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Combining tissue extraction and off-line capillary electrophoresis matrix-assisted laser desorption/ionization Fourier transform mass spectrometry for neuropeptide analysis in individual neuronal organs using 2,5-dihydroxybenzoic acid as a multi-functional agent

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ABSTRACT

In this study we report an improved protocol that combines simplified sample preparation and micro-scale separation for mass spectrometric analysis of neuropeptides from individual neuroendocrine organs of crab *Cancer borealis*. A simple, one-step extraction method with commonly used matrix-assisted laser desorption/ionization (MALDI) matrix, 2,5-dihydroxybenzoic acid (DHB), in saturated aqueous solution, is employed for improved extraction of neuropeptides. Furthermore, a novel use of DHB as background electrolyte for capillary electrophoresis (CE) separation in the off-line coupling of CE to MALDI-Fourier transform mass spectrometric (FT-MS) detection is also explored. The new CE electrolyte exhibits full compatibility with MALDI-MS analysis of neuropeptides in that both the peptide extraction process and MALDI detection utilize DHB. In addition, enhanced resolving power and improved sensitivity are also observed for CE-MALDI-MS of peptide mixture analysis. Collectively, the use of DHB has simplified the extraction and reduced the sample loss by elimination of homogenizing, drying, and desalting processes. In the mean time, the concurrent use of DHB as CE separation buffer and subsequent MALDI matrix offers improved spectral quality by eliminating the interferences from typical CE electrolyte in MALDI detection.

1. Introduction

Mass spectrometry (MS)-based neuropeptidomics has become an increasingly intensive research area due to the importance of neuropeptides as signaling molecules and the rapid development of MS methodologies [1–5]. Because neuropeptides often exist in complex biological matrices at low concentrations, highly specific and sensitive strategies are needed to visualize and characterize the entire complement of endogenous peptides [6]. Additionally, it is often desirable to measure the changes of neuropeptides qualitatively and quantitatively in individual animals upon physiological and environmental perturbations [7,8].

Matrix-assisted laser desorption/ionization (MALDI) facilitates mass spectrometric investigation of single cells [9] and freshly dissected neuronal tissues [10–12]. This MALDI-based *in situ* methodology has evolved into several popular variants such as direct tissue imaging mass spectrometry (IMS) [13–19] and direct tissue peptide profiling [11,12,20,21]. The direct tissue peptide profiling technique can generate a quick snapshot of the peptide profiles of each tissue sample. However, interferences from high salt and lipid content in the tissue and analyte suppression often lead to incomplete and reduced peptidome coverage of a tissue sample.

Alternatively, neuropeptide tissue extraction techniques have the ability to yield peptide-rich samples for MS experiments. Currently, the most common extraction approaches are based on the use of cold acids in combination with mechanical homogenization of the tissue. Numerous enrichment or fractionation techniques [22–25] as well as several modified extraction approaches targeting protease inhibition by means of microwave-assisted irradiation and heat denaturation of protease techniques [26–31] have been developed to improve the neuropeptide analysis of different animal models. However, since most extraction experiments require pooling of multiple organs or neurons, many animals have to be sacrificed in order to obtain a sufficient coverage of the neuropeptidome. At the same time, the multiple steps associated with extraction may cause dilution, sample loss, contamination or chemically induced artifacts. These steps have, to a certain degree, masked the information of neuropeptide changes in an individual neuronal tissue.

Previously, researchers in our lab and others observed that the common MALDI matrix, 2,5-dihydroxybenzoic acid (DHB) could facilitate direct MALDI-MS analysis when used for tissue rinsing

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[11,20,32]. Recently, Romanova et al. [33] reported that the use of saturated DHB in aqueous solution could provide direct extraction of neuropeptides from neuronal tissues ranging from cellular clusters to intact brain. This one-step protocol is effective and can also be used for a long-term preservation of the neuropeptide extracts.

Once peptides have been extracted from tissue samples, the high complexity of an extract does not immediately lend itself to MS analysis. Often further separation steps are required [e.g., one-dimensional (1D) or two-dimensional (2D) HPLC] to achieve maximum neuropeptidome coverage in MS studies. Capillary electrophoresis (CE) has gained increasing attention for the analysis of a variety of signaling molecules, from small amines and amino acids to neuropeptides and larger proteins, due to its high-resolving power and low-sample consumption [34–40]. CE-MS has now become an established technique for bioanalysis of a wide variety of biological samples including neuronal tissue extracts [41,42]. We have recently demonstrated that CE fractionation is promising for global analysis of neuropeptides from complex biological samples [43]. The off-line coupling of CE to MALDI-MS analysis is not only easy to implement but also allows for independent optimization of the separation, deposition, and subsequent MS detection events. Here, we further explore the use of DHB as a multi-functional reagent for neuropeptide analysis in crab *Cancer borealis*. Specifically, DHB is utilized simultaneously as a neuropeptide extraction solvent, a novel background electrolyte for CE separation, and as MALDI matrix for MS detection. The combined use of DHB in three distinct procedures has simplified sample preparation and minimized interference from typical background electrolytes such as phosphate buffer for CE-MS experiments. This new methodology offers excellent analytical sensitivity and spectral quality for the MALDI detection of individual neuronal samples.

2. Experimental

2.1. Materials

Methanol, acetonitrile (ACN), ammonium hydroxide, trifluoroacetic acid (TFA), and acetic acid were purchased from Fisher Scientific (Pittsburgh, PA, USA). Cellulose acetate, 39.7% (w/w), average M_r 50,000, was purchased from Sigma–Aldrich (St. Louis, MO, USA). DHB was obtained from ICN Biomedicals (Costa Mesa, CA, USA). Parafilm "M" was obtained from Pechiney Plastic Packaging (Menasha, WI, USA). ZipTipC_{18} column was manufactured by Millipore and all water used in this study was doubly distilled on a Millipore filtration system (Bedford, MA, USA). The physiological saline consisted of (in mM): NaCl, 440; KCl, 11; $MgCl_2$, 26; $CaCl_2$, 13; Tris (*tris*-hydroxymethylaminomethane), 11; maleic acid, 5; pH 7.45. The sequences and concentrations of nine neuropeptide standards are: PFCNAFTGCamide (crustacean cardioactive peptide, *m*/*z* 956.37), 2.0 μM; SGGFAFSPRLamide (*m*/*z* 1037.55), 2.5 μM; CYFQNCPRGamide ([Arg^8] vasopressin, *m*/*z* 1084.45), 2.5 μM; GAHKNYLRF (*m*/*z* 1105.59), 3.0 μM; IARRHPYFL (kinetensin, *m*/*z* 1172.67), 10.0 μM; DRVYVHPFHL ([Val^5] angiotensin-I, *m*/*z* 1282.67), 7.5 μM; RPKPQQFFGLMamide (Substance P, *m*/*z* 1347.74), 7.5 μM; NFDEIDRSGFGFA (*m*/*z* 1474.66), 1.5 μM; and AGCKNFFWKTFTSC (somatostatin, *m*/*z* 1637.72), 5.0 μM.

2.2. Animal dissection and neuropeptide extraction

Jonah crabs, *C. borealis,* were purchased from the Fresh Lobster Company (Gloucester, MA, USA) and maintained without food in an artificial seawater tank at 10–12 °C. Details of the animal dissection were described previously [11,44]. Animals were cold-anesthetized by packing in ice for 15–30 min prior to dissection and the pericardial organs (POs) were dissected in chilled physiological saline.

For the conventional method, neuropeptides were extracted as described previously [21]. Briefly, the isolated organs were combined, homogenized, and peptides were extracted using ice-cold acidified methanol (methanol:glacial acetic acid:water, 90:9:1, v/v/v). The extract was dried down and resuspended in 10 μL of 0.1% (v/v) formic acid$_{(aq)}$. To extract neuropeptides from neural tissue with saturated aqueous DHB (saDHB) solution, the tissues were dissected out, placed into a vial containing 5–40 μL saDHB solutions, and stored at 4 °C for several hours up to 30 days. Following incubation, the saDHB extracted samples were ready for both direct MALDI and CE-MALDI analysis without the need for further treatment. In contrast, the acidified methanol extracts were desalted by ZipTipC_{18} and eluted in 5 μL of 50:50 ACN:0.1 % TFA(aq) (v/v) solution prior to analysis. The sample application for direct MALDI-MS with Parafilm was described previously [45]. Briefly, Parafilm coating was placed on the sample plate and 150 mg/mL DHB (in methanol:water, 50:50, v/v) was deposited onto its surface. For CE fractions, eluate was collected onto the DHB spots. For direct MALDI analysis, an aliquot of 0.5 μL of sample was applied onto the DHB spots.

2.3. Apparatus

2.3.1. CE

The laboratory-built CE apparatus was previously described [43]. Capillaries (50 μm i.d. × 360 μm o.d.) with varying lengths of 70–80 cm were from Polymicro Technologies (Phoenix, AZ, USA). The CE operated by a high-voltage power supply (HV30KVD, dual-polarity, 0 to ±30 kV, Unimicro Technologies, Pleasanton, CA, USA), was off-line coupled to MALDI-FT-MS with an on-capillary porous ionic joint interface. A pressure-initiated capillary siphoning phenomenon was employed to manipulate the stream of CE runs under a reversed polarity (negative) mode [43]. Subsequent fraction collection was performed on a series of pre-deposited nanoliter volume DHB spots on a Parafilm-coated MALDI sample plate. Prior to use, the new capillary was rinsed with (1) 75:25, NaOH (1.0 M):MeOH (v/v), (2) H_2O, (3) 0.1 M NaOH, (4) air, (5) water again, and (6) running buffer under ~0.5 psi (~3447 Pa) in sequence for 5 min in each step, followed by electrophoretic equilibration with the separation buffer for 10 min prior to injection of the sample. Steps 3–6 were repeated between CE runs to remove any residual peptides adsorbed onto the capillary wall. The sample was injected onto the CE column using a pressure of 0.5 psi for 3 s (~50 nL). Reversed polarity mode CE was then initiated by applying a voltage (−18 kV) between the inlet and the on-column porous ionic joint.

Three background electrolytes were compared for the CE-MALDI-MS in this study: (i) a cocktail of ammonium formate (100 mM):H_2O:ACN with a ratio of 5:4:1 (v/v/v), at pH 2.75; (ii) ammonium phosphate monobasic at 50 mM, adjusted to pH 2.5 with hydrochloric acid; and (iii) a background electrolyte was prepared using saturated DHB (~100 mg DHB in 5 mL water showing excess DHB falling out of solution, initial pH was approximately 2.05 by measurement) as starting material adjusted by adding NH_4OH to a final pH of 2.5–3.0. The resulting solution was then filtered by 0.22 μm Millex syringe driven filter (Millipore). Afterwards ACN was added to the filtrate to form a 95:5 saDHB:ACN (v/v) solution. This solution was then stored in dark and cool (4 °C refrigerator) conditions for less than 1 week.

2.3.2. MALDI-FT-MS

Mass spectra of the CE fractions were acquired on a Varian/IonSpec Fourier transform mass spectrometer (Lake Forest, CA, USA) equipped with a 7.0 T actively shielded superconducting magnet. The FT-MS instrument consisted of an external high-pressure MALDI source. A 355 nm Nd:YAG laser (Laser Science, Franklin, MA, USA) was used to create ions followed by accumulation in the