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Rapid communication

Orcokinins in insects and other invertebrates

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Abstract

Orcokinin (NFDEIDRSGFGFN) and orcokinin homologues are crustacean peptides eliciting potent myotropic effects in gut tissues. Through HPLC purification of brain extract of the cockroach *Blattella germanica*, we isolated the first insect orcokinin (NFDEIDRSGFNS). This insect orcokinin-like peptide do not show myotropic properties in *B. germanica* gut tissues. Gene database search using orcokinin precursor sequences of the crustacean *Procambarus clarkii* led to putative homologues found in non-crustacean groups, including the mosquito *Anopheles gambiae* and the nematode *Caenorhabditis elegans*.

Keywords: Orcokinin; Orcomyotropin; Insects; Crustaceans; *Blattella germanica*; *Anopheles gambiae*; *Caenorhabditis elegans*

1. Introduction

Till now, orcokinins have been considered a family of crustacean neuropeptides. The first orcokinin reported was NFDEIDRSGFGFN, isolated from abdominal nerve cord extracts of the crayfish *Orconectes limosus* (Stangier et al., 1992). Since then, several orcokinin homologues have been identified in hindgut extracts of *O. limosus* (Burdzik et al., 1993), in the thoracic ganglia of *Carcinus maenas* (Bungart et al., 1995), in the olfactory lobe of *Procambarus clarkii* (Yasuda-Kamatani and Yasuda, 2000), in the pericardial organs and within the stomatogastric nervous system of *Cherax destructor* (Skiebe et al., 2002) and in brain-thoracic ganglion extracts of *Cancer borealis* (Huybrechts et al., 2003). Functionally related to orcokinins are the group of orcomyotropins, the first member of which (FDAFTTGF-NH_2) was isolated from hindgut extracts of *O. limosus* (Dircksen et al., 2000).

In molecular characterization, cloning of the cDNA precursor protein in *P. clarkii* (Yasuda-Kamatani and Yasuda, 2000) revealed two genes coding for two very similar pre-pro-orcokinins. Both genes contain copies of four orcokinins and a copy of an orcomyotropin, the peptides having a structure similar to those isolated from *O. limosus* (Dircksen et al., 2000).

The first study on *O. limosus* orcokinins showed that these peptides are potent hindgut myostimulating factors (Stangier et al., 1992). Further studies demonstrated the same biological properties in other orcokinin homologues. For example, Dirksen et al. (2000) described the myotropic activity of orcokinin, [Val^{13}]orcokinin, and orcomyotropin in *O. limosus*, and found that orcomyotropin is more potent than the two orcokinins. Application of exogenous [Ala^{13}] orcokinin to the stomatogastric ganglion of *Homarus americanus* decreased the pyloric rhythm (Li et al., 2002).

Immunocytochemical studies on *H. americanus* and *Astacus astacus* using an antibody against [Asn^{13}]-orcokinin, indicated that orcokinins occur in the nervous system as well as in the haemolymph, suggesting that they act as neurohormones (Bungart et al., 1994). Other observations using the same antibody

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revealed orcokinin-like immunoreactivity in identified neurons of the stomatogastric nervous system, which modulate stomatogastric motor pattern, and in the pericardial organ in *Ch. destructor* (Skiebe et al., 2002), *H. americanus, C. borealis* and *Panulirus interruptus* (Li et al., 2002).

In the present study, we report the purification and structural characterization of the first orcokinin-like peptide found in an insect. It was isolated from brain extracts of the cockroach *Blattella germanica*. In addition, database search found a number of proteins homologous to the orcokinin precursor of *P. clarkii*. These proteins were found in the mosquito *Anopheles gambiae* and in a number of nematodes, including *Caenorhabditis elegans*.

2. Materials and methods

2.1. Insect rearing and tissue collection

Adult females of *B. germanica* were obtained from a colony fed on dog chow (Panlab, Spain) and water, and reared in the dark at 30 ± 1 °C and 60–70% r.h. Brains from 3- to 4-day-old adult virgin females were freed of optic lobes and adhering fat body tissue, and homogenized and sonicated in PBS (0.2 M). Homogenates were immersed in boiling water for 6 min and centrifuged at $10{,}000 \times g$ for 10 min at 4 °C. Then the supernatant was collected. Pellets were resuspended in PBS (0.2 M), homogenized and centrifuged as above. After centrifugation, the two supernatants were pooled and stored at −20 °C until use.

2.2. Peptide purification by HPLC

The orcokinin-like peptide was isolated in the context of a purification of tachykinin peptides from *B. germanica* brain extracts. For this purpose, 1500 brains from 3- to 4-day-old adult females were extracted as described above and purified in 5 consecutive HPLC steps. Steps 1, 2 and 3 were carried out with a Merck–Hitachi (Darmstadt) low-pressure chromatograph with L-6200A pump and L-4200 UV-VIS detector. Steps 4 and 5 were carried out with a Waters (Milford, MA) low-pressure chromatograph with 626 pump, 600S controller and 996 PDA detector. For the first step, the column used was a Waters DeltaPak C_{18}, 300×7.8 mm, 300 Å, 15 μm, and the chromatographic conditions were CH_3CN/0.1% TFA, 1.67%/min, 1.5 ml/min. Fractions were collected every minute and tested in ELISA by means of a polyclonal antiserum raised in rabbits against the insect tachykinin APSGFLGVR-NH_2 (N. Pascual, unpublished). Fractions 26–27 (21.71–23.38% CH_3CN) were separated in a second chromatographic step (column: Waters DeltaPak C_{18}, 300×7.8 mm, 300 Å, 15 μm; conditions: CH_3CN/0.1% TFA, 0.5%/min, 1.5 ml/min). Fractions were collected every minute and tested in the same ELISA. The immunoreactive fractions 37–39 (21.00–22.00% CH_3CN) were pooled and fractionated in a third chromatographic step (column: Merck LiChroCART C_{18}, 125×4 mm, 100 Å, 5 μm; conditions: CH_3CN/10 nM NH_4OAc, 1.67%/min, 1.5 ml/min). Fractions were collected every minute and tested in the same ELISA. Immunoreactive fractions 28–30 (18.75–19.25% CH_3CN) were pooled and led to the fourth chromatographic step (column: Waters DeltaPak C_{18}, 150×2 mm, 300 Å, 5 μm, conditions: CH_3CN/0.1% TFA, 0.25%/min, 0.2 ml/min). In this separation, ELISA tests were performed on isolated peaks. The immunoreactive peak that eluted at 18.05% CH_3CN led to the fifth and last separation (column: Waters DeltaPak C_{18}, 150×2 mm, 300 Å, 5 μm, conditions: CH_3CN/ 0.1% TFA, 0.2%/min, 0.2 ml/min), from which two main peaks, corresponding to pure compounds, were isolated.

2.3. Mass spectrometry and sequencing

One of the two peaks isolated in the last step of HPLC purification was tachykinin-immunoreactive. Its analysis will be reported elsewhere. The other peak, eluting at 16.8% CH_3CN and not showing tachykinin immunoreactivity, was analyzed with an Applied Biosystems (Foster City, CA) Voyager DE-STR matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer. In addition, the amino acid sequence of the purified peptide for this peak was determined by Edman degradation in an Applied Biosystems Procise cLC sequencer.

2.4. Synthetic peptides

Crustacean orcokinin (NFDEIDRSGFGFN) and insect proctolin (RYLPT) were purchased from Bachem AG (Bubendorf, Switzerland). The peptide NFDEIDRSGFNS, corresponding to the sequence determined from the *B. germanica* extracts, was synthesized by Fmoc solid phase methods, purified to near-homogeneity (>95%) by HPLC and characterized by amino acid analysis and MALDI-TOF mass spectrometry.

2.5. Myotropic assay

The synthetic peptides described above were tested on the hindgut and foregut of *B. germanica* females prepared in a standard organ bath, as described elsewhere (Maestro et al., 2001). An FSG-01 transducer (Experimetria Ltd., Budapest, Hungary) was used for isometric recording. The activity was calculated as the

difference of the mean of the force produced by the tissue in the minute after and before the treatment. Proctolin (at 10^{-9} M) was used as a positive control in each experiment.

2.6. Sequence comparison and analysis

Blast searches (Altschul et al., 1990) were carried out using the protein sequence of the longest form of the pre-pro-orcokinins of *P. clarkii* with default parameters. Protein non-redundant database using blastP, and EST database using tblastn were preferentially used. Nucleotide or amino acid alignments were performed with CLUSTAL W (Thompson et al., 1994).

3. Results and discussion

3.1. Identification of an orcokinin-like peptide in the German cockroach

Starting from an extract of 1500 brains from 3- to 4-day-old adult females of *B. germanica*, and after five consecutive HPLC purification steps (described in Material and methods), two peaks corresponding to pure compounds were obtained. The biggest peak, eluting at 16.8% CH_3CN, was analyzed by tandem MS/MS and gave a mass of 1399.6342 Da and a sequence compatible with NFDEL/IDRSGFNS. Due to the ambiguity of the MS assignation of the fifth position (Leu or Ile), the sequence was also determined by Edman degradation analysis, which identified Ile as the residue at the fifth position and confirmed the other residues.

Database search indicated that this peptide was very similar to orcokinin (NFDEIDRSGFGFN), which had been first isolated from the crustacean *O. limosus* (Stangier et al., 1992) as a myoactive peptide acting on gut tissues. Similar peptides have been further described in the same species (Dircksen et al., 2000) and in other crustaceans, such as *C. maenas* (Bungart et al., 1995), *P. clarkii* (Yasuda-Kamatani and Yasuda, 2000), *C. destructor* (Skiebe et al., 2002) and *C. borealis* (Huybrechts et al., 2003) (Table 1). In addition to orcokinins, two related but shorter peptides (FDAFTTGF-NH_2 and FDAFTTGFGHS) called orcomyotropins, have been described in most of these species (Table 1).

Table 1
Orcokinin and orcomyotropin peptides isolated from crustacean species, compared with the sequence isolated in the cockroach *Blattella germanica*

Species	Peptide name	Sequence	Reference
Blattella germanica	NFDEIDRSGFNS	*NFDEIDRSGF*NS	This paper
Orconectes limosus	Orcokinin	*NFDEIDRSGFGFN*	Stangier et al. (1992)
	[Val^{13}]–Orcokinin	*NFDEIDRSGFGF*V	Dircksen et al. (2000)
	Orcomyotropin-I	*FD*AFTT*GF*–NH_2	Dircksen et al. (2000)
Carcinus maenas	[Ser^{9}]–Orcokinin	*NFDEIDRS*S*FGFN*	Bungart et al. (1995)
	[Ala^{13}]–Orcokinin	*NFDEIDRSGFGF*A	Bungart et al. (1995)
	[Val^{13}]–Orcokinin	*NFDEIDRSGFGF*V	Bungart et al. (1995)
Cherax destructor	Orcokinin	*NFDEIDRSGFGFN*	Skiebe et al. (2002)
	[Val^{13}]–Orcokinin	*NFDEIDRSGFGF*V	Skiebe et al. (2002)
	[Ala^{8}–Ala^{13}]–Orcokinin	*NFDEIDR*A*GFGF*A	Skiebe et al. (2002)
	[Thr^{8}–His^{13}]–Orcokinin	*NFDEIDR*T*GFGF*H	Skiebe et al. (2002)
	Orcomyotropin-II	*FD*AFTT*GFG*HS	Skiebe et al. (2002)
Cancer borealis	[Ala^{13}]–Orcokinin	*NFDEIDRSGFGF*A	Huybrechts et al. (2003)
	[Val^{13}]–Orcokinin	*NFDEIDRSGFGF*V	Huybrechts et al. (2003)
	[Ser^{9}–Val13]–Orcokinin	*NFDEIDRS*S*FGF*V	Huybrechts et al. (2003)
	NFDEIDRSGFA	*NFDEIDRSGF*A	Huybrechts et al. (2003)
	Orcomyotropin-II	*FD*AFTT*GFG*HS	Huybrechts et al. (2003)
Procambarus clarkii	Orcokinin	*NFDEIDRSGFGFN*	Yasuda-Kamatani and Yasuda (2000)
	[Val^{13}]–Orcokinin	*NFDEIDRSGFGF*V	Yasuda-Kamatani and Yasuda (2000)
	[Ala^{13}]–Orcokinin	*NFDEIDRSGFGF*A	Yasuda-Kamatani and Yasuda (2000)
	[Thr^{8}–His^{13}]–Orcokinin	*NFDEIDR*T*GFGF*H	Yasuda-Kamatani and Yasuda (2000)
	Orcomyotropin-II	*FD*AFTT*GFG*HS	Yasuda-Kamatani and Yasuda (2000)

The residues conserved with respect to orcokinin have been italicised.

3.2. Biological effects of orcokinin peptides on cockroaches

Given the reported myotropic activity of orcokinins in the crustacean gut, we tested the *B. germanica* peptide (NFDEIDRSGFNS) and crustacean orcokinin (NFDEIDRSGFGFN) on isolated foreguts and hindguts of *B. germanica*. Results showed that neither of these two peptides elicited a significant myotropic effect at concentrations of 10^{-8}, 10^{-7} and 10^{-6} M. Proctolin, tested in the same system as a positive control, did elicit the expected myotropic effect, with ED_{50} values of 1.38 nM (foregut) and 1.16 nM (hindgut).

Previous experiments (Dircksen et al., 2000) had shown that crustacean orcokinin does not induce myotropic effects on hindgut and oviduct of the insect *Locusta migratoria*, which is consistent with the present results. Immunocytochemical studies in the cockroach *Leucophaea maderae* were carried out by Hofer et al. (2003) using an antibody against [Asn^{13}]-orcokinin. Results revealed a series of immunoreactive cells within the accessory medulla, which is considered the site of the circadian clock within the brain. In addition, microinjections of [Asn^{13}]-orcokinin into the medulla of this cockroach elicited phase delays at the end of the subjective day, which were similar to phase delays caused by light pulses applied at this time (Hofer et al., 2003). This suggests that the peptide mediated the transmission of light and stimulated the neurons of the accessory medulla involved in the circadian clock.

3.3. Putative mosquito homologues of the crustacean orcokinin gene

In the crustacean *P. clarkii*, the nucleotide sequence of two orcokinin precursors, pre-pro-orcokinin A and B, have been reported (accession numbers Q9NL82 and Q9NL83, respectively) (Yasuda-Kamatani and Yasuda, 2000). According to the deduced amino acid sequences, pre-pro-orcokinin A contains seven copies of orcokinin, a copy of [Ala^{13}]-orcokinin, two copies of [Val^{13}]-orcokinin, a copy of [Thr^{8}–His^{13}]-orcokinin and a copy of orcomyotropin (Fig. 1). All sequences of these peptides are flanked by cleavage dibasic motifs KK or KR. The organization of pre-pro-orcokinin B is similar, but contains an additional copy of orcokinin (Fig. 1).

Blast searches on GenBank using the protein sequences of pre-pro-orcokinin A as a query encountered several homologues in other taxa. The mosquito *A. gambiae* contains two sequences (accession numbers XP-320317 and XP-306509) with two orcokinin-like units (NFDEIDRFARFNG, NFDEIDRFNG), each flanked by putative cleavage motifs KR, KK or K alone. Although the hits of these sequences are not the most significant, the two peptides are very similar to crustacean orcokinins, showing the typical N-terminal motif NFDEIDR. These *A. gambiae* sequences were from the annotations provided by the Anopheles Genome Sequencing Consortium and lacked the initial Met. Therefore, we completed the DNA and putative amino acid sequences using the flanking genome sequence extracted from the ENSEMBL database (Clamp et al., 2003). No other insect sequences similar

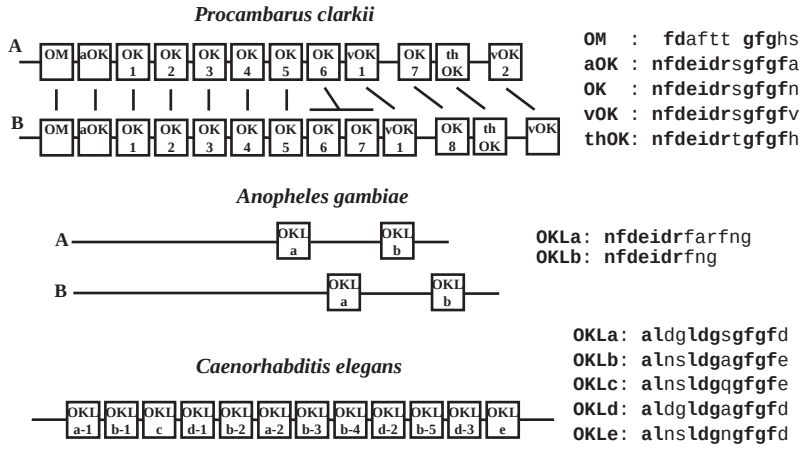

Fig. 1. Schematic representation of the gene organization of orcokinin precursors A and B of *Procambarus clarkii* (accession numbers Q9NL82 and Q9NL83, respectively), genes A and B of *Anopheles gambiae* (accession numbers XP_320317 and XP_306509, respectively), and gene NP_509508 of *Caenorhabditis elegans*. In *Procambarus clarkii*, the peptide units are indicated by the following codes: OM (orcomyotropin), aOK ([Ala^{13}]-orcokinin), OK (orcokinin), vOK ([Val^{13}]-orcokinin) and thOK ([Thr^{8}-His^{13}]-orcokinin). In *A. gambiae* and *C. elegans* the different orcokinin like (OKL) peptide units have been coded by letters. Repeated units of the same peptide in the same sequence have been numbered. Homologous peptide units in genes A and B of *Procambarus clarkii* are indicated by lines.

to *P. clarkii* orcokinin precursor appeared in GenBank. An equivalent search carried out on the genome of *Drosophila melanogaster* gave only one sequence (data base under accession number CG5787-PA) with limited similarity and without the typical NFDEIDR motif of *B. germanica* and *A. gambiae*, which suggests that the fruit fly does not possess orcokinin homologues.

An initial DNA alignment of the two pre-pro-orcokinins of *P. clarkii* was carried out to avoid the ambiguities caused by the identical peptides present. This showed that the extra peptide in orcokinin precursor B is the orcokinin copy 6 or 7, which are identical in DNA terms with each other and with the orcokinin copy 6 of precursor A (see Fig. 1 and below). Accordingly, the corresponding gaps were introduced into the amino acid sequence. This profile was then aligned with the *A. gambiae* sequences and manually adjusted. The final alignment (Fig. 2) shows a clearly significant similarity in the regions corresponding to the orcokinin peptides (Fig. 1). The alignment also shows that the similarity is greater within the two precursors of the same species than between species, which indicates that the gene duplication took place independently in Crustacea and Insecta after the splitting of these two arthropod classes. In addition, the great similarity in DNA between peptide units in A and B genes of *P. clarkii* (only 1 nucleotide substitution in 756 bases; 0.13% difference) and in A and B genes of *A. gambiae* (1.5% difference) indicates that both gene duplications are extremely recent.

3.4. Sequences similar to orcokinin precursor in other invertebrates

Blast searches on GenBank also found three sequences of *C. elegans* that had significant similarities with *P. clarkii* pre-pro-orcokinins (accession numbers, NP_509508, NP_493278 and NP_492158) (Nathoo et al., 2001). The alignment of the *P. clarkii* pre-pro-orcokinin A with the *C. elegans* sequence having the highest significant hit (NP_509508) (Fig. 3) shows that this similarity extends throughout all the sequences. Although the peptides of the *C. elegans* protein show differences from those of *P. clarkii* protein, the number of peptides and their organization within the precursor protein is similar in both species (Fig. 1). The similarity in the peptide units (in particular the motif GFGF) suggests that at least some of them are true homologues. It should be noted, however, that several peptides of *P. clarkii* are very similar to each other. For example, OK2, OK3, OK4, OK5, OK6, OK7 and OK8 form a very tight cluster in a phylogenetic tree (not shown), which indicates that duplication of them is recent. In turn, this suggests that many of the peptide units originated after the separation of the species analyzed here, and that the alignment of *P. clarkii* and *C. elegans* (Fig. 3) may not reflect common descent in the whole length of the protein because it is not sure whether the common ancestor protein contained all the present repeats. The same uncertainty would apply to the alignment of *P. clarkii* and *A. gambiae* (Fig. 2).Though there are other multi-peptide sequences that show significant hits in Blast searches (AAP57098 and AAB27696 in the mollusks *Lymnaea stagnalis* and *Aplysia californica*, respectively, and NP_704535 in the haemosporid *Plasmodium falciparum*), the similarity of the peptide units with crustacean orcokinins is small. Thus, it is difficult to ascertain whether these and the crustacean sequences are homologous.

Blast searches in the EST databases provided more sequences similar to orcokinins. The most significant

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P. clarkii A   MTAQMFTIALLLSLSAIAAAGTIKTAPARTPSTQDDASFPPDGAPVKRFD 50
P. clarkii B   MTAQMFTIALLLSLSAIAAAGTIKTAPARTPSTQDDASFPPDGAPVKRFD 50
A. gambiae A   ----MVKLHLVCGVLLFAFLGWAHGLPVKEQELEEVLTK------LQELK 40
A. gambiae B   ----MVKLHLVCGVLLFAFLGWAHGLPVKEQELEEVLTK------LQELK 40

P. clarkii A   AFTTGFGHSKRNFDEIDRSGFGFAKKNFDEIDRSGFGFNKRNFDEIDRSG 100
P. clarkii B   AFTTGFGHSKRNFDEIDRSGFGFAKKNFDEIDRSGFGFNKRNFDEIDRSG 100
A. gambiae A   RLNDGQ---LKQVNEEKLQQLGGESTNGQRTAGGASSAAAGGIAGAASSG 87
A. gambiae B   RLNDGQ---LKQVNEEKLQQLGGASTNGQRTAGGASSAAAGGIAGAASSG 87

P. clarkii A   FGFNKRNFDEIDRSGFGFNKRNFDEIDRSGFGFNKRNFDEIDRSGFGFNK 150
P. clarkii B   FGFNKRNFDEIDRSGFGFNKRNFDEIDRSGFGFNKRNFDEIDRSGFGFNK 150
A. gambiae A   FKT-KRSLNNSDRRTLLYN---YYSRGLAPM-YDKRNFDEIDRFAR-FNG 131
A. gambiae B   FKT-KRSLNNSDRRTLLYN---YYSRGLAPM-YDKRNFDEIDRFAR-FNG 131

P. clarkii A   ---------------RNFDEIDRSGFGFNKRNFDEIDRSGFGFVKRVWVP 185
P. clarkii B   RNFDEIDRSGFGFNKRNFDEIDRSGFGFNKRNFDEIDRSGFGFVKRVWVP 200
A. gambiae A   K-----------------------------RNLDYEPSYTGSADH-GWNG 151
A. gambiae B   K-----------------------------RNLDYEPSYTGSADHNGWNG 152

P. clarkii A   RYIANLYKRNFDEIDRSGFGFNKRNFDEIDRTGFGFHKRDYDVFPDKRNF 235
P. clarkii B   RYIANLYKRNFDEIDRSGFGFNKRNFDEIDRTGFGFHKRDYDVFPDKRNF 250
A. gambiae A   R----FSKRNFDEIDR----FNGKFAPTAK                     173
A. gambiae B   R----FSKRNFDEIDR----FNGKFASTAKCEIA                 178

P. clarkii A   DEIDRSGFGFVRRNVE                                   251
P. clarkii B   DEIDRSGFGFVRRNVE                                   266
A. gambiae A                                                       173
A. gambiae B                                                       178
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Fig. 2. Alignment of the amino acid sequences of orcokinin precursors A and B of the crustacean *Procambarus clarkii* (accession numbers Q9NL82 and Q9NL83, respectively) and the sequences A and B of the mosquito *Anopheles gambiae* containing orcokinin-like peptides (accession numbers XP_320317 and XP_306509, respectively).

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P. clarkii MTAQMFTLALLLSLSAIAAAGTIKTAPARTPSTQDDASEPPDGAPVKRFDAF 52
C. elegans ---MLHLLVLLVALSSAVTAG----RPRRALDGLDGSGEGFDKRALNSLDG- 44

P. clarkii TTGFGHSKRNFDEIDRSGFGFAKKNFDEIDRSGFGFNKRNFDEIDRSGFGFN 104
C. elegans -AGFGFEKRALNSLDGQGFGFEKRALDGLDGAGFGFDKRALNSLDGAGFGFE 95

P. clarkii KRNFDEIDRSGFGFNKRNFDEIDRSGFGFNKRNFDEIDRSGFGFNKRNFDEI 156
C. elegans KRALDGLDGSGFGFDKRALNSLDGAGFGFEKRALNSLDGAGFGFEKRALDGL 147

P. clarkii DRSGFGFNKRNFDEIDRSGFGFVKRVYVPRYIANLYKRNFDEIDRSGFGFNK 208
C. elegans DGAGFGFDKRALNSLDGAGFGFEK------------RALDGLDGAGFGFDK 186

P. clarkii RNFDEIDRTGFGFHKRDYDVFPDKRNFDEIDRSGFGFVRRNVE          251
C. elegans RALNSLDGNGFGFDKRTFKHSSNK--LRSVFRNLKGFKQH            224
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Fig. 3. Alignment of the amino acid sequences of orcokinin precursor A of the crustacean *Procambarus clarkii* (accession number Q9NL82), and sequence NP_509508 of the nematode *Caenorhabditis elegans*.

hits came from 17 nematode species, indicating the widespread distribution of orcokinin-like peptides in this invertebrate class. In contrast, no orcokinin-like sequences were found among the EST sequences available from a number of isopteran, hemipteran, coleopteran, hymenopteran, lepidopteran and dipteran insects (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). Obviously, this does not rule out the possibility that orcokinin-like sequences occur in these species, given that their genomes have not been completely sequenced. What is clear is that the genome of the fruitfly *D. melanogaster* does not contain orcokinin coding genes, which indicates that orcokinins have been lost at least in this highly modified dipteran species.

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