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Pyrokinin neuropeptides in a crustacean

Isolation and identification in the white shrimp *Penaeus vannamei*

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Identification of substances able to elicit physiological or behavioural processes that are related to reproduction would greatly contribute to the domestication of commercially important crustaceans that do not reproduce easily in captivity. Crustaceans are thought to release urine signals used for chemical communication involved in courtship behaviour. In contrast to insects, very little is known about the endocrinological processes underlying this phenomenon. Therefore, an extract of 3500 central nervous systems of female white shrimp *Penaeus vannamei* was screened for myotropic activity in order to purify pyrokinin-like peptides that belong to the pyrokinin/PBAN neuropeptide family. Members of this family regulate reproductive processes in insects, including pheromone biosynthesis. Purification of these pyrokinins was achieved by a combination of reversed-phase and normal-phase chromatography. Subsequent characterization by mass spectrometry, Edman degradation and peptide synthesis resulted in the elucidation of two novel peptides. Pev-PK 1 has the primary sequence DFAFSPRL-NH_2 and a second peptide (Pev-PK 2) is characterized as the nonapeptide ADFAFNPRL-NH_2. Pev-PK 1 contains the typical FXPRL-NH_2 (X = G, S, T or V) C-terminal sequence that characterizes members of the versatile pyrokinin/PBAN family. Pev-PK 2 displays an Asn residue at the variable X position of the core pyrokinin sequence. These crustacean pyrokinins are the first to be found in a noninsect. The synthetic peptides display myotropic activity on the *Leucophaea maderae* as well as on the *Astacus leptodactylus* hindgut.

Keywords: Crustacea; neuropeptide; pyrokinin; reproduction.

The pyrokinins are a family of neuropeptides with members in several insect orders. The first, leucopyrokinin, was isolated from the cockroach *Leucophaea maderae* [1]. Subsequently, six pyrokinins were identified in the locust *Locusta migratoria* through their ability to induce *Leucophaea maderae* hindgut contraction [2–6]. The same bioassay was used to isolate two pyrokinins from the locust *Schistocerca gregaria* [7]. Six additional members were isolated from the American cockroach *Periplaneta americana* by monitoring the contractile activity of the hyperneural muscle [8–10]. Pyrokinins are characterized by the C-terminal pentapeptide sequence FXPRL-NH_2 (with X = G, S, T or V) and by the fact that they were isolated for their myotropic activity (Table 1). Pyrokinins are members of the larger pyrokinin/PBAN peptide family that all share this pentapeptide core. Members of this pyrokinin/PBAN family have been found to regulate a variety of physiological and behavioural processes in insects such as myotropic activity of the cockroach and locust hindgut [2,11], contraction of the locust oviduct [2], egg diapause in the silkworm [12], acceleration of pupariation in fleshfly larvae [13], stimulation of sex pheromone biosynthesis in female moths [14–16], and melanization and reddish coloration in moth larvae [17,18]. The peptides show cross-reactivity in these different physiological processes suggesting homologous features of the receptor sites. The pentapeptide FXPRL-NH_2 fragment proved to be the active core required for activity in all these different processes [11,18–21]. Insect pyrokinins were found to be concentrated in and around the corpora allata [10], the neurohaemal organ that synthesizes and secretes juvenile hormone (JH). In most insect species, JHs play a central role in reproduction. In crustaceans, methyl farnesoate (MF) is the major juvenile hormone-like product [22] and seems to be a reproductive hormone [23]. Any factor that is involved in the regulation of MF would be of great importance. It is clear that pyrokinins are implicated in the regulation of critical reproductive processes in arthropods, making them a potential target compound in pest-control research in insects.

So far, members of the pyrokinin/PBAN family have only been identified in insects. The present study on a crustacean (the penaeid shrimp *Penaeus vannamei*) investigates whether this neuropeptide family has a wider distribution in Arthropoda than has been thought. Furthermore, identification of substances potentially capable of hormonally regulating reproductive processes could greatly contribute to the domestication of commercially important species, such as penaeid shrimp, that do not reproduce easily in captivity.

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Abbreviations: AUFS, absorption units full scale; CNS, central nervous system; EtOH, ethanol; JH, juvenile hormone; MALDI-TOF, matrix assisted laser desorption time of flight; MF, methyl farnesoate; MT, myotropin; PBAN, pheromone biosynthesis activating neuropeptide; PK, pyrokinin.

Enzymes: aminopeptidase M (3.4.11.2).

Note: departmental web sites available at http://www.kuleuven.ac.be/bio/fysio and at http://www.cenaim.espol.edu.ec.

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Table 1. Sequence comparison of insect pyrokinins with those of the crustacean *Penaeus vannamei*. Amino acids that are conserved throughout the family are in boldface. pQ indicates a pyroglutamic acid residue.

Class	Species	Peptide name	Sequence	Reference
Insecta	*L. maderae*	Lem-PK	pQTS**FTPRL-NH$_2$**	1
	L. migratoria	Lom-PK I	pQDSGDEWPQQP**F**V**PRL-NH$_2$**	2
		Lom-PK II	pQSVPT**FTPRL-NH$_2$**	3
		Lom-MT I	GAVPAAQW**FS**PRL-NH$_2$**	4
		Lom-MT II	EGD**FTPRL-NH$_2$**	5
		Lom-MT III	RQQP**F**V**PRL-NH$_2$**	6
		Lom-MT IV	RLHQNGMP**FSPRL-NH$_2$**	6
	S. gregaria	Scg-MT I	GAAPAAQ**FSPRL-NH$_2$**	7
		Scg-MT II	TSSLFPH**PRL-NH$_2$**	7
	P. americana	Pea-PK-1	HTAG**F**I**PRL-NH$_2$**	8
		Pea-PK-2	SPP**F**A**PRL-NH$_2$**	8
		Pea-PK-3	LVP**F**R**PRL-NH$_2$**	9
		Pea-PK-4	DHLPHVY**SPRL-NH$_2$**	9
		Pea-PK-5	GGGGSGETSGMW**F**G**PRL-NH$_2$**	9
		Pea-PK-6	SESEVPGMW**F**G**PRL-NH$_2$**	10
Crustacea	*P. vannamei*	Pev-PK 1	DFA**FSPRL-NH$_2$**	This study
		Pev-PK 2	ADFA**F**N**PRL-NH$_2$**	This study

MATERIALS AND METHODS

Animals

Mature female specimens of the white shrimp *Penaeus vannamei* 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 and kept at the indoor maturation and reproduction facilities of the CENAIM (Centro Nacional de Acuicultura e Investigaciones Marinas) research centre until use.

Leucophaea maderae cockroaches were taken from stock colonies maintained at 27 °C and fed dry dog food pellets and water *ad libidum*.

Specimens of the crayfish *Astacus leptodactylus* were purchased from a local seafood dealer (Colette, Belgium). They were maintained in freshwater tanks where the water was recirculating constantly through sand filtration units. The crayfish were kept at room temperature.

Tissue extraction

The central nervous systems (brain, stomatogastric and thoracic ganglion, ventral nerve cord) of 3500 specimens were dissected. Prior to dissection, females were kept in ice-cold seawater for 30 min. The dissected CNSs were placed in ice cold extraction fluid [methanol/water/acetic acid (90 : 9: 1, v/v/v)] and homogenized. The resulting solution was centrifuged at 8000 r.p.m. for 10 min. The pellet was re-extracted and the supernatants were pooled. Methanol was removed using a rotary evaporator. The remaining solution was washed with ethyl acetate and hexane. The supernatants were dried and transported to the Zoological Institute (Leuven, Belgium) for further purification. The extract was resuspended in 0.1% trifluoracetic acid, loaded on Megabond Elute cartridges, and eluted with 50% CH_3CN/0.1% trifluoracetic acid and used in the further purification by HPLC.

Chromatography

A combination of reversed-phase and normal-phase HPLC was carried out to further process the prepurified extract. A Gilson HPLC system with Waters 486 detector, set at 214 nm, was used. The separations of the biologically active compounds were performed on (a) a preparative Bondapak C18 cartridge (Waters Associates); solvent A: 0.1% trifluoracetic acid in water; solvent B: 50% CH_3CN in 0.1% aqueous trifluoracetic acid; conditions: 100% A for 10 min followed by a linear gradient to 100% B in 150 min, flow rate: 6 mL·min^{-1}; detector range: 2.0 absorption units full scale (AUFS). (b) A semipreparative Pep-S C2-C18 column (Pharmacia); solvents A and B as in (a); conditions: a linear gradient from 0% to 25% B in 60 min, then to 50% B in 60 min, flow rate: 1.5 mL·min^{-1}, detector range: 1.0 AUFS. (c) An analytical Vydac Diphenyl column (The Separations Group); solvents A and B as in (a); conditions: a linear gradient starting with 10% to 15% B, depending on the predicted elution position of the peptide, increasing to 40% B in 60 min, flow rate: 1.5 mL·min^{-1}, detector range: 0.5 AUFS. (d) An LC8DB column (Supelco); solvents A and B as in (a); conditions: a linear gradient from 35% to 50% B in 80 min, flow rate 1.5 mL·min^{-1}, detector range 0.2 AUFS. (e) A Biosep-Sec-S-2000 size exclusion column (Phenomenex); solvent A: 95% CH_3CN/0.01% trifluoracetic acid, solvent B: 50% CH_3CN/0.01% trifluoracetic acid; conditions: a linear gradient from 0% B to 50% B in 60 min, flow rate 1 mL·min^{-1}, detector range: 0.1 AUFS. Fractions were collected every 2 min for (a) and (b). Peaks were collected manually for (c) (d) and (e).

Bio-assay

The peptide purifications were monitored by a simple and very fast bioassay, the *Leucophaea maderae* hindgut muscle preparation [24]. The same assay was used for sequence verification of the synthetic peptides. Potential myotropic activity in a crustacean was evaluated in a similar gut assay. The hindgut of the crayfish *Astacus leptodactylus* was dissected, and the bioassay was set up in a similar way as for the *Leucophaea* hindgut assay. The chamber in which the hindgut was mounted was filled with crayfish saline [25].

Enzymatic degradation and deblocking

Since Edman degradation based amino-acid sequencing of peptides with a blocked N-terminus is not possible, an aliquot

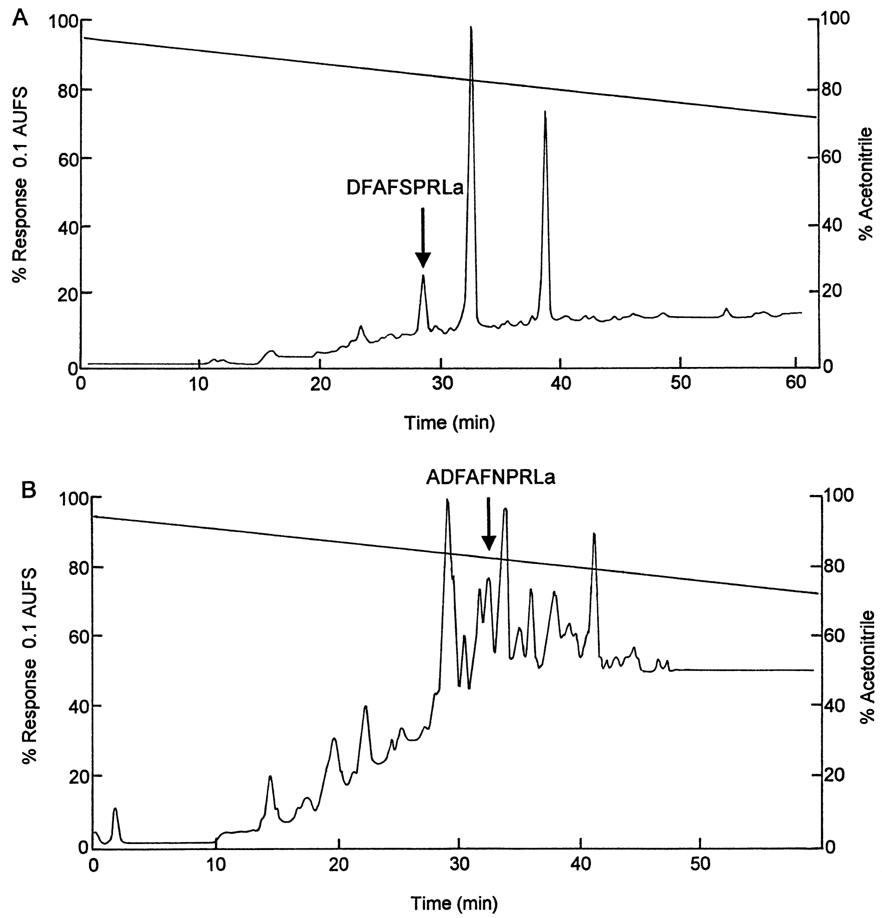

Fig. 1. HPLC chromatograms of the final (fifth) purification steps of pyrokinins from *Penaeus vannamei*. Both runs were performed on a Biosep-Sec-S-2000 size exclusion column (300 × 7.8 mm, 5 μm, 145 Å) run in normal phase. Peaks were eluted with a decreasing CH_3CN/0.01% trifluoracetic acid gradient at a flow rate of 1 mL·min^{-1}. Arrows indicate bioactive fractions. (A) Chromatogram of the final purification step of Pev-PK 1. The myoactive material that was loaded eluted from the LC8DB (fourth) column at 34 min (20.7% CH_3CN). The manually collected fraction eluting at 29 min (84.1% CH_3CN) is Pev-PK 1. (B) Chromatogram of the final purification step of Pev-PK 2. The myoactive material that was loaded eluted from the LC8DB column at 39 min (21% CH_3CN). The manually collected fraction eluting at 33 min (82.6% CH_3CN) is Pev-PK 2.

of each purified peptide sample was digested in 0.5 mL cockroach saline, containing 0.1 unit of aminopeptidase M (Pierce). Following incubation for 2 h at 37 °C, the gel was removed from the suspension by centrifugation, and the supernatant was removed for bioassay.

Structure determination

About one-tenth of the active peak was concentrated by rotary evaporation and subjected to mass analysis on a Micromass Tofspec matrix assisted laser desorption time of flight mass spectrometer (MALDI-TOF MS). One μL was mixed with 1 μL matrix solution [α-cyano-4-hydroxycinnamic acid in CH_3CN/EtOH/trifluoracetic acid (60 : 39.9 : 0.1)], applied on the multi sample target and allowed to dry. The samples were measured either in the linear (acceleration voltage 24 kV) or in the reflectron mode (acceleration voltage 24 kV, reflectron voltage 28.5 V). The laser energy was reduced until an optimal resolution and signal-to-noise ratio was obtained. The result of 10–20 shots was averaged to obtain the final spectrum.

N-Terminal amino-acid sequence analysis was carried out on a PerkinElmer/Applied Biosystems Procise 492 microsequencer running in the pulsed liquid mode.

The obtained primary structures were synthesized using the Fmoc/*t*-Bu strategy on a 2-chlorotrityl resin in which the Rink-linker was esterified. The synthetic peptides were used for verification of the sequence.

Table 2. Amino acid sequence, elution characteristics (i.e. elution time with corresponding percentage CH_3CN between parentheses), and molecular mass of the peptides investigated. The average molecular mass was calculated with the addition of a hydrogen and an amide group for the N and C termini, respectively.

Peptide		Column material retention time (min) and corresponding percentage CH_3CN (%)					Average mass (Da)	
Name	Sequence	Bondapak	C2–C18	Di-phenyl	LC8DB	Biosep Sec- S-2000	Calc.	Obs. natural $(M + H)^+$
Pev-PK 1	DFAFSPRL-NH_2	66–68 (22–23)	56–60 (12–12.5)	26 (13)	34 (20.7)	29 (84.1)	951.1	952.6
Pev-PK 2	ADFAFNPRL-NH_2	66–68 (22–23)	60–65 (12.5–13.5)	30–36 (14–15)	39 (21)	33 (82.6)	1049.2	1051.0

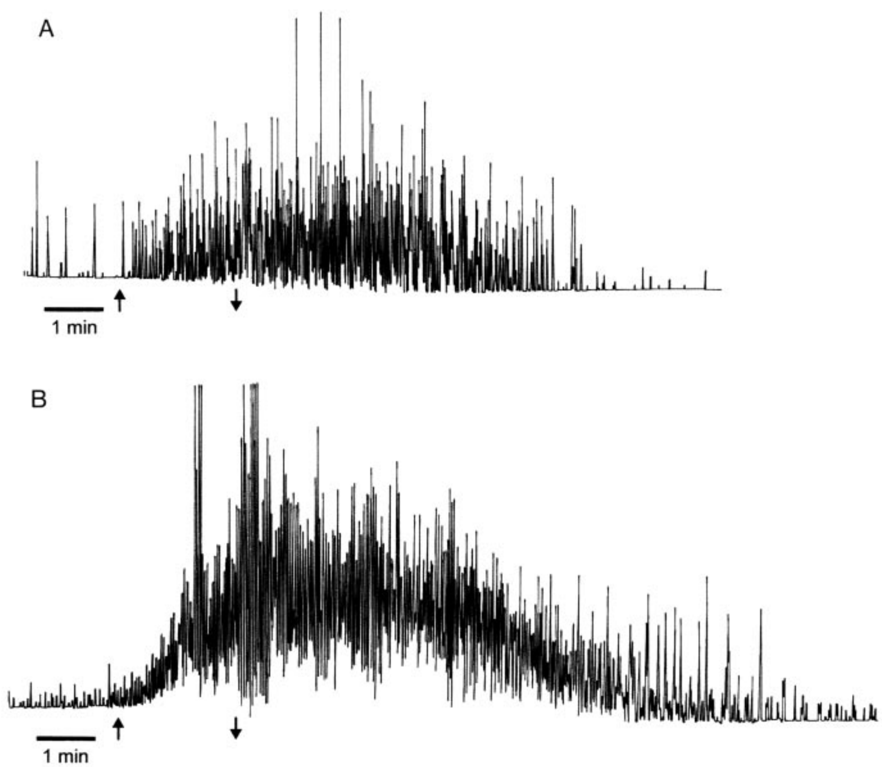

Fig. 2. Representative records of the hindgut-stimulating activities of Pev-PK 1 from *Penaeus vannamei* on the *Astacus* hindgut. Upward arrows indicate application of Pev-PK 1 and downward arrows indicate start of washout with saline. Contractions were measured using an isotonic transducer. The signal from the transducer was displayed on an oscillograph recorder. (A) Hindgut-stimulating activity of Pev-PK 1 at 3.5×10^{-9} M (threshold concentration). (B) Hindgut-stimulating activity of Pev-PK 1 at 10 times threshold concentration (3.5×10^{-8} M).

RESULTS

We report here the isolation of two neuropeptides from the central nervous system of the white shrimp *Penaeus vannamei* (Fig. 1). Table 2 shows their retention times in the five HPLC steps that were used. Aliquots of the purified substances were incubated with aminopeptidase M. After incubation, a loss of bioactivity was observed, which indicates that the peptides were not blocked at the N-terminus. They were identified by MALDI-TOF mass spectrometry and by sequence analysis through automated Edman degradation.

The first component was characterized as an octapeptide with the sequence DFAFSPRL-NH_2 and a mass of 951.6 Da. It is designated as Pev-PK 1 (*Penaeus vannamei* pyrokinin 1) because it contains the typical C-terminal FXPRL-NH_2 (X being S) that characterizes members of the pyrokinin/PBAN family. The second component was sequenced as ADFAFNPRL-NH_2 (1050 Da). Because of its C-terminal sequence similarity with the pyrokinin/PBAN family it was designated as Pev-PK 2 (*Penaeus vannamei* pyrokinin 2). The final yields of Pev-PK 1 and Pev-PK 2, based on the sequencing data, were approximately 180 and 220 pmol, respectively.

Both peptides were synthesized as an amide and coeluted with the native myotropic fractions under the HPLC conditions of the last purification step. Both synthetic peptides are potent stimulators of the spontaneous contractions of the hindgut of *Leucophaea maderae*: Pev-PK 1 with threshold concentrations of 1.4×10^{-8} M and Pev-PK 2 with threshold concentrations of 2.2×10^{-8} M. At these minimal concentrations, an increase in amplitude and frequency of the spontaneous contractions was observed. At higher concentrations, these inotropic and chronotropic effects were accompanied by a strong tonic response. Because living penaeid shrimp were not available in Belgium, the synthetic peptides were tested for myotropic activity on the hindgut of the crustacean *Astacus leptodactylus*. Both Pev-PK 1 and Pev-PK 2 displayed stimulating activity with threshold concentrations of 3.5×10^{-9} and 9.5×10^{-9} M, respectively. At threshold concentrations pyrokinins were able to produce an increase in amplitude and frequency of the spontaneous contractions (Fig. 2A). Higher concentrations elicited a tonic effect in addition to the inotropic and chronotropic effects (Fig. 2B).

DISCUSSION

We have successfully identified two peptides containing the C-terminal sequence FXPRL-NH_2 in the central nervous system of the penaeid shrimp *P. vannamei*. These neuropeptides are the first of the versatile pyrokinin/PBAN family isolated from Crustacea, all previous studies having been related to Insecta.

An amide residue at the C-terminus blocks the two isolated neuropeptides, which could be expected because the amidated C-terminus is required for bioactivity [26,27].

Pev-PK 1 complies with the common pyrokinin C-terminal sequence FXPRL-NH_2 (X = V, T, S or G). It has the same typical C-terminal FSPRL-NH_2 sequence as all members of the insect PBAN family, as well as that of the locust pyrokinins Lom-MT I, Lom-MT IV and Scg-MT I. This core FSPRL-NH_2 sequence is all that is needed for myotropic and pheromonotropic [6,11] activity in insects. The eight amino-acids long Pev-PK 1 is smaller compared to the PBAN and locust pyrokinin peptides, consisting, respectively, of 32–33 and 12–13 amino acids.

Pev-PK 2 differs from Pev-PK 1 at the N-terminus, where it has an extra alanine residue. At the variable X position of the pyrokinin active core, Pev-PK 2 has an asparagine residue instead of the typical threonine, valine, glycine or serine residue. In both *Leucophaea* and *Astacus* hindgut assays, Pev-PK 2 displays similar threshold concentrations to

Pev-PK 1, indicating that this variable amino acid can be replaced by an asparagine residue without substantial loss of activity. Experiments with insect pyrokinin analogues showed that the amino acid occupying the variable X position of the pentapeptide demonstrates the most tolerance to alanine substitution in a pupariation accelerating assay [28].

Previously reported peptides isolated from *Penaeus vannamei* showed a strong difference in stimulating potency between the *Leucophaea* and *Astacus* assays [29]. Pev-kinins 1 and 2, and Pev-tachykinin are potent stimulators of cockroach hindgut contractions, whereas only Pev-kinin 2 proves to be active at physiological concentrations in the crayfish hindgut assay. However, both pyrokinins prove to be active at physiological concentrations in both insect and crustacean hindgut assays, displaying threshold concentrations at nanomolar range. Pyrokinins are more potent on the *Astacus* hindgut than on the *Leucophaea* hindgut.

In crustaceans, urine-borne pheromones play an important role in sexual behaviour. Male individuals are thought to release sex pheromones that stimulate ovarian development [30]. Although previous studies suggest that sex pheromones produced by females are important in courtship [31], no female sex pheromones have been chemically identified to date. In contrast with insects, little is known about the endocrinological processes underlying these phenomena in crustaceans. The isolation and identification of Pev-PK 1 and Pev-PK 2, the first crustacean members of the pyrokinin/PBAN neuropeptide family, may give a great impetus towards aquaculture in general and crustacean sex-pheromone research in particular.

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