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Discovering neuropeptides in *Caenorhabditis elegans* by two dimensional liquid chromatography and mass spectrometry ☆

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Abstract

Completion of the *Caenorhabditis elegans* genome sequencing project in 1998 has provided more insight into the complexity of nematode neuropeptide signaling. Several *C. elegans* neuropeptide precursor genes, coding for approximately 250 peptides, have been predicted from the genomic database. One can, however, not deduce whether all these peptides are actually expressed, nor is it possible to predict all post-translational modifications. Using two dimensional nanoscale liquid chromatography combined with tandem mass spectrometry and database mining, we analyzed a mixed stage *C. elegans* extract. This peptidomic setup yielded 21 peptides derived from formerly predicted neuropeptide-like protein (NLP) precursors and 28 predicted FMRFamide-related peptides. In addition, we were able to sequence 11 entirely novel peptides derived from nine peptide precursors that were not predicted or identified in any way previously. Some of the identified peptides display profound sequence similarities with neuropeptides from other invertebrates, indicating that these peptides have a long evolutionary history.
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Neuropeptides are naturally occurring signaling molecules that interact with cell surface receptors to trigger an intracellular transduction pathway. Not only are they structurally diverse, their signaling cascades are highly variable, so there is a tremendous potential of different effects on living cells. Because of their critical signaling role, peptides are attractive for pharmaceutical use, since they can be used as therapeutics or as indirect targets via their cognate receptors or processing enzymes. Current estimates suggest that as much as one-third of the human world population harbors one or more species of parasitic helminths [1]. In this context, the important role of neuropeptides in the biology of nematodes has captivated the interest of many parasitologists. Understanding the role of neuropeptides is unfortunately often hindered by the absence of primary sequence information and knowledge about their post-translational modifications. This sequence information has arrived very slowly, due to the huge efforts required in tissue collection and purification to ultimately isolate and functionally characterize a peptide. Although 23 FMRFamide-like peptide (*flp*) genes [2,3] and 32 neuropeptide-like protein (*nlp*) genes [4] have been predicted from the genome sequence of *Caenorhabditis elegans*, only 12 FMRFamide-related peptides (FaRPs) have been biochemically isolated and identified to date [5–10].

☆ *Abbreviations:* 2D, two dimensional; nanoLC, nanoscale liquid chromatography; SCX, strong cation exchange; Q-TOF, quadrupole time-of-flight; MS/MS, tandem mass spectrometry; *nlp* (NLP), neuropeptide-like protein; *flp* (FLP), FMRFamide-like peptide; FaRP, FMRFamide-related peptide; MIP, myoinhibiting peptide; ACN, acetonitrile; TFA, trifluoroacetic acid; FA, formic acid.

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The concept for the simultaneous identification of naturally occurring peptides, derived from protein precursors in vivo within a peptide producing cell, differs from that of tryptic peptides, which in contrast are artificially derived from proteins on the laboratory bench. The first approach is called 'peptidomics' to clearly distinguish it from 'proteomics.' We recently introduced this concept of peptidomics, which aims at the simultaneous visualization and identification of all the expressed peptides with their post-translational modifications of a cell, tissue or organism [11]. In this way, the neuropeptidome of *Drosophila melanogaster* was already successfully explored and we demonstrated that a two dimensional-LC separation is more suited than a one dimensional-LC separation in order to identify the peptidome as complete as possible [12,13]. In this paper, we describe for the first time a high throughput peptidomic analysis of a nematode, i.e., *C. elegans*, by the use of specific extraction and sample preparation procedures, followed by two dimensional nanoscale liquid chromatography–quadrupole time-of-flight tandem mass spectrometry (2D-nanoLC-Q-TOF MS/MS).

Materials and methods

Animals. Wild-type *C. elegans* (variety Bristol N_2) were raised at 20 °C, under standard laboratory conditions, on conventional nematode growth media (NGM) plates, cultured with the *E. coli* strain OP-50.

Peptide extraction and sample preparation. Mixed stage worms from 10 fully grown petri dishes (diameter 90 mm), free from bacteria and dead animals by flotation on 30% sucrose, were placed in an ice-cold methanol/water/acetic acid (90:9:1, v/v/v) solution. After homogenization, sonication, and centrifugation at 500*g* for 15 min, the pellet was discarded. The methanol was evaporated and the remaining aqueous residue was delipidated by re-extraction with ethyl acetate and *n*-hexane. The organic solvent layer was decanted and the aqueous solution was subsequently desalted by using a SepPak C18 cartridge (Waters, Milford, MA, USA). The peptides were eluted with 50% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA). One-fifth of this fraction was further dried in a Speedvac concentrator, redissolved in 20 μL of 2% ACN with 0.1% formic acid (FA), and subsequently filtered through a 22 μm spindown filter (Ultrafree-MC, Millipore, Bedford, USA) prior to analysis.

2D-nanoLC MS/MS. The two dimensional nanoscale liquid chromatography tandem mass spectrometry experiments were conducted on an *Ultimate* HPLC pump, a *Switchos* column-switching device, and a *Famos* autosampler (all LC Packings, The Netherlands), coupled to a Q-TOF hybrid Quadrupole Time-Of-Flight mass spectrometer (Micromass, UK). Twenty microliters of the sample, corresponding to two fully grown NGM plates, was injected on a strong cation exchange (SCX) column (Bio-SCX, 500 μm × 15 mm, LC-Packings, The Netherlands), which was on-line with a C18 pre-column (μ-guard column MGU-30 C18, LC-Packings, The Netherlands). Sample loading was done at a flow rate of 30 μl/min with water containing 2% ACN and 0.1% FA. After loading the sample, the SCX column was switched off-line and the reversed phase pre-column was rinsed for 5 min. Next, this reversed phase trapping column was switched on-line with the nanoscale Atlantis C18 column (3 μm, 100 μm × 100 mm, Waters, USA). The peptides were separated using a 50 min gradient from 2 to 50% ACN containing 0.1% FA at a flow rate of 200 nl/min. The second fraction of peptides was eluted from the SCX column by injection of 20 μl of a 20 mM ammonium acetate solution. The eluting peptides were again concentrated and desalted on the C18 pre-column prior to the nanoscale HPLC and MS analysis. This procedure was performed 10 times with successive concentrations of ammonium acetate (0, 20, 50, 100, 200, 400, 600, 800, 1000, and 2000 mM) that were used to elute peptide fractions from the SCX column. The 2D-LC system was directly connected with the electrospray interface of the Q-TOF mass spectrometer. The column eluent was directed through a stainless steel emitter (Proteon, Denmark) and nitrogen was used as nebulising gas. The mass spectrometer was set to automatic data-dependent MS to MS/MS switching when the intensity of the doubly and triply charged parent ions increased above 15 counts/s. The applied collision energy of the argon gas was chosen automatically between 25 and 40 eV depending on the number of charges and the mass range of the selected parent ion.

Peptide identification. The MS/MS data of all 10 SCX fractions were transformed into peak list files using the Proteinlynx software (Micromass, UK). These text files were subsequently submitted to a Mascot search on an in-house server to identify the peptides. This bioinformatics program matches the fragmentation data from the peak list files against any FASTA format protein database [14]. A home-made *C. elegans* database was constructed, containing the known FLP and NLP precursors. To identify possible unknown neuropeptide precursors, the NCBInr database with taxonomy set on *C. elegans* was also applied. Pyroglutamic acid (Glu), carboxyterminal amidation, acetylation (aminoterminal, Lys), sulfatation (Tyr), and oxidation (Met), which are all common post-translational modifications of neuropeptides, were selected as variable modifications in the Mascot program. Individual ions with *Probability Based Mowse Scores* above the threshold required for identity or extensive homology ($p < 0.05$) were further manually explored. First, we checked whether the fragmentation spectrum shows extensive similarity to the theoretical fragment ions of the presumed peptide sequence. If this was the case, we further checked whether this peptide meets all requirements to correspond to a naturally occurring peptide. This means that the peptide must be flanked in the precursor by dibasic (or basic) cleavage sites or N-terminally flanked by the signal sequence, since this is the case for all presently known endogenous bioactive peptides. If the presumed peptide sequence carboxyterminally contains an amidation, this means that in the precursor sequence a glycine must be present as carboxyterminal amidation is always derived from a glycine. Next the presumed peptide precursor should contain a signal peptide, a common feature of all secreted proteins. Therefore, newly identified precursors were further analysed by SignalP 3.0 [15] to identify the signal sequence. The presence of a signal sequence, the presence of dibasic (or basic) cleavage sites amino- and carboxyterminally located from the peptide (which are used by the endogenously present convertases), and the presence of a glycine located aminoterminally from the cleavage place in case of carboxyterminal amidation were handled as three additional rules to identify endogenous, naturally occurring, peptides.

The few peptides with individual ion scores just below the indicated threshold, but having satisfying fragmentation spectra and meeting all requirements to correspond to an endogenous peptide, all originated from a precursor of which another peptide had already been successfully identified (a score above the threshold). This is a strong indication that these peptides, although below the threshold, do correspond to an endogenous peptide. These peptides are indicated by a footnote in Table 1 which lists all the presently identified *C. elegans* peptides.

Results

In the 2D-nanoLC experiment, different peptide fractions eluting from the strong cation exchange column in each salt step underwent a subsequent nanoscale HPLC separation on an analytical C18 column. Each

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