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Discovery and Characterization of the Crustacean Hyperglycemic Hormone Precursor Related Peptides (CPRP) and Orcokinin Neuropeptides in the Sinus Glands of the Blue Crab *Callinectes sapidus* Using Multiple Tandem Mass Spectrometry Techniques

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Abstract

The crustacean sinus gland (SG) is a well-defined neuroendocrine site that produces numerous hemolymph-borne agents including the most complex class of endocrine signaling molecules—neuropeptides. Via a multifaceted mass spectrometry (MS) approach, 70 neuropeptides were identified including orcokinins, orcomyotropin, crustacean hyperglycemic hormone (CHH) precursor-related peptides (CPRPs), red pigment concentrating hormone (RPCH), pigment dispersing hormone (PDH), proctolin, RFamides, RYamides, and HL/IGSL/IYRamide. Among them, 15 novel orcokinins, 9 novel CPRPs, one novel orcomyotropin, one novel Ork/Orcomyotropin-related and one novel PDH were *de novo* sequenced via collision induced dissociation (CID) from the SG of a model organism *Callinectes sapidus*. Electron transfer dissociation (ETD) was used for sequencing of intact CPRPs due to their large size and charge state. Capillary isoelectric focusing (CIEF) was employed for separation of members of the orcokinin family which is one of the most abundant neuropeptide families observed in the SG. Collectively, our study represents the most complete characterization of neuropeptides of the SG and provides a foundation for future investigation of the physiological function of neuropeptides in the SG of *C. sapidus*.

Keywords

De novo sequencing; *Callinectes sapidus*; sinus glands; mass spectrometry; neuropeptides; CPRP; Orcokinins; capillary isoelectric focusing; CIEF; ETD; CID

Introduction

Neuropeptides are the largest and most diverse group of endocrine signaling molecules in the nervous system. These signaling peptides are critical for initiation and regulation of numerous physiological processes such as feeding, reproduction and development.^{1, 2} Mass spectrometry (MS), with unique advantages such as high sensitivity, high throughput,

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chemical specificity and the capability of *de novo* sequencing with limited genomic information, is well suited for the detection and sequencing of neuropeptides in endocrine glands, and simultaneously provides the potential for information on post-translational modifications.^{3–6}

The sinus glands (SG) are located between the medulla interna and medulla externa of the eyestalk in *C. sapidus*. It is a well-known neuroendocrine site that synthesizes and secretes peptide hormones regulating various physiological activities such as molting, hemolymph glucose levels, integument color changes, contraction on visceral muscles, eye pigment movements and hydro-mineral balance.⁷ Our previous neuropeptidomic studies of SG in several crustacean species including *Cancer borealis*,^{8–11} *Carcinus maenas*,¹² and *Homarus americanus*^{13, 14} used multiple MS strategies combining MALDI-based high resolution accurate mass profiling, biochemical derivatization, and nanoscale separation coupled to tandem MS, for *de novo* sequencing. In addition, several previous studies reported a limited number of neuropeptides in *C. sapidus*.^{15–17} In the current study, we explore the neuropeptidome of the SG in the blue crab *C. sapidus*, a vital species of economic importance on the seafood market worldwide. In total, 70 neuropeptides are identified including 27 novel neuropeptides, and among them, the crustacean hyperglycemic hormone precursor-related peptides (CPRPs) and orckinins represent major neuropeptide families known in the SG. Therefore, we focus our effort on the comprehensive characterization of these two highly important classes of signaling peptides in the present study.

Orcokinins were originally purified from the crayfish *Orconectes limosus*.¹⁸ They are comprised of a family of neuropeptides with the conserved N-terminus sequence NFDEIDR and C-terminal variations that can occur at residues eight, nine, or thirteen.^{19, 20} Orcokinins have been identified in a large number of crustaceans^{18, 19, 21–27} and several insect species.^{28–30} These studies indicate that this peptide family has been conserved throughout invertebrates and suggest their potential roles in complex physiological processes. Further physiological studies show the effect of orckinins in the stomatogastric ganglion of the lobster *Homarus americanus*²³ and their myotropic effects in the hindgut of crayfish *O. limosus*.^{18, 31} As one of the most abundant neuropeptides in crustacean SG,^{8, 21, 27} comprehensive profiling and characterization of the orckinin family represents an important first step toward a better understanding of the functional roles of this peptide family. However, given their conserved amino acid sequences and similar molecular weights, the large number and chemical complexity of orckinins in the SG requires the use of highly efficient separation techniques prior to MS detection for comprehensive characterization.

Among the separation techniques which have been coupled with MS, capillary isoelectric focusing (CIEF) is an emerging tool for the focusing and separation of proteins and peptides.^{32–36} Here, we present a modified CIEF system for neuropeptide analysis by adding two membrane-coated joints near both ends of the capillary to prevent carrier ampholyte band shifts and interferences from gas bubbles to retain high separation efficiency with high sensitivity. The combination of this modified membrane assisted CIEF (MA-CIEF) system with MALDI-FTMS provides a superior analytical platform to enrich and separate orckinins for efficient detection.

The crustacean hyperglycemic hormone (CHH) and its precursor-related peptide (CPRP) are produced concurrently during the cleavage of CHH from the CHH preprohormone protein.³⁷ The CPRP peptide is localized between the signal peptide and the CHH sequence and is separated from the CHH by a dibasic cleavage site. The functions of CPRPs are currently unknown.³⁸ However, the complete characterization of CPRPs provides a foundation for future experiments elucidating their functional roles in crustaceans. The cDNA of CHH

preprohormone in SG of *C. sapidus* has been characterized,³⁹ but the direct detection of CPRP has not been reported due to its relatively large size and possible post-translational modifications. While small fragments of CPRPs can be identified using nanoLC-ESI-Q-TOF tandem mass spectrometry, the intact peptide is difficult to sequence due to the large molecular weight of CPRPs.

Peptidomics traditionally uses collision induced dissociation (CID) for tandem MS experiments for *de novo* sequencing. Recently, an alternative peptide fragmentation method has been developed using the transfer of an electron termed electron transfer dissociation (ETD).^{40, 41} ETD involves a radical anion with low electron affinity to be transferred to peptide cation, which results in more uniform cleavage along the peptide backbone and reduced sequence discrimination and thus provides improved sequencing for larger peptides compared to CID.⁴² Specifically, for an ion trap ETD, the fluoranthene radical anion is allowed to react with peptide cations in the three dimensional trap. The resulting dissociation cleaves at the N-C α bond to produce c- and z[•]-type product ions. The peptidome of model organisms like *C. sapidus* can be further explored and expanded utilizing ETD as a complementary fragmentation technique to CID. An improved coverage for peptidomic analysis has been reported by using ETD as an additional fragmentation method.⁴³ It was observed that enzymatically produced peptides with a higher *m/z* produced improved sequence coverage using ETD. This observation, and subsequent development termed decision tree analysis, determined that a charge state of ≥ 6 all peptides, endogenous or enzymatic, should be fragmented via ETD.⁴⁴ In the present study, the highly charged peptide CPRP with an intact molecular weight of 3837 Da readily produces plus six charge states, which is highly amenable to fragmentation by ETD and produces remarkably improved fragmentation and thus improved sequence coverage when compared to CID. In this report, we developed a MS-based platform utilizing multiple tandem mass spectrometry techniques and enhanced separation methods to demonstrate the discovery and characterization of CPRP and orckinin neuropeptides in the sinus glands of the blue crab *C. sapidus*.

Experimental Section

Chemical and materials

Acetic acid, sodium hydroxide, acetonitrile (ACN) and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Formic acid and hydroxypropyl cellulose (HPC) were from Sigma-Aldrich (St. Louis, MO). 2,5-dihydroxybenzoic acid (DHB) was obtained from Alfa Aesar. (Ward Hill, MA). Parafilm "M" was obtained from Pechiney Plastic Packaging (Menasha, WI). Fused-silica capillary with 75 μ m i.d. and 360 μ m o.d. was purchased from Polymicro Technologies (Phoenix, AZ). Millipore C₁₈ Ziptip column was used for sample cleaning, and all water used in this study was deionized water (18.2 M Ω -cm) prepared from a Milli-Q Millipore system (Billerica, MA). The physiological saline consisted of 440 mM NaCl, 11mM KCl, 26 mM MgCl₂, 13 mM CaCl₂, 11 mM Trizma base, and 5 mM maleic acid in pH 7.45.

Animals and dissection

C. sapidus (blue crab) were obtained from commercial food market and maintained without food in artificial sea water at 10–12 °C. Animals were cold-anesthetized by packing on ice for 15–30min before dissection. The optic ganglia, with the SG attached, was dissected in chilled (~4°C) physiological saline and individual SGs were isolated from the optic ganglia by micro-dissection and immediately placed in acidified methanol (90% methanol, 9% glacial acetic acid, and 1% water) and stored at –80°C until tissue extraction.

Direct tissue preparation and tissue homogenization

SGs were dissected and prepared for MALDI MS analysis, followed by rinsing in 10 mg/mL of 2, 5-dihydroxybenzoic acid (DHB), to remove the extracellular salts associated with the tissue sample. Tungsten needles and fine forceps were used to dissect and transfer small pieces of tissue onto a MALDI sample plate followed by the application of 150mg/ml DHB matrix solution (in 50:50 acetone: water, v/v) for MALDI FTMS and 5 mg/ml α -cyano-4-hydroxycinnamic acid (CHCA) for MALDI-TOF/TOF MS. Acidified methanol was used during the homogenization of SGs. The homogenized SGs were immediately centrifuged at 16,100 $\times$ g using an Eppendorf 5415D tabletop centrifuge (Eppendorf AG). The pellet was washed using acidified methanol and combined with the supernatant and further dried in a Savant SC 110 SpeedVac concentrator (Thermo Electron Corporation). The resulting dried sample was re-suspended with a minimum amount (20 μ L) of 0.1% formic acid

Fractionation of homogenates using reversed phase (RP)-HPLC

The re-suspended 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 (deionized water containing 0.1% formic acid) and Solution B (acetonitrile containing 0.1% formic acid). About 20 μ L of extract was injected onto a Macrosphere C₁₈ column (2.1 mm i.d. $\times$ 250 mm length, 5 μ m particle size; Alltech Assoc. Inc., Deerfield, IL). The separation consisted of a 120 minute gradient of 5–95% Solution B. Fractions were automatically collected every two minutes using a Rainin Dynamax FC-4 fraction collector (Rainin Instrument Inc., Woburn, MA). The fractions were then concentrated using in Savant SC 110 SpeedVac concentrator (Thermo Electron Corporation) and re-suspended with minimum amount of 0.1% formic acid.

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 an external 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.

Off-line analysis of HPLC fractions was performed by spotting 0.3 μ L of each individual HPLC fraction of interest on the MALDI sample plate and adding 0.3 μ L of the saturated DHB. The resulting mixture was allowed to crystallize at room temperature. The MALDI-FTMS analysis was then performed as described above.

MALDI-TOF/TOF

A model 4800 MALDI-TOF/TOF analyzer (Applied Biosystems, Framingham, MA) equipped with a 200 Hz, 355 nm Nd:YAG laser was used for direct peptide profiling in SG sample and HPLC fraction screening. Acquisitions were performed in positive ion reflectron mode. Instrument parameters were set using the 4000 Series Explorer software (Applied Biosystems). Mass spectra were obtained by averaging 1000 laser shots covering mass range m/z 500–4000. MS/MS was achieved by 1 kV collision induced dissociation (CID) using air. For sample analysis, 0.4 μ L of sample was spotted on MALDI plate first and allowed to dry followed by the addition of 0.4 μ L matrix which consists of a saturated solution of CHCA in 70% acetonitrile.

Nano-LC-ESI-Q-TOF MS/MS

Nanoscale LC-ESI-Q-TOF MS/MS was performed using a Waters nanoAcquity UPLC system coupled to a Q-TOF Micro mass spectrometer (Waters Corp., Milford, MA). Chromatographic separations were performed on a homemade C₁₈ reversed phase capillary column (75 μ m internal diameter $\times$ 100 mm length, 3 μ m particle size, 100 $\text{\AA}$). The mobile phases used were: 0.1% formic acid in deionized water (A); 0.1% formic acid in ACN (B). An aliquot of 5.0 μ l of a tissue extract or HPLC fraction was injected and loaded onto the trap column (Zorbax 300SB-C₁₈ Nano trapping column, Agilent Technologies, Santa Clara, CA) using 95% mobile phase A and 5% mobile phase B at a flow rate of 10 μ l/min for 10 minutes. For neuropeptides, the linear gradient was from 5% to 45% buffer B over 90 min. A data dependent acquisition was employed for the MS survey scan and the selection of three precursor ions and subsequent MS/MS of the selected parent ions. The MS scan range was from m/z 400–1800 and the MS/MS scan was from m/z 50–1800.

NanoLC coupled to MS/MS by CID and ETD

Setup for RP nanoLC separation—The setup for nanoLC consisted of an Eksigent nanoLC Ultra (Dublin, CA) with a 10 μ L injection loop. Mobile phase A consisted of H₂O in 0.1% formic acid and mobile phase B consisted of ACN (Fisher Scientific, Optima LC/MS grade, Fair Lawn, NJ). Injections consisted of 5 μ L of prepared tissue extract onto an Agilent Technologies Zorbax 300 SB-C₁₈ 5 μ m, 5 $\times$ 0.3 mm trap cartridge (Santa Clara, CA) at a flow rate of 5 μ L/min for 5 minutes at 95% A/5% B. Separation was performed on a Waters HPLC column with 3 μ m Atlantis dC₁₈ 75 μ m $\times$ 150 mm (Milford, MA) on a gradient from 5 to 45% mobile phase B at 250 nL/min, over 90 minutes at room temperature. Emitter tips were pulled from 75 μ m inner diameter 360 μ m outer diameter capillary tubing (Polymicro Technologies, Phoenix, AZ) using a commercial laser puller model P-2000 (Sutter Instrument Co., Novato, CA).

Mass spectrometry data acquisitions—An ion-trap mass spectrometer (amaZon ETD, Bruker Daltonics, Bremen, Germany) equipped with an on-line nanospray source was used for mass spectrometry data acquisition. Hystar (Version 3.2, Bruker Daltonics, Bremen, Germany) was used to couple and control Eksigent nanoLC software (Dublin, CA; Version 3.0 Beta) for MS acquisition for all experiments. Smart parameter setting (SPS) was set to m/z 700 for CID and m/z 500 for ETD, compound stability and trap drive level were set at 100%. Optimization of the nanospray source resulted in dry gas temperature, 125°C, dry gas, 4.0 L/min, capillary voltage, –1300 V, end plate offset, –500 V, MS/MS fragmentation amplitude, 1.0V, and SmartFragmentation was set at 30–300%. Data were generated in data dependent mode with strict active exclusion set after two spectra and released after one minute. CID MS/MS fragmentation was set to fragment the six most abundant MS peaks with a preference for doubly charged ions and excluded singly charged ions. ETD MS/MS fragmentation was set to fragment the four most abundant MS peaks with no preference for specifically charged ions except for excluding singly charged ions. For MS generation the ion charge control (ICC) target was set to 200,000, maximum accumulation time, 50.00 ms, four spectral average, acquisition range of m/z 300–1500, and scan speed (enhanced resolution) of 8,100 m/z s^{–1}. For MS/MS generation the ICC target was set to 200,000, maximum accumulation time, 50.00 ms, three spectral averages, acquisition range of m/z 100–2000, and scan speed (Ultrascan) of 32,000 m/z s^{–1}. The ETD reagent parameters were set to 400,000 ICC target for the fluoranthene radical anion. For ETD MS/MS acquisition the fluoranthene radical anion has an m/z of 210 and therefore the m/z 210 was removed from the resulting ETD MS/MS spectra, and m/z 160 was set as the MS/MS low m/z cut-off.

Peptide prediction, *de novo* sequencing and database searches—*De novo* sequencing using ESI-Q-TOF was performed via a combination of MassLynx[™] 4.1 PepSeq

software (Waters) and manual sequencing. Tandem mass spectra acquired from the Q-TOF were first deconvoluted using MaxEnt 3 software (Waters) to convert multiply charged ions into their singly charged forms. The resulting spectra were pasted into the PepSeq window for sequencing analysis. The candidate sequences generated by the PepSeq software were compared and evaluated for homology with previously known peptides. The online program blastp (National Center for Biotechnology Information, Bethesda, MD; <http://www.ncbi.nlm.nih.gov/BLAST/>) was used to search the existing NCBI crustacean protein database, using the candidate peptide sequences as queries. For all searches, the blastp database was set to non-redundant protein sequences (*i.e.* nr) and restricted to crustacean sequences (*i.e.* taxid: 6657). For each of the proteins putatively identified via blastp, the BLAST score and BLAST-generated E-value for significant alignment are provided in the appropriate subsection of the results. Peptides with partial sequence homology were selected for further examination by comparing theoretical MS/MS fragmentation spectra generated by PepSeq with the raw MS/MS spectra. If the fragmentation patterns did not match well, manual sequencing was performed. For CID and ETD data via amaZon ion-trap mass spectrometer, the MS data were processed with DataAnalysis (Version 4.0, Bruker Daltonics Bremen, Germany). Deviations in parameters from the default Protein Analysis in DataAnalysis were as follows: intensity threshold, 1000, maximum number of compounds, 1E7, and retention time window 0.5 minutes. These parameter changes assisted in providing better quality spectra for sequencing. Identification of peptides was performed using Mascot (Version 2.3, Matrix Science, London, U.K.). Searches were performed against a custom crustacean database; no enzyme selected; amidated C-terminal as variable modifications; peptide tolerance mass error 1.2 Da; MS/MS mass error tolerance was 0.6 Da.

Fabrication of CIEF system—75 μm i.d. $\times$ 60 cm fused-silica capillary was flushed by 1 M NaOH for 10 min and dried with air before treatment. Hydroxypropyl cellulose (HPC) was dissolved in water to the final concentration of 5% (w/w) and was used to coat the capillary inner wall in an oven by heating the capillary from 60 $^{\circ}\text{C}$ to 140 $^{\circ}\text{C}$ over 16 minutes followed by 20 minute stabilization. Immediately following this procedure, the capillary was removed from the oven and washed with water and dried before further processing. Cellulose acetate membrane-coated porous joints were made 3 cm from both ends of the HPC-coated capillary as we have previously described.⁴⁵ Briefly, a small fracture section was made on the capillary fixed on a 1 cm $\times$ 0.3 cm plastic slide and was further covered by cellulose acetate solution (12% in acetone, w/v). Under a gentle stream of air a uniform cellulose acetate membrane could form over the fracture section. This porous joint was then placed into a plastic vial (Fisher Scientific, Pittsburgh, PA) with about 2 cm of capillary stretched out of the vial from a small hole on the bottom. The 1% acetic acid solution was filled in the vial and the electrode wire was inserted into the vial to form an electrical connection.

CIEF procedure and sample collection—CIEF was performed by using TriSep-2100 HV power supplier from Unimicro Technologies (Pleasanton, CA). The 1% acetic acid solution and 1% sodium hydroxide solution were chosen as anolyte and catholyte, respectively. The 1% acetic acid solution was filled into the plastic vials containing membrane-coated joints, and electrodes were placed into these vials so that a circuit was formed via the porous joints, as shown in Figure 1. Prior to CIEF, the capillary was flushed with water and dried under air flow in sequence. A constant voltage of 20 kV was then applied to the capillary for 10–12 min depending to the samples. After focusing, the fractions were mobilized by air pressure. A MALDI plate pre-coated with Parafilm M was used for sample collection as described previously.⁴⁶ The matrix was pre-spotted on the MALDI plate with 120 mg/ml of DHB. For each of the spots, 250 nL of matrix solution was

mixed with approximately 150 nL of an individual collected fraction, and co-crystallized before MALDI-FTMS analysis.

Results and Discussion

Comprehensive Neuropeptidomic Study in the Sinus Gland of *C. sapidus* Using Complementary Mass Spectral Techniques

In this study, the high resolution and mass accuracy of MALDI-FTMS, high sensitivity of MALDI-TOF/TOF and capability of *de novo* sequencing of nanoLC-ESI-Q-TOF were used to identify 70 neuropeptides consisting of 12 neuropeptide families from the SG. Although 43 neuropeptides were previously identified in *C. borealis* and *C. maenas*, here we report the successful identification of 27 novel peptides *de novo* sequenced for the first time from the SG of *C. sapidus*, as shown in Table 1. Multiple complementary mass spectrometric instruments have been employed, each offering overlapped yet distinct coverage due to different ionization preference and sequencing capabilities. As a comparison, 47 peptides were detected and sequenced using nanoESI-Q-TOF, 38 peptides were detected by MALDI-TOF/TOF and 53 were observed via MALDI-FTMS, while only 18 were commonly detected by all three instruments in the SG of *C. sapidus*. In addition, multiple sample preparation methods including direct tissue analysis, tissue extraction, off-line HPLC separation were used in this study to obtain a more complete neuropeptidome coverage. Direct tissue analysis provides a quick snapshot of the peptide profile as well as accurate mass measurements to identify numerous previously known neuropeptides; acidified methanol extraction offers a more complete profile of the peptides present in the whole tissue. Off-line reversed phase HPLC prior to MS analysis reduces the chemical complexity and thus expands the dynamic range of detectable neuropeptides using MS. Improved sample preparation results in enhanced neuropeptide detection from the HPLC fractions compared to crude extraction due to its significant signal suppression from lipids and chemical complexity. As an example, 8 peptides were detected from a single direct tissue, 21 were detected in crude extract and 53 were detected from HPLC fractions using MALDI-FTMS.

Among these novel peptides, there are 9 CPRPs and 15 orckinins. Small pieces of detected CPRP fragments taken together cover the amino acid sequence of the intact CPRP RSAEGLGRMGRLASKSDTVTPLRGFEGETGHPL. Thirty-two orckinins were sequenced from SG of *C. sapidus*, suggesting that the SG is rich in orckinins. Fifteen novel orckinins including 9 full length orckinins such as DFDEIDRSGFG, DFDEIDRSSFG, DFDEIDRSSFA, NFDEIDRSSFGF, DFDEIDRSGFGFA, DFDEIDRSGFGFV, DFDEIDRSSFGFA, DFDEIDRSSFGFV and DFDEIDRSSFGFN, and 6 truncated orckinins such as EIDRSGFGF, NFDEIDRSA, FDEIDRSGFG, FDEIDRSGFA, FDEIDRSSFA and DEIDRSGFGFA were sequenced by ESI-Q-TOF MS/MS. A spectrum of a novel orckinin, DFDEIDRSGFGFA (m/z 1475.56), is shown in Figure 2(a). In addition, several novel peptides from other peptide families were sequenced including one orckinin-related peptide TPRDIANLY (m/z 1062.56), one pigment dispersing hormone (PDH)-related peptide pQELHVPAREA (m/z 1190.58) and one orcomyotropin FPAFTTGFGSH (m/z 1168.54). The MS/MS fragmentation spectra of these three peptides sequenced by ESI-Q-TOF MS/MS are shown in Figure 2(b-d). As shown in Figure 3, one truncated orckinin FDEIDRSSFA (m/z 1186.54) was detected in neuropeptide profile of the direct tissue analysis, suggesting that these truncated peptides are endogenously processed orckinins in the SG due to minimal sample preparation involved in direct tissue analysis method as opposed to conventional tissue extract analysis. Collectively, our data not only significantly expand the catalog of peptide hormones known to be present in *C. sapidus*, but also provide a foundation for future studies of peptide functions in this important commercial species.

Identification and Characterization of an Intact CPRP Using ETD

Using ESI-Q-TOF, 9 truncated CPRPs fragments were sequenced to cover the amino acid sequence of the intact CPRP peptide RSAEGLGRMGRLLASLKSDTVTPLRGFEGETGHPLE. Several truncated CPRP fragments such as CPRP [1–9] 975.52 (RSAEGLGRMa, data not shown) and CPRP [1–13] 1415.79 (RSAEGLGRMGRLL) were detected in direct tissue analysis as shown in Figure 3 by MALDI TOF/TOF, suggesting the potential cleavage on prohormone involved in posttranslational proteolytic processing. More truncated CPRP fragments were expected to exist in the SG but were not detected in single tissue analysis, presumably due to their relatively lower abundances or poor ionization efficiency. Furthermore, the truncated CPRP fragments such as CPRP [14–25] ASLKSDTVTPLR resulted from a typical cleavage site on Arg as well. A representative CID MS/MS spectrum of CPRP [14–25] ASLKSDTVTPLR, is shown in Figure 4(a). CID using ESI-Q-TOF mainly produces fragmentation for doubly and triply charged molecules, which is adequate to sequence peptides with relatively small molecular weight (less than 2000 Da). However, CID fragmentation is insufficient to sequence the larger intact CPRP in a complex sample whose molecular mass is 3837 Da and therefore it is extremely difficult to directly sequence by traditional CID via ESI-Q-TOF. ETD is an attractive fragmentation alternative to sequence the highly charged CPRP peptide. In ETD, a protonated peptide reacts with an anion (fluoranthene radical) where the anion transfers an electron to the peptide cation and the resulting fragmentation occurs along the N-C α bond by free-radical-driven cleavage. The large size of CPRP and numerous basic amino acid residues offer multiple sites to sequester charge make it well-suited for ETD fragmentation technique. Unlike CID which suffers from sequence-dependent fragmentation, ETD has no preferred cleavage sites except proline, which leads to a more uniform distribution of the fragment ions, thus providing superior sequence coverage for larger peptides.⁴⁷ As shown in Figure 4(c, d), the extensive fragmentation patterns of ETD is observed compared to the limited and biased CID fragmentation in Figure 4(b). As evident from the comparison, ETD offers more uniform and extensive fragmentation than CID, thus providing enhanced coverage for large intact peptides such as CPRPs. Specifically, we observe 12.5% of the b- and y- cleavages for CID as compared to an average of 53.5% of c- and z^{*}- type fragments. More than a four-fold increase in fragments using ETD suggests the utility of this relatively new tandem MS fragmentation method as a complementary technique for *de novo* sequencing of large intact endogenous peptides and novel neuropeptide discovery endeavors.

Figure 5 compares the sequences of CPRP in *C. sapidus* to those of other crustacean species. Significant sequence homology is observed at both the N- and C-termini of the CPRP sequences of these related species, with several amino acid residues being completely conserved among all of the crustacean species examined (highlighted with boxes). The extensive sequence homology among the CPRPs supports the hypothesis that they serve an important biological role and that this role is conserved. Although previous studies showed that the CPRP can act as a circulating hormone in the hemolymph and has a relatively long lifetime,^{38, 48} the precise physiological role(s) it plays in crustaceans remains unknown. Further investigations are needed to understand the functions of various CPRPs in multiple crustaceans.

Discovery of N-terminal Asp-Orcokinin

The first orcokinin NFDEIDRSGFGFN was isolated from abdominal nerve cord extracts of the crayfish *Orconectes limosus* as a myoactive peptide acting on gut tissues.¹⁸ Subsequently, numerous orcokinins have been discovered from crustaceans, insects and other invertebrates^{22, 28, 49–51} with the conserved N-terminal motif NFDEIDR. Unlike previously reported orcokinins, most of the novel full sequence orcokinins identified from

this study, share the conserved N-terminal sequence of DFDEIDR except NFDEIDRSSFGF. One plausible explanation for this observation could be caused by deamidation of Asn during complex sample preparation procedures including tissue extraction, peptide purification, and separation. Alternatively, conserved amino-terminal Asp-containing orckinins could be caused by a change of a single codon in orckinins for this species; however, this is a hypothesis due to the lack of genetic information of pre-proorckinin for this specific species. To investigate the origins of these amino-terminal Asp-containing orckinins, direct tissue analysis was used to examine the resulting peptide profiles with minimal sample preparation involving only matrix application. As shown in Figure 3, via direct tissue analysis of SG by MALDI-TOF/TOF, 8 orckinins were observed, including both more commonly found N-terminal Asn-containing orckinins such as NFDEIDRSGFGFA (m/z 1474.66) and NFDEIDRSGFGFV (m/z 1502.52), and N-terminal Asp-containing orckinins such as DFDEIDRSSFA (m/z 1301.56) and DFDEIDRSGFGFA (m/z 1475.56). A reduced number of N-terminal Asp-containing orckinins were detected in the direct tissue analysis due to their low abundances and sample complexity in a single SG tissue. Nonetheless, the detection of DFDEIDRSSFA and DFDEIDRSGFGFA provided validation of N-terminal Asp-containing sequences in orckinins detected in the SG of *C. sapidus*.

Profiling of Orckinin Family Neuropeptides from the Sinus Gland of *Callinectes sapidus* Using MA-CIEF Separation Coupled to MALDI FTMS Detection

Orckinin (NFDEIDRSGFGFN) and orckinin homologues were first discovered as crustacean peptides with numerous physiological effects such as potent hindgut myostimulating factors,¹⁸ inhibiting the pyloric rhythm,²³ and neurohormones in hemolymph,⁵² to name a few. Therefore, it is important to comprehensively identify and discover new orckinins in complex animal nervous systems. However, due to the existence of multiple acidic amino acids such as D and E, orckinins are less easy to ionize in the positive ion mode, which makes them difficult to detect. As shown in Figure 6(a), most orckinins have relatively low peak intensity around 20% or lower by direct MALDI FTMS profiling. As shown in Table 1, a total of 32 orckinin isoforms are identified from *C. sapidus* SG by combining multiple sample preparation methods, including direct tissue analysis, crude extraction, capillary electrophoresis and offline HPLC separation. With the large number of orckinins in *C. sapidus* and their relatively poor ionization efficiencies, a reliable separation method with high efficiency is required. Membrane-assisted capillary isoelectric focusing (MA-CIEF) provides the separation needed for the large number of orckinins in *C. sapidus* to be detected by MALDI-FTMS. MA-CIEF is a modified CIEF system developed in our lab, which provides great potential in MS-based proteomics⁵³ and neuropeptide analysis.⁵⁴ Compared with regular CIEF, the MA-CIEF method offers much faster separation and higher peak intensities. Orckinin family neuropeptides feature unique pI values that are different from other neuropeptide families. It was found that with the MA-CIEF/MALDI-FTMS based platform, MS signals were significantly improved, and more orckinins were detected compared with those by direct MALDI-FTMS profiling without any separation.⁵⁴ As shown in Figure 6(a), only 11 orckinins including FDEIDRSSFA (m/z 1186.59), NFDEIDRSGF (m/z 1199.65), NFDEIDRSGFG (m/z 1256.63), NFDEIDRSGFA (m/z 1270.65), NFDEIDRSSFG (m/z 1286.63), NFDEIDRSGFGFA (m/z 1474.75), DFDEIDRSGFGFA (m/z 1475.85), NFDEIDRSGFGFV (m/z 1502.79), NFDEIDRSSFGFA (m/z 1504.75), NFDEIDRSSFGFV (m/z 1532.81) and NFDEIDRSSFGFN (m/z 1547.74) were identified from 0.3 μ L tissue extraction of SG using MALDI-FTMS with relatively low intensity. After separation by MA-CIEF, 2.5 μ L of tissue extraction resulted in 24 fractions with each one containing around 100 nL of tissue extraction and 15 orckinins were identified from a single fraction at a relatively high sensitivity. In Figure 6(b), the 7 peaks marked with stars are orckinins not identified by

direct profiling of tissue extract, including EIDRSGFGF (m/z 1027.48), EIDRSGFGFA (m/z 1098.53), FDEIDRSGFA (m/z 1156.58), NFDEIDRSSFA (m/z 1300.60), NFDEIDRSGFGF (m/z 1403.65), NFDEIDRSSFGF (m/z 1433.64), and DFDEIDRSGFGFV (m/z 1503.69). The profiling of all 24 fractions enabled identifications of 23 orckinins, including all of the isoforms previously identified by direct profiling and 12 additional orckinins which were not detected via direct profiling. Furthermore, only 2 novel peptides were detected by direct profiling of tissue extraction, in contrast 5 novel peptides were detected in a single fraction and a total of 9 novel peptides were detected by combining results from all the fractions by CIEF separation, highlighting the unique advantages of CIEF in discovery of novel peptides. After CIEF separation, isoforms of the orckinin family with similar pI values were separated from other neuropeptides and focused to a narrow band corresponding to their similar pI values. Most neuropeptides in the crustacean nervous systems are basic, for example, RFamides have pI values higher than 12, and tackykinins have pI values higher than 14 (calculated from <http://innovagen.net>). Orckinins, unlike other neuropeptides, are the most acidic neuropeptide family with pI value around 3–4, therefore they can be easily concentrated to a narrow section of the CIEF capillary and separated from other neuropeptides, which make CIEF extremely suitable for concentrating and separating orckinins.

Conclusions

In this study, the neuropeptides in the SG of *C. sapidus* were identified and sequenced using multifaceted MS-based platforms. Collectively, 70 neuropeptides have been sequenced including 15 novel ones. ETD was performed to improve the sequence coverage of large endogenous neuropeptides with higher charge and larger size. The intact CPRP from *C. sapidus* was identified and characterized with 68% sequence coverage by MS for the first time. The discovery of N-terminal Asp-containing orckinins was reported and discussed. A large number of orckinins were discovered and a novel MA-CIEF configuration was employed to facilitate the separation of acidic amino acid-containing orckinins. Our study not only significantly expands the catalog of peptide hormones known to be present in *C. sapidus*, but also provides a foundation for future studies of peptide function in this important aquaculture species.

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Figure 1. Schematic illustration of the membrane-assisted CIEF setup enhanced with cellulose acetate membrane-coated porous joints

The sample was mixed with 0.5% of Pharmalyte and filled into the capillary, which was then inserted into the anolyte and catholyte reservoirs with acid and base, respectively. A high voltage power supply was connected via the two membrane-coated joints near capillary ends to form a circuit, so that the shadowed sections were free from electrical field during focusing and formed “plugs” to prevent sample loss. After focusing, the reservoirs were removed, and the fractions were mobilized by air pressure and collected on a MALDI plate with pre-spotted matrix.

Figure 2.

MS/MS fragmentation spectra of several novel neuropeptides in *C. sapidus* SG by ESI-Q-TOF: (a) orcokinin (DFDEIDRSGFGFA m/z 1475.64), all y-ions and b-ions are detected; (b) Ork/Orcomytropin-related peptide (TPRDIANLY m/z 1062.56), almost all b-ions from b_2 to b_8 are detected and are very abundant compared to the incomplete y-ion series; (c) orcomytropin (FPAFTTGFGSH m/z 1168.54), y_1 - y_8 , b_1 - b_8 except b_7 are detected with the most abundant fragment ions being a_3 and a_4 ; (d) PDH-related peptide (pQELHVPEREA m/z 1190.57), y_1 - y_8 , b_2 - b_4 and b_8 are detected.

Figure 3. Neuropeptide profiling of the sinus gland by direct tissue analysis using MALDI-TOF/TOF. The mass spectral insets with higher magnification highlight the existence of two N-terminal Asp-containing orckinin neuropeptides: DFDEIDRSSFA (m/z 1301.56) and DFDEIDRSGFGFA (m/z 1475.56). A truncated CPRP fragment CPRP [1–13] RSAEGLGRMGRL (m/z 1415.79) was labeled in blue.

<i>C. sapidus</i> _SG_CPRP	RS	AE	GL	RM	GR	LL	AS	LK	SS	---	DT	VT	PL	RG	FE	GE	TH	HP	LE	36	
<i>C. maenas</i> _SG_CPRP_I	RS	TQ	GY	RM	DR	LL	AAL	KT	SP	ME	PS	AA	-	AV	EN	GT	TH	HP	LE	38	
<i>C. maenas</i> _SG_CPRP_II	RS	TQ	GY	RM	DR	LL	AAL	KT	SP	ME	PS	AA	-	AV	QH	GT	TH	HP	LE	38	
<i>C. borealis</i> _SG_CPRP_I	RS	AQ	GL	KM	ER	LL	AS	YR	GA	-	LE	PNT	PL	GD	LS	GS	VG	HP	VE	38	
<i>C. borealis</i> _SG_CPRP_II	RS	AQ	GL	KM	ER	LL	AS	YR	GA	-	LE	PNT	PL	GD	LS	GS	LG	HP	VE	38	
<i>C. borealis</i> _SG_CPRP_III	RS	AQ	GL	KM	EH	LL	AS	YR	GA	-	LE	PNT	PL	GD	LS	GS	LG	HP	VE	38	
<i>C. borealis</i> _SG_CPRP_IV	RS	AQ	GL	KM	ER	LL	VS	YR	GA	-	VE	PNT	PL	GD	LS	GS	LG	HP	VE	38	
<i>C. pagurus</i> _SG_CPRP	RS	AQ	GM	KM	ER	LL	AS	YR	GA	-	LE	PST	PL	GD	LS	GS	LG	HP	VE	38	
<i>C. produtus</i> _SG_CPRP_I	RS	AQ	GM	GK	M	EH	LL	AS	YR	GA	-	LES	NT	PT	GD	LP	GG	LV	HP	VE	38
<i>C. produtus</i> _SG_CPRP_II	RS	AQ	GM	GK	M	EH	LL	AS	YR	GA	-	LES	NT	PL	GD	LP	GG	LV	HP	VE	38
<i>C. produtus</i> _SG_CPRP_III	RS	AQ	GM	GK	M	EH	LL	AS	YR	GA	-	LES	NT	PL	GD	LP	GG	LV	HP	VE	38
<i>C. produtus</i> _SG_CPRP_IV	RS	AQ	GM	GK	M	ER	LL	AS	YR	AA	-	VE	PNT	PL	GD	LP	GG	LV	HP	VE	38
<i>O. limosus</i> _SG_CPRP_A	RS	VE	GS	SR	ME	RL	LS	SG	-	SS	---	SSE	PL	S	FL	SQD	---	QS	VS		33
<i>O. limosus</i> _SG_CPRP_B	RS	VE	GS	SR	ME	RL	LS	SG	-	SS	---	SSE	PL	S	FL	SQD	---	QS	VN		33
<i>H. americanus</i> _SG_CPRP_A	RS	VE	GA	SR	ME	EK	LS	SS	NS	P	---	SST	PL	G	FL	SQD	---	HS	VN		34
<i>H. americanus</i> _SG_CPRP_B	RS	VE	GV	SR	ME	EK	LS	SS	IS	P	---	SST	PL	G	FL	SQD	---	HS	VN		34

Figure 5.

Amino acid sequence alignment of 16 CPRPs from seven crustacean species. Amino acid residues exhibiting 100% conservation are boxed with solid lines. Amino acid residues with significant, but less than 100%, conservation are boxed with dashed lines, indicating significant sequence homology among CPRPs in various crustacean species.

Figure 6. Analysis of orckinin family neuropeptides in the sinus gland (SG) of *C. sapidus* is enhanced by the use of membrane-assisted (MA)-CIEF/MALDI-FTMS. Orckinins peptides are marked with molecular weight on the mass spectra. (a) Control sample without CIEF separation. (b) Fraction 16 which is rich in orckinin family peptides after CIEF separation. Peptides with stars were only found with CIEF separation, and peptides labeled in red are novel peptides.

Neuropeptides identified in the sinus gland (SG) of *Callinectes sapidus* SG by multiple MS approaches

Table 1

Neuropeptide family	m/z	Peptide sequence	Q-TOF	FT	TOF/TOF
CabTRP	934.4927	APSGFLGMRa	+	+	-
	950.4921	APSGFLGM(O)Ra	+	-	-
	980.5027	TPSGFLGM(O)Ramide	-	+	-
CCAP	956.3752	PFCNAFTGCa	+	+	-
CPRP[1-9]	975.5153	RSAEGLGRMa	+	+	+
CPRP[1-10]	1033.5207	RSAEGLGRMG	+	+	+
CPRP[15-25]	1216.6896	SLKSDTVTPLR	+	-	+
CPRP[18-28]	1221.6110	SDTVTPLRGFE	+	+	-
CPRP[14-25]	1287.7267	ASLKSDTVTPLR	+	+	+
CPRP[1-12]	1302.7059	RSAEGLGRMGRL	+	+	+
CPRP[13-25]	1400.8108	LASLKSDTVTPLR	+	+	-
CPRP[1-13]	1415.7900	RSAEGLGRMGRL	+	+	+
CPRP[22-36]	1639.8075	TPLRGFEGETGHPLE	+	+	-
Orcomyotropin	1168.5422	FPAFTTGFGSH	+	+	-
	1186.5164	FDFTTGFGHS	+	+	+
Ork	1027.4843	EIDRSFGFG	+	+	-
	1066.4800	NFDEIDRSA	+	+	-
	1098.5214	EIDRSFGFA	+	+	-
	1142.5113	FDEIDRSFG	+	-	-
	1156.5269	FDEIDRSGFA	+	+	-
	1186.5375	FDEIDRSSFA	+	+	-
	1198.5487	NFDEIDRSFGa	-	+	-
	1199.5327	NFDEIDRSGF	-	+	+
	1213.5484	DEIDRSFGGFA	-	+	+
	1228.5593	NFDEIDRSSFa	+	+	-
	1256.5542	NFDEIDRSFG	+	+	+
	1257.5382	DFDEIDRSFG	+	+	-

Neuropeptide family	m/z	Peptide sequence	Q-TOF	FT	TOF/TOF
	1270.5699	NFEIDRSFGFA	+	+	+
	1271.5539	DFEIDRSFGFA	+	+	—
	1286.5648	NFEIDRSSFG	+	+	+
	1287.5488	DFEIDRSSFG	+	—	+
	1300.5804	NFEIDRSSFA	+	+	—
	1301.5644	DFEIDRSSFA	+	+	+
	1360.6168	FDEIDRSFGFA	—	+	—
	1403.6226	NFEIDRSFGF	+	+	+
	1433.6332	NFEIDRSSFGF	+	+	+
	1474.6597	NFEIDRSFGFA	+	+	+
	1475.6437	DFEIDRSFGFA	+	+	+
	1502.6910	NFEIDRSFGFV	+	+	+
	1503.6750	DFEIDRSFGFV	+	+	+
	1504.6703	NFEIDRSSFGFA	+	+	+
	1505.6543	DFEIDRSSFGFA	+	+	—
	1532.7016	NFEIDRSSFGFV	+	+	+
	1533.6856	DFEIDRSSFGFV	+	—	—
	1547.6761	NFEIDRSSFGFN	+	+	+
	1548.6601	DFEIDRSSFGFN	+	—	+
Ork/Orcomyotropin-related	1554.6972	NFEIDRTGFGFH	—	+	+
	1062.5578	TPRDIANLY	+	—	—
Others	844.4788	HLGSLYRa	—	+	+
	902.5458	KIFEPLR	—	+	—
	1259.7106	KIFEPLRDKN	—	—	+
PDH	1190.5762	pQELHVPEREa	+	—	—
proctolin RFa	1927.1800	NSELINSILGLPKVMNDaa	+	—	—
	649.3668	RYLPT	—	+	+
	925.4890	ETNFLRFa	—	—	+
	1005.5741	GRPNFLRFa	—	+	—

Neuropeptide family	m/z	Peptide sequence	Q-TOF	FT	TOF/TOF
	1029.5629	DHVPFLRFa	-	+	-
	1045.5802	GHRNFLRFa	-	-	+
	1059.5959	AHRNFLRFa	-	+	-
	1104.6061	GAHKNYLRFa	+	+	+
	1116.5473	YEQDFLRFa	-	+	-
	1146.6055	GYSKNYLRFa	-	+	+
	1172.6323	AYNRSFLRFa	-	+	+
	1174.6368	FTSKNYLRFa	-	-	+
	1209.6123	DENRNFLRFa	-	+	-
	1273.6436	YGSDRNFLRFa	-	-	+
	1314.7753	DARTAPLRLRFa	-	+	-
RPCH	930.3443	pELNFSPGWa	+	-	-
RYa	1030.4703	pEGFYSQRYa	+	-	+
SIFa	1381.7375	GYRKPPENGSIa	-	+	-

Peptides identified by *de novo* sequencing using ESI-Q-TOF, MALDI-FTMS and MALDI-TOF/TOF. Neuropeptides listed in red are novel peptides sequenced for the first time in this study. Lower case “a” at the C-terminal peptide indicates C-terminal amidation. CabTRP: *Cancer borealis* tachykinin-related peptide; CCAP: Crustacean cardioactive peptide; CPRP: CHH precursor-related peptide; PDH: pigment dispersing hormone; RPCH: red pigment concentrating hormone; Ork: Orcokinin