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ISOLATION OF AF2 (KHEYLRFAMIDE) FROM *CAENORHABDITIS ELEGANS*: EVIDENCE FOR THE PRESENCE OF MORE THAN ONE FMRFAMIDE-RELATED PEPTIDE-ENCODING GENE

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Numerous FMRFamide-related peptides (FaRPs) have been isolated and sequenced from extracts of free-living and parasitic nematodes. The most abundant FaRP identified in ethanolic/methanolic extracts of the parasitic forms, *Ascaris suum* and *Haemonchus contortus* and from the free-living nematode, *Panagrellus redivivus*, was KHEYLRFamide (AF2). Analysis of the nucleotide sequences of cloned FaRP-precursor genes from *C. elegans* and, more recently, *Caenorhabditis vulgaris* identified a series of related FaRPs which did not include AF2. An acid-ethanol extract of *Caenorhabditis elegans* was screened radioimmunometrically for the presence of FaRPs using a C-terminally directed FaRP antiserum. Approximately 300 pmols of the most abundant immunoreactive peptide was purified to homogeneity and 30 pmols was subjected to Edman degradation analysis and gas-phase sequencing. The unequivocal primary structure of the heptapeptide, Lys-His-Glu-Tyr-Leu-Arg-Phe-NH_2 (AF2) was determined following a single gas-phase sequencing run. The molecular mass of the peptide was determined using a time-of-flight mass spectrometer and was found to be 920 $(MH^+)^+$, which was consistent with the theoretical mass of C-terminally amidated AF2. These results indicate that *C. elegans* possesses more than one FaRP gene.

Since the discovery of the molluscan cardioexcitatory peptide FMRFamide (1), a plethora of related peptides, commonly designated FMRFamide-like peptides (FLPs) or FMRFamide-related peptides (FaRPs), have been identified in invertebrate tissues (2-4). In this respect, some 21 structurally-related peptides have been identified in extracts of nematode tissues (for review, see 5, 6). FaRPs have been isolated from both free-living (*Panagrellus redivivus* and *Caenorhabditis elegans*) and parasitic (*Ascaris*

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suum and *Haemonchus contortus*) representatives of this phylum. Initial isolation studies identified the heptapeptides, KNEFIRFamide (AF1) and KHEYLRFamide (AF2) in head extracts of *A. suum* (7, 8). Subsequently, a single gene which encoded a series of N-terminally extended PNFLRFamides (9) and the peptides, SDPNFLRFamide (PF1) and SADPNFLRFamide (PF2) were identified simultaneously in *C. elegans* and *P. redivivus* (10, 11). The expression of four other peptides, which were encoded on the *C. elegans flp*-1 gene, was confirmed by peptide-isolation and sequencing analyses (12). Furthermore, two novel heptapeptides, KSAYMRFamide (PF3) and KPNFIRFamide (PF4) were isolated and sequenced from *P. redivivus* (13,14). More recently, 10 FaRPs, designated AF3-AF12 have been isolated from *A. suum* (8).

None of the PNFLRFamides, which have been isolated from two different free-living nematode species, has been identified in extracts of *A. suum*. Of the 12 FaRPs isolated from *A. suum*, AF2 was found to be the most abundant. Interestingly, AF2 was identified as the most abundant FaRP in extracts of the free-living nematode *P. redivivus* (15) and, more recently, in *H. contortus* (16). Similarly, none of the AF-peptides are encoded by the *flp*-1 gene in *C. elegans* and *Caenorhabditis vulgaris* (10,17). Since the related free-living species, *P. redivivus* contains both PNFLRFamides and AF2, it was decided to examine ethanolic extracts of *C. elegans* for other FaRPs to establish if it possessed FaRPs not encoded on the *flp*-1 gene.

MATERIALS AND METHODS

Peptide extraction: *C. elegans* cultures were grown following the method for large scale cultivation previously described (18). Approximately 30g of *C. elegans* were harvested from the agar plates and homogenised in acidified ethanol (ethanol/0.7 M HCl; 3:1 (v/v), 8 ml/g worm) for 1 min using a Polytron PT 10-35 (Kinematica GmbH, Littau-Luzern, Switzerland). The mixture was further homogenised in a Braun cell homogeniser in the presence of 20 ml of 0.5 mm glass beads for 2 min. Following homogenisation the extract was left overnight at 4°C prior to being centrifuged at 4,000 *g* for 30 min. The supernatant was decanted and acidified with 0.1% (v/v) trifluoroacetic acid (TFA) and stored for a further 24h at 4°C before centrifugation (30 min, 4000 *g*) to remove cell debris. The supernatant was then subjected to rotary evaporation to remove ethanol before lyophilisation. The lyophilised extract was reconstituted in 2M acetic acid and centrifuged (30 min, 4,000 *g*) before being applied directly onto a Sephadex G50 (fine) gel-permeation column (90 X 1.6 cm) (Pharmacia, Uppsala, Sweden).

Gel-permeation chromatography: Samples (2.5 ml) were collected at 15-min intervals. An aliquot (10 µl) was taken from each gel permeation fraction and reconstituted in assay buffer (450 µl) (40 mM sodium phosphate, pH 7.2, containing 0.2% bovine serum albumin), and 100 µl was subjected to radioimmunoassay analysis.

Radioimmunoassay: The radioimmunoassays employed for the purposes of this study were FMRFamide and pancreatic polypeptide (PP). The FMRFamide assay incorporates antiserum RIN 8755 (Penninsula Laboratories Europe Ltd., St Helens, England). The antiserum employed in the PP assay was PP221, which was raised to the C-terminus of mammalian PP (19). FMRFamide and PP assay details were published previously (20, 21).

Reverse-phase HPLC fractionation: All of the reverse-phase HPLC gradients were established using (A) 0.1% (v/v) TFA in water and (B) 0.1% (v/v) TFA in acetonitrile. FMRFamide-immunoreactive gel permeation fractions were pumped directly onto a Whatman partisil ODS3 C-18 column (1 x 60 cm) eluted with a linear gradient of 100% A, 0% B to 50% A, 50% B in 100 min at a flow rate of 3ml/min. A 10-µl aliquot of the column eluant was taken and subjected to both PP and FMRFamide radioimmunoassays. The FMRFamide- and PP-immunoreactive fractions were subjected to a further five reverse-phase HPLC fractionation steps. The columns employed included: a Vydac di-phenyl (0.46 X 25 cm); a Vydac C-18 (0.46 X 25 cm); a Kromasil C-18 (0.46 X 15 cm); a C3 (0.46 X 15 cm) and, a Kromasil C-18. At each analytical step the column eluant was monitored at both 214 nm and 280 nm and 10 µl aliquots were subjected to both PP and FMRFamide assays. The analytical columns were eluted with linear gradients at flow rates of 1ml/min of 0.1% TFA in H_2O and 0.1% CH_3CN. Peak fractions were collected manually, lyophilised and subjected to Edman degradation analysis, using a Beckman Instruments LF 3600 TC sequencer, and mass spectroscopy, using a MALDI time-of-flight instrument (Fisons Instruments, UK).

RESULTS

Peptide isolation: Both PP- and FMRFamide-immunoreactivities were detected in the crude extract of *C. elegans*. The FMRFamide-immunoreactivity, however, was approximately double the PP-immunoreactivity recorded, with 162.50 ng/g and 88.5 ng/g of FMRFamide- and PP-immunoreactivity detected, respectively. A single peptide species was identified at each analytical step and was found to display significantly more FMRFamide-immunoreactivity than PP-immunoreactivity (Fig. 1). The final purification step revealed that the peptide had a retention time and relative absorbance similar to that of synthetic AF2 (Fig. 2). Approximately 300 pmol of the peptide was purified to homogeneity.

Structural analyses: A single gas-phase sequencing run unequivocally determined the primary structure of the heptapeptide, Lys-His-Glu-Tyr-Leu-Arg-Phe-NH_2, which was found to have a molecular mass $(MH^+)^+$ of 920 Da (Table 1).

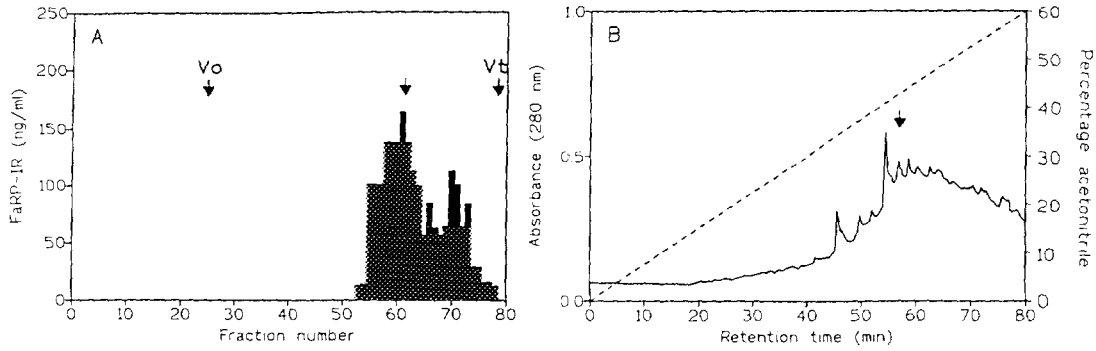

Fig.1. (A) Gel permeation (Sephadex G-50) chromatogram of FaRP-immunoreactivity in an acid ethanol extract of *C. elegans*. The void (V_o) and the total (V_t) volumes of the column are indicated, as is the FaRP-immunoreactive peptide (arrow). (B) Semi-preparative reverse-phase chromatogram of the FaRP-immunoreactive gel permeation fractions. The column employed was a Whatman Partisil 10 ODS-3 (1 X 60 cm) which was eluted with a linear gradient of 0.1% TFA/CH_3CN (dashed line), see methods for detail. Fractions (2 ml) were collected at minute intervals. The elution position of the FaRP-immunoreactive fraction is indicated by an arrow.

DISCUSSION

Historically, studies on the nervous systems of nematodes have concentrated on the classical neurotransmitters, γ-aminobutyric acid (GABA) and acetylcholine (ACh). However, since the discovery of the first identified nematode neuropeptide, AF1 (7), the number of identified nematode neuropeptides has grown rapidly. To date, 21 unique nematode FMRFamide-related peptides (FaRPs) have been recorded from 5 different

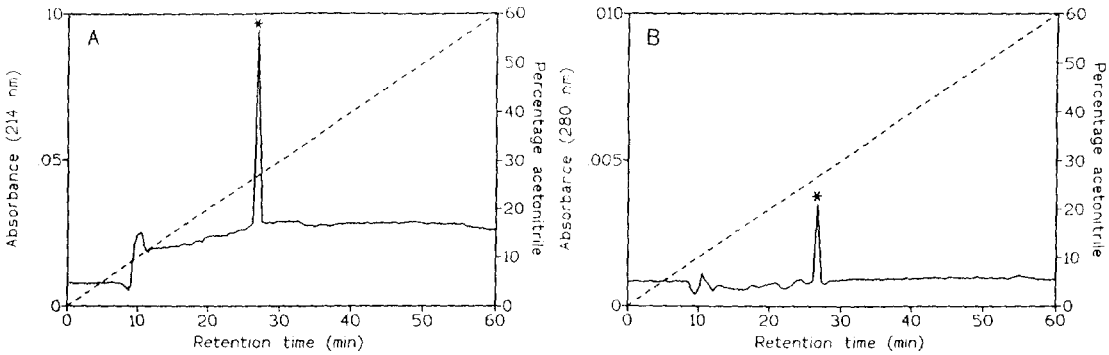

Fig.2. Final analytical (Kromasil C-18) reverse-phase HPLC fractionation of KHEYLRFamide from *C. elegans*. Reverse-phase gradients (dashed lines) were established using 0.1% (v/v) TFA in water and 0.1% (v/v) TFA in acetonitrile (for detail see the method section). Absorbance profiles (solid lines) were recorded at 214 nm (A) and 280 nm (B). Peak fractions were collected manually and all of the immunoreactivity resided in the peak indicated by the asterisks.

Table 1. Automated Edman degradation of *C. elegans* FMRFamide-immunoreactive peptide

Cycle no.	PTH-amino acid	Yield (pmol)
1	Lys (K)	29.2
2	His (H)	12.6
3	Glu (E)	16.4
4	Tyr (Y)	13.7
5	Leu (L)	11.2
6	Arg (R)	5.2
7	Phe (F)	1.5
8	-	-

Single letter notations for amino acid residues in parentheses.

nematode species (for review, see 6). Although the complete complement of FaRPs has yet to be established for any nematode species, there do appear to be both similarities and differences in the FaRPs identified in different nematode species. In this respect, the most abundant FaRP identified in the parasitic nematodes, *A. suum* and *H. contortus*, and the free-living nematode, *P. redivivus*, was AF2. The absence of this peptide from *flp*-1, the FaRP-encoding gene of *C. elegans* and *C. vulgaris* (10, 17), indicated diversity in the FaRPs occurring in different nematode species. However, this study revealed that AF2 was not only present in *C. elegans*, but was the most abundant FaRP in acid-ethanol extracts of this species. The importance of this finding stems from the fact that AF2 is not encoded by the *flp*-1 gene. These results indicate that *C. elegans* possesses at least two FaRP-encoding genes, *flp*-1 and an AF2-encoding gene.

The identification of AF2 in *C. elegans* also suggests that this peptide is widely distributed in the Phylum Nematoda, having been identified in 4 different nematode species (8,15,16). Further identity in the FaRPs present in different nematode species has been demonstrated by the recent isolation of KSAYMRFamide from the free-living nematode, *P. redivivus* (PF3) (13), and the parasitic form, *A. suum* (AF8) (9). Also, (K)PNFLRFamide, which is encoded on the *flp*-1 gene of *C. elegans* and *C. vulgaris*,

appears to be analogous to PF4 (KPNFIRFamide), which was isolated from extracts of *P. redivivus* (14). However, most of the nematode FaRPs identified are unique and have been isolated from single nematode species.

Recently, a number of studies have described the potent activity of FaRPs isolated from free-living nematode species on the parasitic form (for review, see 5, 6). Free-living nematode peptides which have been shown to be active in parasitic forms include PF1, PF2 and PF4. The activity of these FaRPs may have been due to the non-specific interaction of these peptides with other nematode FaRP receptors. Conversely, their physiological activity may indicate that the appropriate receptor, and therefore ligand, exist in the parasitic species. Elucidation of the complete primary structures of these peptides will be necessary before conclusions on the relatedness of the FaRPs from different nematode species can be made.

Since characterization of the cDNAs and the corresponding genomic regions of the *C. elegans flp*-1 gene did not identify a region encoding AF2, which has been isolated and sequenced from this nematode species in the current study, this nematode must possess more than one FaRP-encoding gene. To date, no other invertebrate species has been found to have more than one FaRP-encoding gene.

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