Peptides 36 (2012) 230–239

Contents lists available at SciVerse ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Mass spectrometric elucidation of the neuropeptidome of a crustacean neuroendocrine organ

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ARTICLE INFO

Article history:
Received 2 April 2012
Received in revised form 14 May 2012
Accepted 14 May 2012
Available online 22 May 2012

Keywords:
Callinectes sapidus
Pericardial organ
De novo sequencing
Neuropeptidome
Neuropeptides
Chemical derivatization

ABSTRACT

The blue crab *Callinectes sapidus* has been used as an experimental model organism for the study of regulation of cardiac activity and other physiological processes. Moreover, it is an economically and ecologically important crustacean species. However, there was no previous report on the characterization of its neuropeptidome. To fill in this gap, we employed multiple sample preparation methods including direct tissue profiling, crude tissue extraction and tissue extract fractionation by HPLC to obtain a complete description of the neuropeptidome of *C. sapidus*. Matrix-assisted laser desorption/ionization (MALDI)–Fourier transform mass spectrometry (FTMS) and MALDI-time-of-flight (TOF)/TOF were utilized initially to obtain a quick snapshot of the neuropeptide profile, and subsequently nanoflow liquid chromatography (nanoLC) coupled with electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) tandem MS analysis of neuropeptide extracts was conducted for *de novo* sequencing. Simultaneously, the pericardial organ (PO) tissue extract was labeled by a novel *N,N*-dimethylated leucine (DiLeu) reagent, offering enhanced fragmentation efficiency of peptides. In total, 130 peptide sequences belonging to 11 known neuropeptide families including orcomyotropin, pyrokinin, allatostatin A (AST-A), allatostatin B (AST-B), FMRFamide-like peptides (FLPs), and orcokinin were identified. Among these 130 sequences, 44 are novel peptides and 86 are previously identified. Overall, our results lay the groundwork for future physiological studies of neuropeptides in *C. sapidus* and other crustaceans.

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1. Introduction

Crustacean neurosecretory systems synthesize and secrete a diverse class of peptide hormones that play important roles in regulating physiological activities such as reproduction, development, molting, growth, aggression, and adaptation [7,23,30–32,39]. Blue crabs, *Callinectus sapidus*, are a model organism that is frequently used to study the effects of neuropeptides on many physiological processes. For example, previous research using blue crabs as the experimental model system showed that the rhythmic contractions of heart are neurogenic, driven by rhythmic motor patterns generated by the cardiac ganglion (CG) and could be modulated by neurotransmitters and neuropeptides [10–12,18]. In another study, blue crabs were employed to study the induction of courtship display behavior by multiple neuropeptides and neuromodulators [43,44]. Comprehensive neuropeptidomic information would greatly facilitate these studies. In addition to being an important experimental model organism, blue crabs have also long served as a major commercial species in fisheries and aquaculture. However, due to overharvesting and environmental contamination, adult populations of blue crabs are decreasing [1,29]. A better understanding of the basic biology involved in the blue crab lifecycle and behavior will contribute to improving the blue crab aquaculture and replenishing the declining population. Therefore, profiling the neuropeptidome of blue crabs is essential to expanding our knowledge of neuropeptides implicated in blue crab neurobiology.

The crustacean pericardial organ (PO) is a well-defined neuroendocrine site that controls the secretion of various crustacean neuropeptides [4,9,15,35,36]. Many studies have reported the identification of specific neuropeptide families in this essential

Abbreviations: 2D RP-LC, two dimensional reversed-phase liquid chromatography; ACN, acetonitrile; AST-A, allatostatin A; AST-B, allatostatin B; CCAP, crustacean cardioactive peptide; CG, cardiac ganglion; CHCA, α-cyano-4-hydroxy-cinnamic acid; DHB, 2,5-dihydroxybenzoic acid; DiLeu, dimethylated leucine; FLPs, FMRFamide-like peptides; HPLC, high performance liquid chromatography; MALDI–TOF/TOF, matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometer; MALDI-FTMS, matrix-assisted laser desorption/ionization Fourier transform mass spectrometer; MS, mass spectrometry; MS^2, tandem mass spectrometry; nanoLC–ESI-Q-TOF, nanoflow liquid chromatography electrospray ionization quadrupole time-of-flight; PO, pericardial organ; RPCH, red pigment concentrating hormone.

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0196-9781/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.peptides.2012.05.007

endocrine organ, such as FLPs, orcokinin and allatostatins [6,7,13,14,22,24–28,36,37]. Previous studies of neuropeptide families in decapod crustacean provide a foundation and methodologies for blue crab PO neuropeptide profiling using multifaceted mass spectrometric strategies [15,27]. In this study, we employ two dimensional reversed-phase liquid chromatography (2D RP-LC) and dimethylated leucine (DiLeu) chemical derivatization to complement and augment the aforementioned methodologies. 2D RP-LC provides better neuropeptide separation for MS analysis than 1D RP-LC [41,46], while derivatization using DiLeu facilitates neuropeptide fragmentation upon tandem MS (MS^2) which makes *de novo* sequencing less complicated compared to unlabeled neuropeptides [16,17]. DiLeu, a new type of 4-plex isobaric tandem mass (MS^2) tag, was recently developed in our lab. MS^2 spectra of labeled neuropeptides exhibit intense signature reporter ions (m/z 114) that can be used as a check mark of a labeled neuropeptide. DiLeu labeling also improves neuropeptide fragmentation that is beneficial for *de novo* sequencing [40,45].

In this paper, blue crab PO neuropeptidome characterization was carried out by a multifaceted mass spectrometric strategy facilitated with DiLeu labeling. PO tissue and tissue extract as well as fractions of reversed-phase HPLC separation of tissue extract were screened using a high throughput matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI–TOF/TOF) mass spectrometer. Accurate masses of neuropeptides were determined by a high-resolution, high mass accuracy MALDI Fourier transform mass spectrometer (FTMS). Subsequently, tissue extract and HPLC fractions as well as DiLeu labeled tissue extract were analyzed with nanoflow liquid chromatography electrospray ionization quadrupole time-of-flight (nanoLC–ESI-Q-TOF) mass spectrometer to generate tandem mass spectra for *de novo* sequencing. Using this combined approach, 130 peptides were identified from the blue crab PO including 44 new peptides to this species. Our data greatly expand the catalog of peptide hormones known to be present in *C. sapidus* and also provide a foundation for future studies of peptide functions in this species.

2. Materials and methods

2.1. Materials

Methanol (Catalog No.: AC61009-0040 HPLC Grade), acetonitrile (ACN, Catalog No.: AC610010040 HPLC Grade for HPLC and A955-4 Optima for UPLC), formic acid (Catalog No.: AC14793-2500 99% for HPLC and A117-50 Optima for UPLC) and acetic acid (Catalog No.: A38S-212) were purchased from Fisher Scientific (Pittsburgh, PA). Gelatin was purchased from BD (Franklin Lakes, NJ) (Catalog No.: 214340). 2,5-Dihydroxybenzoic acid (DHB) was obtained from MP Biomedicals, Inc. (Solon, OH) (Catalog No.: 212011). α-Cyano-4-hydroxy-cinnamic acid (CHCA) was purchased from Sigma–Aldrich (St. Louis, MO) (Catalog No.: 28166-41-8). Acidified methanol was prepared using 90% methanol, 9% glacial acetic acid, and 1% water. All water used in this study was doubly distilled on a Millipore filtration system (Bedford, MA). C_{18} ziptips were purchased from Millipore (Billerica, MA) (Catalog No.: ZTC18S096). Peptide standards were synthesized by the Peptide Synthesis Facility at the Biotechnology Center, University of Wisconsin at Madison (Madison, WI).

2.2. Animals and dissection

C. sapidus (blue crabs) were obtained from commercial food market and maintained without food for seven days in artificial sea water at 10–12 °C. Animals are cold-anesthetized by packing on ice for 15–30 min before dissection. The animal was then pinned ventral side up in a Sylgard-lined dissection dish to expose the pericardial cavity. The POs were identified visually as an iridescent web of nerves branching over the muscles surrounding the pericardial cavity and dissected free. All dissection was carried out in chilled (approximately 4 °C) physiological saline (composition in mM: NaCl, 440; KCl, 11; $MgCl_2$, 26; $CaCl_2$, 13; Trizma base, 11; maleic acid, 5; pH 7.45).

2.3. Direct tissue analysis and tissue extract analysis

Small pieces of POs were dissected, followed by brief rinsing in acidified methanol for peptide extraction and subsequently rinsing in 10 mg/mL of 2,5-dihydroxybenzoic acid (DHB), to remove the extracellular salts associated with the tissue samples. For direct tissue analysis, small pieces of tissue were transferred onto a MALDI sample plate followed by application of 150 mg/mL DHB matrix solution (in 50/50 methanol/water) for MALDI–FTMS and 5 mg/mL CHCA (in 50/50 ACN/water) for MALDI–TOF/TOF MS. For tissue extract analysis, acidified methanol was used for homogenization of neural tissues, followed by centrifugation at 16,000 × g using Eppendorf 5415D tabletop centrifuge (Eppendorf AG). The pellet was washed with acidified methanol, supernatants were combined for further drying in Savant SC 110 SpeedVac concentrator (Thermo Electron Corporation) and re-suspended with 50 μL of 0.1% formic acid in water.

2.4. Fractionation of tissue extracts using reversed phase (RP)-HPLC

The resuspended extracts were then vortexed and briefly centrifuged. The resulting supernatants were subsequently fractionated via high performance liquid chromatography (HPLC). HPLC separations were performed using a Rainin Dynamax HPLC system equipped with a Dynamax UV-D II absorbance detector (Rainin Instrument Inc., Woburn, MA). The mobile phases included: Solution A (de-ionized water containing 0.1% formic acid) and Solution B (ACN containing 0.1% formic acid). About 50 μL of extract was injected onto a Macrosphere C_{18} column (2.1 mm i.d. × 250 mm length, 5 μm particle size; Alltech Assoc. Inc., Deerfield, IL). The separations consisted of a 120 min gradient of 5–95% Solution B. Fractions were automatically collected every two minutes using a Rainin Dynamax FC-4 fraction collector. Fractions were further dried in a Savant SC 110 SpeedVac concentrator (Thermo Electron Corporation) and resuspended with 10 μL of 0.1% formic acid.

2.5. MALDI–FTMS and direct tissue analyses

MALDI–FTMS experiments were performed on a Varian/IonSpec ProMALDI Fourier transform mass spectrometer (Lake Forest, CA) equipped with a 7.0 Tesla actively-shielded superconducting magnet. The FTMS instrument contains a high pressure MALDI source where the ions from multiple laser shots can be accumulated in the external hexapole storage trap before the ions are transferred to the ICR cell via a quadrupole ion guide. A 355 nm Nd:YAG laser (Laser Science, Inc., Franklin, MA) was used to create ions in an external source. The ions were excited prior to detection with an rf sweep beginning at 7050 ms with a width of 4 ms and amplitude of 150 V base to peak. The filament and quadrupole trapping plates were initialized to 15 V, and both were ramped to 1 V from 6500 to 7000 ms to reduce baseline distortion of peaks. Detection was performed in broadband mode from m/z 108.00–2500.00.

Off-line analysis of HPLC fractions was performed by spotting 0.3 μL of HPLC fractions on the MALDI sample plate and adding 0.3 μL of the saturated DHB. The resulting mixture was allowed

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