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In vitro metabolism of an insect neuropeptide by homogenates of the nematode *Caenorhabditis elegans*

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Abstract

The cytosolic fraction of homogenates from the free-living soil nematode *Caenorhabditis elegans* is capable of metabolizing the insect neuropeptide adipokinetic hormone, a decapeptide blocked at the N-terminus by a pGlu residue. Analysis of digests by RP-HPLC and LC-MS revealed that an initial endoproteolytic cleavage step produced a heptapeptide with an unblocked N-terminus that can serve as a substrate for aminopeptidases. The aminopeptidase activity is depressed in the presence of the inhibitor amastatin; the initial product of the endoproteolytic step accumulates during incubation, and expected aminopeptidase product peptides are reduced in amount, as assessed by chromatographic peak size. The absence of some expected peptide fragments in the reaction mixtures suggests that multiple proteases contribute to short peptide half-lives. Comparison of the adipokinetic hormone digestion in *C. elegans* to that reported previously for insects reveals the same general pattern of peptide fragment production.

Introduction

Neuropeptides play key roles in all aspects of invertebrate physiology, and have been studied in a number of invertebrate groups including crustaceans (Keller, 1992), molluscs (Muneoka & Kobayashi, 1992), insects (Nassel, 1993), and nematodes (Davis & Stretton, 1995; Shaw, 1996). Genetic and physiological studies demonstrate varied roles for neuropeptides, including neurotransmission, muscular activity, and egg laying (Nelson *et al.*, 1998; Brownlee & Fairweather, 1999; Day & Maule, 1999; Waggoner *et al.*, 2000). There is also evidence for the presence in nematodes of specific neuropeptides similar to those long the subjects of study in other invertebrates, such as insect allatostatin (Smart *et al.*, 1995) and adipokinetic hormone (Davenport *et al.*, 1991, 1994), a key regulator of lipid metabolism in insects. These and other reports for both free-living and parasitic nematodes suggest a highly complex neurohormonal signalling system in these animals (Reinitz *et al.*, 2000; Davis & Stretton, 2001; Kimber *et al.*, 2001).

An essential and well-documented component of normal neuropeptide activity is the regulated attenuation of neuropeptide signals through coordinated and limited proteolysis. This has been the subject of some study in parasitic nematodes (Sajid & Isaac, 1995; Sajid *et al.* 1996, 1997; Masler *et al.*, 2001) and free-living nematodes (Baset *et al.*, 1998). Endopeptidase and aminopeptidase activities are involved in nematode neuropeptide metabolism, and at least one gene, for *Caenorhabditis elegans* AP-1, has been cloned (Baset *et al.*, 1998). With regard to invertebrate neuropeptides, the biochemistry of adipokinetic hormone metabolism in particular is well characterized in insects (Rayne & O'Shea, 1992; Masler *et al.*, 1996), with at least one study in nematodes (Masler, 2002). In the current

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report, evidence is provided for the presence of endopeptidase and aminopeptidase enzymes in extracts of the free-living nematode *C. elegans*, which coordinate to metabolize adipokinetic hormone. Comparisons are made between the profiles of adipokinetic hormone metabolism in insects and the nematode.

Materials and methods

Caenorhabditis elegans were reared in liquid culture at 22°C (Chitwood *et al.*, 1995). Worms were exhaustively washed with water to remove culture medium prior to extraction, and then processed in 10–20 volumes of water by disruption with a Polytron homogenizer (Brinkman Instruments, Westbury, New York). After centrifugation (48,000 × *g*, 30 min, 10°C), the supernatant was collected, and the pellet was re-extracted with the Polytron in 4–5 volumes of water and centrifuged as above. The two supernatant solutions were pooled, aliquots were dried by vacuum centrifugation, and stored at −15°C. Aliquots were dissolved in 100 mM Tris-HCl, pH 8.1 (assay buffer) as needed. Total protein was estimated using the microBCA assay (Pierce Chemical, Rockford, Illinois) following the manufacturer's instructions.

Samples used in the chromatographic assay were prepared by incubating worm extract (5–6 μg) alone, locust adipokinetic hormone only (pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr; Peninsula Laboratories, Belmont, California), extract plus 2 μg adipokinetic hormone, or extract, adipokinetic hormone and 1 mM of the aminopeptidase inhibitor amastatin (Sigma Chemical, St Louis, Missouri) in assay buffer, 5 μl total volume, 37°C, for 4 h. Reactions were stopped by the addition of 50 μl of 0.1% trifluoroacetic acid (TFA) in acetonitrile (CH_3CN). Duplicate reactions were run. The mixtures were dried under vacuum, dissolved in 0.1% TFA, centrifuged (9800 × *g*, 1 min, 26°C) and injected onto the column. The chromatographic system used a reversed-phase C_8 column (Hewlett-Packard 5 mm id × 150 mm, 5 μm particle size, Hewlett-Packard, Avondale, Pennsylvania), flow rate = 0.5 ml min^{-1}. A linear gradient of 5% to 45% CH_3CN in 0.1% TFA was run for 20 min (2% CH_3CN min^{-1}). Absorbance was monitored at 210 nm or 280 nm and retention times determined by on-board integration software (ChemStation, Hewlett-Packard). The LC-MS system comprised a microbore RP-HPLC column (Magic C_{18}, Biochrom Resources, 0.3 mm id × 150 mm), flow rate = 8 ul min^{-1}. A linear gradient of 2% to 65% CH_3CN in 0.2% CHO_2H and 0.005% heptafluorobutyric acid was run for 30 min (2.1% CH_3CN min^{-1}). The HPLC system was coupled to a Finnigan Model LCQ tandem mass spectrometer (ThermoFinnigan, Piscataway, New Jersey) equipped with an electrospray interface (ESI). Source conditions were 170°C capillary temperature, 22 units gas flow and 4.2 kV ESI voltage. Data were analysed with BioExplore and SEQUEST programs (Finnigan) and fragment ions were searched against a database constructed from the sequence of the adipokinetic hormone peptide.

Results

The cytosolic fraction of *C. elegans* homogenates contained proteases that used the insect neuropeptide adipokinetic hormone as a substrate. A prominent chromatographic peak eluting at 14.5 min was detected in the 210 nm profile of the incubation of adipokinetic hormone with *C. elegans* extract (fig. 1C). Extract alone incubated under identical conditions exhibited a peak at the same retention time (14.5 min; fig. 1A, 'E') but of a significantly smaller size than that seen in extracts containing adipokinetic hormone (fig. 1C). No peak was observed at 14.5 min in samples containing adipokinetic hormone alone (fig. 1B). The adipokinetic hormone peak eluted at 18.2 min (AKH, fig. 1B). At 280 nm (fig. 2) the 14.5 min peak absorbed strongly in the extract-adipokinetic hormone sample (fig. 2C), suggesting the presence of aromatic residues. In comparison, the 14.5 min peak in the extract-only sample, while absorbing at 280 nm (fig. 2A), was only 25% the size of the corresponding peak in the adipokinetic hormone-extract sample. As in the 210 nm profile, no peak was detected at 14.5 min with adipokinetic hormone alone. Based upon these observations, a product of adipokinetic hormone digestion eluted at the 14.5 min position. The size of the 14.5 min peak at 210 nm was markedly reduced in the presence of amastatin (fig. 1D) when compared with the size of the peak when amastatin was absent (fig. 1C). These differences in peak size were also evident at 280 nm (fig. 2C,D). Thus, material eluting at 14.5 min increased in absorbance in the presence of adipokinetic hormone, decreased when amastatin was present, and appeared to possess aromatic amino acids.

A prominent peak, observed at both 210 nm and 280 nm, eluted at 15.8 min in profiles of complete incubations (extract, adipokinetic hormone, amastatin; figs 1D, 2D). The peak was totally absent from extract or adipokinetic hormone alone (figs 1A,B and 2A,B) and was present only at trace levels in samples containing extract and adipokinetic hormone but no amastatin (figs 1C, 2C; arrows). Its strong absorbance at 280 nm (fig. 2D) indicates the present of aromatic amino acids. An examination by LC-MS of the contents of adipokinetic hormone digest incubations revealed that, in addition to adipokinetic hormone, the C-terminal heptapeptide Phe-Thr-Pro-Asn-Trp-Gly-Thr, the hexapeptide Thr-Pro-Asn-Trp-Gly-Thr and the pentapeptide Pro-Asn-Trp-Gly-Thr were present. The expected tripeptide pGlu-Leu-Asn, the complementary fragment to Phe-Thr-Pro-Asn-Trp-Gly-Thr, was not detected.

Based upon these observations, a model for the digestion of adipokinetic hormone by *C. elegans* can be constructed (fig. 3). An initial endoproteolytic cleavage (fig. 3, En1) occurred at Asn_3-Phe_4 yielding Phe-Thr-Pro-Asn-Trp-Gly-Thr (fig. 3, II). In the presence of amastatin, this fragment was partially protected from N-terminal degradation and accumulated in the reaction mixture, as evidenced by the large peak at 15.8 min. The absorbance of this peak at 280 nm (fig. 2D), is likely to have resulted from the presence of the aromatic residue tryptophan. In the absence of amastatin, this product peptide, Phe-Thr-Pro-Asn-Trp-Gly-Thr, served as substrate for an initial aminopeptidase cleavage (fig. 3, Ap1) to yield

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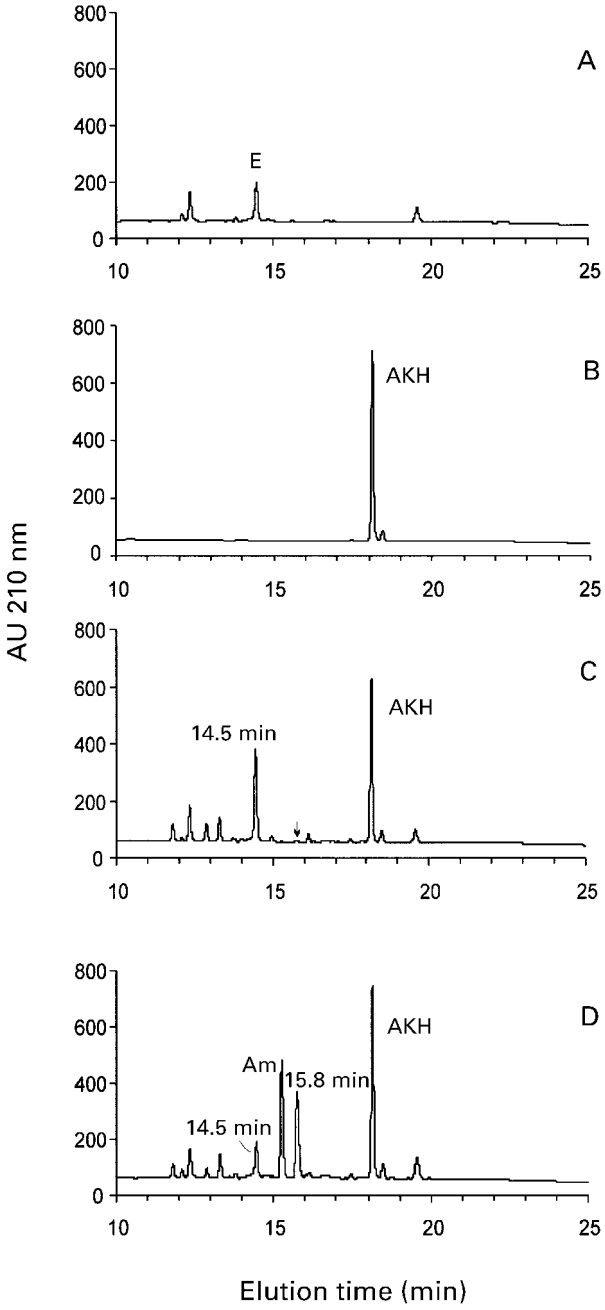

Fig. 1. Chromatographic profiles of 4-h digests of locust adipokinetic hormone by the cytosolic fraction of *Caenorhabditis elegans* homogenates. Extracts, incubations and RP-HPLC conditions are as described in the text. Detection was at 210 nm. A. Extract only. B. Adipokinetic hormone only. C. Extract plus adipokinetic hormone. D. Extract, adipokinetic hormone and amastatin. AKH, adipokinetic hormone; Am, amastatin; E, endogenous UV absorbing material. 14.5 min and 15.8 min are retention times of peaks of interest. Arrow in panel C indicates suspected trace 15.8 min peak.

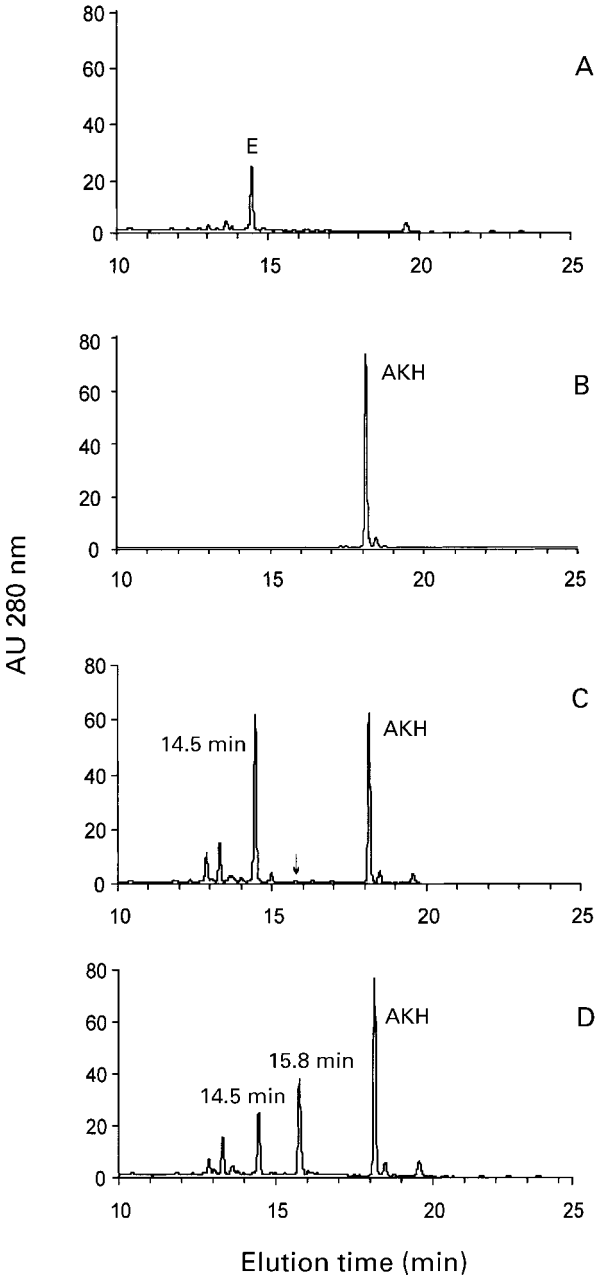

Fig. 2. Chromatographic profiles of 4-h digests of locust adipokinetic hormone by the cytosolic fraction of *Caenorhabditis elegans* homogenates. Extracts, incubations and RP-HPLC conditions are as described in the text. Detection was at 280 nm. A. Extract only. B. Adipokinetic hormone only. C. Extract plus adipokinetic hormone. D. Extract, adipokinetic hormone and amastatin. AKH, adipokinetic hormone; E, endogenous UV absorbing material. 14.5 min and 15.8 min are retention times of peaks of interest. Arrow in panel C indicates suspected trace 15.8 min peak.

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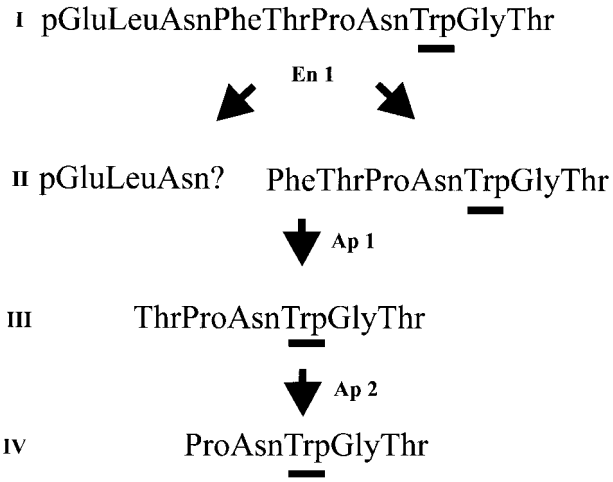

Fig. 3. Likely digestion pattern of locust adipokinetic hormone by *Caenorhabditis elegans* extract. I, adipokinetic hormone; II, initial products of endoprotease step En1; III, product of aminopeptidase step Ap1; IV, product of aminopeptidase step Ap2. The aromatic tryptophan is underlined.

Thr-Pro-Asn-Trp-Gly-Thr (fig. 3, III). This peptide in turn serves as the substrate for a second aminopeptidase step (fig. 3, Ap2) to yield Pro-Asn-Trp-Gly-Thr (fig. 3, IV). The peak at 14.5 min may represent either Thr-Pro-Asn-Trp-Gly-Thr or Pro-Asn-Trp-Gly-Thr, since each is a peptide more polar than Phe-Thr-Pro-Asn-Trp-Gly-Thr. This results in an elution time prior to the Phe-Thr-Pro-Asn-Trp-Gly-Thr peptide (14.5 min vs. 15.8 min). When aminopeptidase digestion of Phe-Thr-Pro-Asn-Trp-Gly-Thr is inhibited (by amastatin), the levels of Thr-Pro-Asn-Trp-Gly-Thr and Pro-Asn-Trp-Gly-Thr are reduced, causing a decrease in the size of the 14.5 min peak (figs. 1D and 2D) compared with peak size and peptide levels in the absence of amastatin (figs. 1C and 2C).

Discussion

In insects, digestion of adipokinetic hormone by tissue preparations has been described for at least two different species (Rayne & O'Shea, 1992; Masler *et al.*, 1996). These reports showed that adipokinetic hormone is attacked initially by a membrane-bound endopeptidase with the resulting production of pGlu-Leu-Asn and Phe-Thr-Pro-Asn-Trp-Gly-Thr. In the *C. elegans* cytosolic preparation, the same initial endoproteolysis occurs. This initial internal cleavage of adipokinetic hormone by the *C. elegans* soluble fraction may be by an endopeptidase similar in substrate requirements to neprilysin, a member of the zinc metalloendopeptidase family (Turner *et al.*, 2001). The neprilysin-like enzyme occurs in insects (Lamango & Isaac, 1993; Masler *et al.*, 1996) and in the nematode *Ascaris suum*, where it cleaves adipokinetic hormone at the Asn_3-Phe_4 bond (Sajid & Isaac, 1995), as reported in insects (Rayne & O'Shea, 1992; Masler *et al.*, 1996). Interestingly, the genomes of *Drosophila melanogaster* and *C. elegans* each possess families of multiple neprilysin-like genes (Isaac *et al.*, 2000). In all reports to date, neprilysin is membrane associated and, at least in mammals, is an integral membrane protein (Turner *et al.*, 2001). In the present study, the enzyme source is the soluble fraction, and under the conditions of extract preparation it is doubtful that an integral membrane protein would have been released to the cytosol. However, there is some evidence that membrane associated, neprilysin-like activity in *C. elegans* is not due to an integral membrane protein (Isaac *et al.*, 2000). The *C. elegans* neprilysin genes are expressed in a tissue-specific manner, presumably to perform different physiological functions (Isaac *et al.*, 2000), and in both soluble and insoluble forms. Therefore, the soluble endoprotease activity responsible for adipokinetic hormone metabolism detected in the present study may represent a neprilysin-like enzyme.

Amastatin-sensitive aminopeptidase activity has been reported in *C. elegans* homogenates (Masler *et al.*, 2001) and primarily occurs in the cytosol. A recombinant *C. elegans* aminopeptidase (AP-1, Baset *et al.*, 1998), suggested to have a role in neuropeptide degradation, was characterized as a cytosolic enzyme. Adipokinetic hormone was identified as a potential substrate of *C. elegans* aminopeptidase (Masler, 2002). It is not surprising, then, that a soluble *C. elegans* homogenate fraction provides both endopeptidase and aminopeptidase digestion of adipokinetic hormone. As in insects, amastatin inhibited further metabolism in *C. elegans* of the C-terminal fragment of the initial endoproteolytic cleavage between Asn_3 and Phe_4. Similarly, Thr-Pro-Asn-Trp-Gly-Thr and Pro-Asn-Trp-Gly-Thr, the prominent products of first and second aminopeptidase cleavages, respectively, occur in digests with *C. elegans* and insect reaction mixtures. Laurent *et al.* (2001) described a cytoplasmic aminopeptidase in *C. elegans* similar to mammalian aminopeptidase P (EC 3.4.11.9). The enzyme cleaves the N-terminal X-Pro bond and is sensitive to aminopeptidase inhibitors. They suggest that this nematode aminopeptidase P has a physiological role in the metabolism of proline-containing peptides. In the present study, Pro-Asn-Trp-Gly-Thr was absent from preparations containing amastatin. The production of Pro-Asn-Trp-Gly-Thr from Thr-Pro-Asn-Trp-Gly-Thr in *C. elegans* may indeed be the result of the action of an aminopeptidase P-like enzyme. There may be at least two, and probably more, aminopeptidases involved in adipokinetic hormone catabolism in *C. elegans*. Further, it is likely that multiple endopeptidases are involved. Not all theoretical peptide fragments were found in the digests examined by LC-MS, suggesting that extensive peptide digestions by additional proteases may have occurred, resulting in very short half-lives for some products. This should not be surprising since a search of the MEROPS enzyme database revealed 246 different peptidase genes in *C. elegans* compared with 325 genes in *Homo sapiens* (Coates *et al.*, 2000). The potential for biochemical variety in the worm is clearly quite immense.

It is striking that the digestion patterns of adipokinetic hormone for the cytosolic *C. elegans* preparations and the insect microsomal systems (Rayne & O'Shea, 1992; Masler *et al.*, 1996) are the same. If adipokinetic hormone is present in nematodes as an endogenous peptide, as a number of reports have suggested, then it is not unreasonable to expect that its metabolic degradation in

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nematodes would be similar to other organisms in which it may have a physiological role. Despite the wide variety of proteolytic enzymes predicted to be present in nematodes and other invertebrates, there appears to be a conservation of the function of proteases in their regulation of neuropeptide signals in invertebrates (Isaac *et al.*, 2000). Additional studies on an increased number of different neuropeptides in nematodes is required to support this observation further, but the area of nematode neuropeptides is continuing to expand (Davis & Stretton, 2001).

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