	Gly-Ser-Gly-Phe-Ser-Ser-Trp-Gly-NH_2
	pGlu-Ser-Ser-Phe-His-Ser-Trp-Gly-NH_2
	Asp-Pro-Ala-Phe-Ser-Ser-Trp-Gly-NH_2
	Gly-Ala-Ser-Phe-Tyr-Ser-Trp-Gly-NH_2
Locustakinin	Ala-Phe-Ser-Ser-Trp-Gly-NH_2
Achetakinins 1-5	Ser-Gly-Ala-Asp-Phe-Tyr-Pro-Trp-Gly-NH_2
	Ala-Tyr-Phe-Ser-Pro-Trp-Gly-NH_2
	Ala-Leu-Pro-Phe-Ser-Ser-Trp-Gly-NH_2
	Asn-Phe-Lys-Phe-Asn-Pro-Trp-Gly-NH_2
	Ala-Phe-His-Ser-Trp-Gly-NH_2
Culekin 1-3	Asn-Pro-Phe-His-Ser-Trp-Gly-NH_2
	Asn-Asn-Ala-Asn-Val-Phe-Tyr-Pro-Trp-Gly-NH_2
	Trp-Lys-Tyr-Val-Ser-Lys-Gln-Phe-Phe-Ser-Trp-Gly-NH_2
Aedes leuckinin 1-3	Asn-Ser-Lys-Tyr-Val-Ser-Lys-Gln-Lys-Phe-Tyr-Ser-Trp-Gly-NH_2
	Asn-Pro-Phe-His-Ala-Trp-Gly-NH_2
	Asn-Asn-Pro-Asn-Val-Phe-Tyr-Pro-Trp-Gly-NH_2
Heliokinin 1-3	Tyr-Phe-Ser-Pro-Trp-Gly-NH_2
	Val-Arg-Phe-Ser-Pro-Trp-Gly-NH_2
	Lys-Val-Lys-Phe-Ser-Ala-Trp-Gly-NH_2

FIG. 4. Comparison of the primary structures of *Penaeus* kinin 1 and 2 with the insect kinins [18, 26, 27]. Bold amino acids are shared by Pev-kinin 1 and other kinins.

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