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One-Step Sampling, Extraction, and Storage Protocol for Peptidomics Using Dihydroxybenzoic Acid

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The isolation and extraction of natively occurring signaling peptides (SPs) from tissue is a critical first step in characterizing these peptides. Recent studies have outlined several approaches designed to preserve and extract SPs from tissue. Here, we demonstrate a surprisingly simple method to extract SPs from tissue samples, ranging from cell clusters to brain punches to intact brain regions, using a matrix often employed in matrix-assisted laser desorption/ionization mass spectrometry—2,5-dihydroxybenzoic acid (DHB). DHB allows for the effective extraction of endogenous peptides from tissue as well as long-term preservation of tissue samples and extracts. Using the mouse pituitary gland as a model, the extraction protocol effectively recovers 24 known and many additional putative peptides from individual samples. Peptide extracts stored in the DHB extraction media are stable for years without freezing. The approach is also effective for other neuronal tissues; the complement of neuropeptides in bag cell neuron clusters from the *Aplysia* central nervous system, the rat cerebellum, and rat dorsal striatum also have been examined. Advantages of this new extraction procedure are its technical simplicity, reproducibility, ease of remote preparation of samples, and long-term sample preservation without freezing.

Peptides evolved as signaling molecules early in the evolutionary process.[1] A variety of signaling peptides (SPs) are found in the nervous systems of all metazoa, from the simple hydra to the complex mammal. These molecules are often responsible not only for transfer of information inside individual organisms but also between them; for example, yeasts rely on SPs to direct mating.[1] Perhaps in no region of an animal is the SP content more complex and difficult to study than in the brain; some SPs are present throughout extended regions and at relatively high concentrations, and others are present at low levels in only a few cells. Thus, efficient characterization of a fraction of the SPs in an organism can be daunting. Despite these challenges, advances in neuropeptidomics have demonstrated tremendous gains over the past decade.[2−9]

Although methods to characterize SPs have significantly advanced as a result of improved mass spectrometers and bioinformatic tools, often the success of a neuropeptidomics experiment depends on the sample preparation and SP extraction processes. In the brain, once a peptide is released, it is can be rapidly degraded via a range of extracellular enzymatic steps. These same enzymes can degrade the peptides during extraction; thus, a number of approaches have been developed to maximize peptide extraction and recovery. Svensson et al.[10] reported that the use of focused microwave irradiation for animal sacrifice aided in the detection of endogenous neuropeptides instead of protein degradation products. Che et al.[11] have described methods to denature this enzymatic activity soon after animal death via microwave fixation. One of the most common approaches used to store and transport tissues before analysis—freezing and subsequent thawing—often contributes to further sample degradation and artifactual peptide/protein modification. The microwave approaches, when coupled with freezing, have been shown to be effective for a variety of peptidomics experiments.[10,11] However, these methods are applied to the entire brain, making manipulations of the brain before sampling difficult. For example, if tissue sections or punches are required from specific anatomical regions, or if a brain slice is first subjected to biochemical treatments or physiological recordings, these microwave approaches may not

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be practicable as it can be difficult to isolate precise brain regions after such treatments.

Perhaps the most common approach for peptide extraction is based on the use of organic solvents and strong acids in combination with mechanical disruption of the tissue.[12] This approach has proved successful for SP analyses of different animal models including mammals, insects, mollusks, and worms.[4,13−17] Over the last 15 years, we have isolated small tissue samples and individual neurons using a 2,5-dihydroxybenzoic acid (DHB) rinsing protocol for matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS).[18] In our original approach, brain tissue was briefly rinsed in DHB before cell isolation. Isolated cells were then placed on the MALDI target where peptides were extracted by an application of MALDI matrixes such as DHB or hydroxycinnamic acid. This approach produces intense peptide signals from small samples.[19−21]

Expanding on our prior approach, we report a simple and effective method for extracting and stabilizing SPs while preserving gross tissue morphology during prolonged storage, thereby eliminating the need for freezing during sample preparation. Using the mouse pituitary as a model, we demonstrate that by exposing a tissue sample to a saturated aqueous solution of the common MALDI matrix (and effective antioxidant) DHB, numerous known and putative SPs are extracted. We validate the method by applying it to profiling peptides in the morphologically defined and biochemically well-investigated bag cell neurons of the marine mollusk *Aplysia californica*. In addition, the extraction method has been tested for sample-limited applications such as individual tissue-punch biopsies from morphologically defined rat brain regions, the cerebellum and dorsal striatum.

An advantage of our approach is that a tissue sample placed into a solution of DHB is processed for SP extraction immediately after tissue isolation, with no additional steps required. Although the process is similar to on-plate SP extraction used in direct tissue MALDI MS, here it is performed on a larger scale with increased incubation/extraction times. Boosting the incubation time to hours or even days allows efficient SP transfer into solution from an intact (mechanically undisrupted and temperature untreated) brain sample such as an entire pituitary. Resulting neuropeptide extracts have a shelf life of at least 3 years, even when stored at room temperature. Qualitatively, the efficiency of peptide extraction by DHB solution is comparable to the microwave irradiation approach described elsewhere[10,11] but has an added advantage of technical simplicity and long-term sample preservation.

EXPERIMENTAL SECTION

Tissue Sample Preparation. Adult C57BL/6 mice, bred in the UIUC animal facility from stock mice obtained from The Jackson Laboratory (Bar Harbor, ME) were sacrificed by quick decapitation,[22] and pituitaries quickly surgically removed as previously described.[23] Samples of rat dorsal striatum (dSTR) from male Sprague−Dawley rats bred in the UIUC animal facility from stock obtained from Harlan (Indianapolis, IN) were collected in duplicate from symmetrical regions of 1 mm thick coronal brain slices using 2 mm i.d. tissue biopsy punches.

A. californica were obtained from Charles M. Hollahan (Santa Barbara Marine Bio., Santa Barbara, CA). Animals were maintained in constantly circulated, aerated, and chilled to 14 °C artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH). Symmetrical bag cell clusters, comprised of ∼400 neurons each, from the *Aplysia* central nervous system (CNS) were manually dissected from the abdominal ganglia as described elsewhere.[24] All procedures on animals were in accordance with animal use protocols approved by the UIUC IACUC and the UIUC guidelines on animal research.

Chemicals. Sigma-Aldrich (St. Louis, MO) was the source for all chemicals, unless stated otherwise.

Peptide Extraction and MALDI MS Samples. Immediately after isolation, individual mouse pituitaries were rinsed with an excess of physiological saline and placed in 60−100 μL of saturated aqueous solution of DHB for varying periods of time. Each *Aplysia* bag cell cluster was placed in 500 μL of saturated aqueous solution of DHB and incubated for 48 h to allow for peptide extraction. Samples of the rat dSTR and cerebellum were obtained from freshly sectioned brain slices as described above and incubated in 30 μL of aqueous saturated DHB for 48 h prior to analysis.

MALDI MS Analysis of Natively Occurring Peptides. For MALDI MS analysis, 0.7 μL of DHB peptide extract was spotted on a stainless steel MALDI target and cocrystallized with 0.7 μL of freshly prepared concentrated DHB matrix (50 mg/mL 50% acetone). Initially, MALDI mass spectra were acquired with a commercially available time-of-flight (TOF) mass spectrometer, the Voyager-DE STR BioSpectrometry Workstation (Applied Biosystems, Foster City, CA). Instrument settings were optimized for maximal sensitivity in reflectron mode. External calibration spanned the mass range from 600 to 6000 Da. After the 3 year storage period, and for the other experiments, peptide extracts were evaluated using a Bruker Ultraflex II mass spectrometer (Bruker Daltonics, Bremen, Germany) in reflectron mode with external calibration covering the 600−4000 Da mass range. Each mass spectrum shown is a sum of four acquisitions taken from the same sample; each acquisition is obtained with 100 laser shots. Selected molecular ions observed in extracts using reflectron mode and corresponding by mass to known peptides were fragmented for sequence verification using MALDI-TOF/TOF. Fragmentation of precursor ions was induced by laser without the use of collision gas.

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Scanning Electron Microscopy. Portions of brain tissue were rinsed with physiological saline and immersed in excessive volumes of DHB solution, 20 mg/mL, for 1 h. After rinsing with 0.1 M phosphate buffer (PB), tissue samples were fixed with modified Karnovsky's fixative (2% glutaraldehyde/2% paraformaldehyde in 0.1 M PB) overnight at 4 °C. Fixative was washed with several changes of 0.1 M PB for at least 30 min. Following a brief rinse with distilled water, specimens were dehydrated in a series of graded ethyl alcohol solutions, 10 min each, 33%, 50%, 70%, 95%, and three changes of 100%. Specimens immersed in ethanol were transferred into Teflon porous vials and subjected to critical point drying using the Samdri-PVT-3D drier (Tousimis, Rockville, MD). The texture and microstructure of DHB-treated specimens were compared to that of freshly isolated and fixed brain tissue samples processed in an analogous manner. For scanning electron microscopy (SEM) imaging, a 5 nm coat of palladium/gold was applied onto dried samples using a sputter coater. Images were acquired on an environmental scanning electron microscope, the XL30 ESEM-FEG (FEI Company, Hillsboro, OR), in normal SEM mode.

RESULTS AND DISCUSSION

Extraction of Peptides with an Aqueous Solution of 2,5-Dihydroxybenzoic Acid. Over the past decade, the ability of MALDI MS to investigate biological tissues, cells, and even organelles directly has resulted in the discovery of a number of important constituents in these structures, as well as determined changes in their molecular complement during different physiological and pathological events.[3,4,23,25] Such progress has been achieved, in part, due to the unique ability of MALDI matrix solutions to extract analytes from a specimen and incorporate the constituents into MALDI matrix crystals upon sample drying. A majority of direct MALDI MS applications for single cell analysis and MS imaging and profiling rely on a brief exposure of a small (or thin) sample to a MALDI matrix solution. Quick on-probe extraction of peptides and proteins allows detection of the most abundant molecular species by MALDI MS. Here we show that similar approaches can be used effectively with tissue samples on a several 100-fold larger scale.

We investigate extraction of peptides from multiple mouse pituitaries treated individually with aqueous DHB solution immediately after isolation. In contrast to standard acid-based extraction protocols, the described procedure is technically simple. The tissue sample is immersed in a ≥10× volume of aqueous DHB solution (20 mg/mL) promptly after dissection and incubated at +4 °C for various intervals to determine the optimal incubation time. The acidity of the DHB solution (pK = 3) denatures proteolytic enzymes, thus preserving ubiquitous proteins and bioactive peptides from degradation during incubation. Detectable levels of peptides are observed in the extract after 2 h incubations; however, the intensities of signal, signal-to-noise ratio, and the number of observed peptides dramatically increase over at least 24 h incubation periods. We find that 48 h is an optimal period for extraction; incubation times extending beyond 48 h do not bring substantial improvements in peptide detection in the tissue extract. Addition of fresh DHB matrix in large excess (50 mg/mL, 50% acetone, 50% water) directly onto the MALDI plate during MS sample preparation is important for obtaining high-quality spectra from raw tissue extracts prepared by this method. Although the extracts contain DHB in concentrations sufficient for MALDI applications, DHB crystals from the extracts containing the tissue, when placed on the MALDI sample plate, produce deformed crystals compared to standard DHB crystals, and peptide signals recorded from such samples are poor.

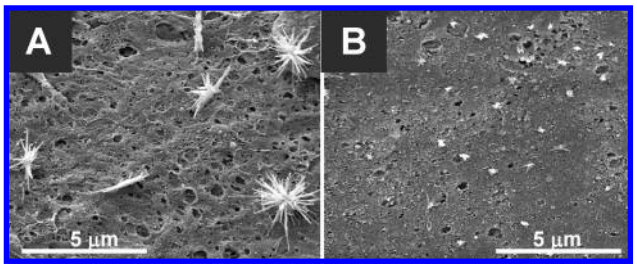

Figure 1. Scanning electron microscopy images show increased roughness and porosity of the (A) DHB pretreated brain slices relative to (B) the freshly fixed control samples.

What is the role of DHB in peptide extraction? As shown in Figure 1, a brain slice pretreated in the DHB solution for 1 h has an increased surface roughness and contains a number of pores. As seen on the electron micrograph, the pore sizes span a large range. It may be that these pores are the result of uneven tissue collapse upon exposure to an acidic pH. We speculate that these pores may facilitate the penetration of the extraction solvent into deeper layers of the tissue sample, thus making possible the extraction of peptides from tissue without grinding; the extracted peptides may diffuse outside the tissue through the pores. Advantageous for peptidomics experiments, we do not observe peaks in the mass region above 7000 Da, which may be due to the porous nature of the DHB-exposed tissue. In contrast to another common MALDI matrix, 3,5-hydroxycinnamic acid, an additional benefit of using DHB for peptide extraction is its high solubility in water: ~20 mg/mL. The use of aqueous extraction conditions favors peptide extraction, with significantly reduced lipid extraction simplifying the mass spectra and preserving the cell membranes. We observe few lipid peaks in the 400−800 Da mass range as judged by the shape of the isotopic clusters.

Analysis of Mouse Pituitary Peptides by MALDI-TOF MS. Well suited as a model to study peptide extraction from biological tissues and effects from prolonged storage of the resulting extracts, the pituitary contains a broad range of known physiologically active peptides originating from several prohormones, including pro-opiomelanocortin (POMC) protein (P01193). Many of these peptides are posttranslationally modified, allowing the stability, not only of the peptides, but also of labile posttranslational modifications such as acetylation and phosphorylation, to be followed using the DHB extraction and storage approach.

In this study, we used entire mouse pituitaries to investigate the performance of DHB as an extraction media. Pituitaries were dissected and immediately immersed in small volumes of saturated aqueous DHB solution. The effect of the DHB solution was almost immediately observed visually as a discoloration of the immersed tissue. Mass spectrometric analyses of the extraction media, whether performed in several hours or several years, demonstrate that a variety of SPs are both extracted and preserved by the DHB solution. Masses matching peptides encoded by the POMC

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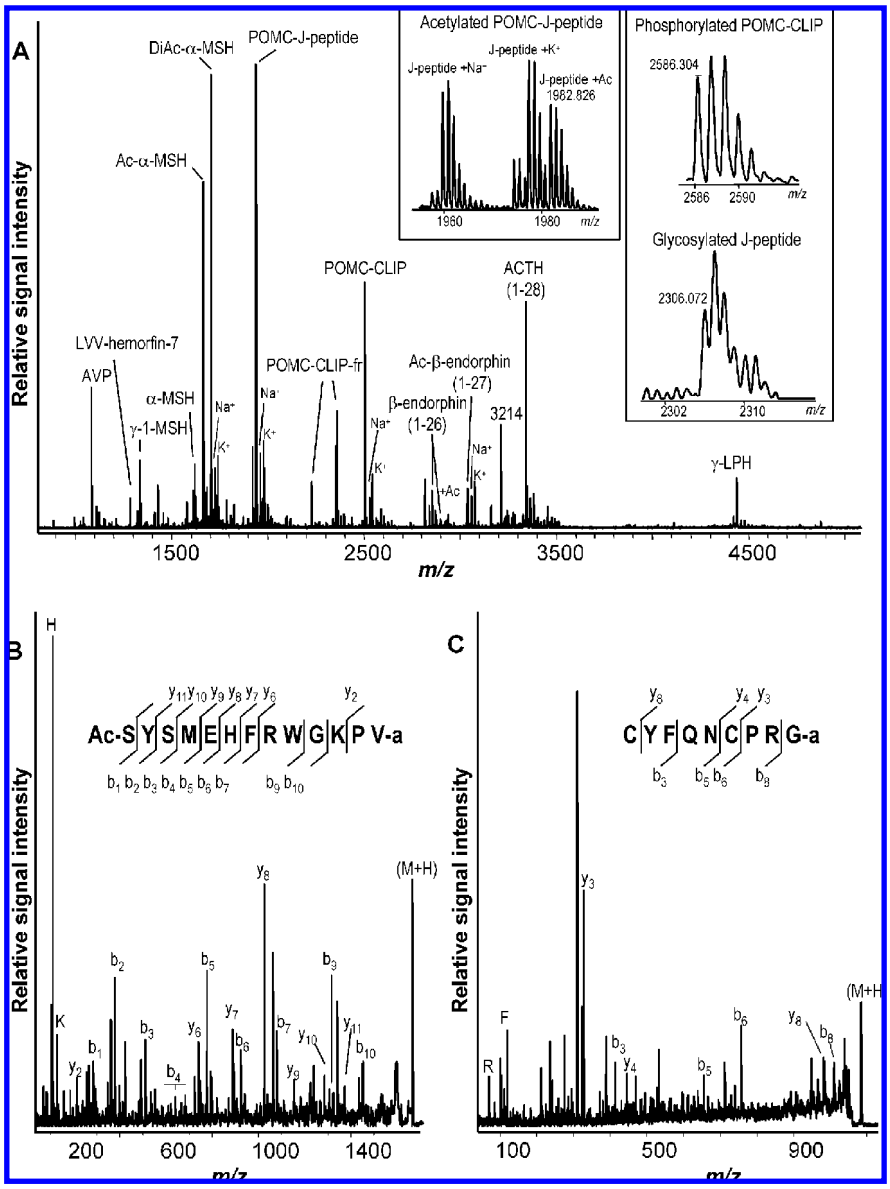

Figure 2. (A) Representative MALDI MS spectrum of an individual mouse pituitary extract prepared using DHB solution as an extraction solvent. Known peptides are labeled: Ac, acetylated; fr, fragment. POMC- and provasopressin-derived peptides are assigned on the principle of peptide mass fingerprinting after selected molecular ions are verified using MALDI-TOF/TOF MS. (B) Tandem MS spectrum confirming the assignment of Ac-α-MSH peptide from POMC precursor. (C) Tandem MS spectrum confirming the assignment of AVP peptide from provasopressin.

precursor are detected in all samples, with average mass errors of less than 30 ppm (Figure 2 and Table 1). Tandem MS performed on selected molecular ions from raw extracts (Figure 2, parts B and C), as well as the detection of multiple peptides resulting from known POMC prohormone proteolytic processing steps, adds confidence to our assignments, similar to the way the detection of multiple peptides from the tryptic processing of a protein improves the confidence of the protein identification.[26] Among the peptides detected are β-lipotropin (β-LPH), known to be the primary product of posttranslational processing in anterior pituitary, and opiate β-endorphin (1−31), produced by hypothalamic arcuate neurons regulating secretion of pituitary SPs via a hormonal pathway. Intermediate pituitary SPs, α-melanocyte-stimulating hormone (α-MSH), acetylated β-endorphin, and its truncated forms, β-endorphin (1−27) and (1−26), reported previously in wild-type mice,[27] also were detected. Adrenocorticotropic hormone (ACTH) synthesized in anterior pituitary was observed as a possibly truncated form: ACTH (1−28). To our knowledge, the existence of ACTH (1−28) peptide has not been previously reported.

Oxytocin and vasopressin peptides are localized in the posterior pituitary (pars nervosa, or neural lobe).[28] Peaks corresponding to peptide arginine vasopressin (AVP) originating from provasopressin prohormone and vasopressin-neurophysin (P35455),[29] as

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Table 1. Known Peptides Detected by MALDI MS in Raw Extract of Individual Mouse Pituitary[a]

precursor	peptide	(M + H) obsd	(M + H) calcd	mass error, ppm
hemoglobin β	LVV-hemorphin-7	1324.57	1324.72	−114
POMC [76−85]	γ-MSH	1338.53	1338.66	−100
POMC [126−136]	Ac-α-MSH (3−13)	1414.59	1414.72	−93
POMC [126−136]	di-Ac-α-MSH (3−13)	1456.61	1456.77	−113
POMC [125−136]	AC-α-MSH (2−13)	1577.66	1577.79	−82
POMC [124−136]	α-MSH	1622.70	1622.79	−59
POMC [124−136]	Ac-α-MSH	1664.71	1664.8	−54
POMC [124−136]	di-Ac-α-MSH	1706.73	1706.81	−47
POMC [103−120]	J-peptide	1940.81	1940.86	−27
POMC [103−120]	Ac-J-peptide	1982.83	1982.86	−15
POMC [102−120]	J-peptide + N-terminal Arg	2096.93	2096.99	−30
POMC [141−160]	CLIP (1−20)	2230.13	2230.14	−4
POMC [103−120]	glycosylated J-peptide	2306.00	2306.01	−4
POMC [141−161]	CLIP (1−21)	2359.20	2359.17	11
POMC [141−162]	CLIP	2506.30	2506.26	16
POMC [141−162]	phosphorylated CLIP	2586.30	2586.19	41
POMC [205−230]	Ac-β-endorphin (1−26)	2900.64	2900.6	15
POMC [205−231]	Ac-β-endorphin (1−27)	3037.74	3037.58	54
POMC [142−151]	ACTH (1−28)	3344.92	3344.92	0
POMC [170−202]	γ-LPH (10−38)	3456.64	3456.65	−2
POMC [165−202]	γ-LPH	4437.90	4437.14	172
provasopressin	vasopressin	1084.33	1084.45	−115
provasopressin	154−end	1635.75	1635.83	−51
provasopressin	151−end	1976.01	1976.06	−26
av mass error (ppm)				−27

[a] Monoisotopic masses of protonated ions are listed as (M + H). Peptides encoded by POMC and provasopressin are assigned on the principle of peptide mass fingerprinting after the sequences of a selected peptides from the same prohormone were confirmed via tandem MS.

well as two previously reported provasopressin end peptides,[11] are present in almost every mass spectrum (Figure 2C). Processed AVP is secreted by the nerve endings in the neural lobe of the pituitary, which explains the peptide presence in our samples. The proteolytic product of another prohormone thought to colocalize with proAVP, oxytocin-neurophysin 1 (P35454), was not detected in these extracts.

The DHB extraction protocol resulted in spectra that lacked numerous previously reported fragments of ubiquitous proteins. For example, known degradation products of hemoglobin and chromogranin were not observed, even though these are commonly reported in MS studies of the mouse pituitary peptidome that have used other common homogenization/extraction approaches.[11,30] Euthanasia by decapitation, as used in this study, minimizes stress but has the disadvantage of leaving a substantial volume of blood in the brain and in other organs, including the pituitary. The lack of hemoglobin peaks indicates a reduction in proteolytic degradation products and selective extraction of SPs using this approach. A few observed, unidentified signals may result from peptide hypothalamic releasing factors usually found in the portal blood system that bathes the anterior pituitary, controlling the secretion of pituitary hormones.

Pituitary adenylyl-cyclase-activating polypeptide (PACAP) is reported to be expressed in the pituitary and produces two known bioactive peptides, PACAP (1−27) and PACAP (1−38); neither were observed in this study. These PACAP peptides have not been observed in other peptidomic studies of mouse or rat pituitary.[11,23,30−33] However, PACAP (1−38), as measured by radioimmunoassay, has been reported to be present at about 45 fmol/mg wet tissue in diencephalons of the rat brain.[34] The low level of PACAP may be the reason PACAP-related peptides have not been detected; for example, the easily detected α-MSH peptide from POMC precursor has been found at 10−20 pmol per individual pituitary[35] or approximately 2000 pg α-MSH/μg protein.[36] One of the unassigned peaks in our mass spectra, 3213.8 m/z, corresponds by mass to a protonated ion of the PACAP-related peptide (82−109) and is tentatively assigned as such.

Interestingly, we observe fewer fragments of the J-peptide and several other POMC peptides reported by others[11] resulting from cleavages at an Asp−X bond that is especially sensitive to acid-induced cleavage.[37] These cleavages have been thought to occur during disruption of tissue by sonication, treatment with strong acid and/or heat, or during microwave irradiation.[11] In addition to its unmodified form, we detect masses corresponding to J-peptide with N-terminal acetylation, and with a recently reported O-glycosylation of Ser[15] with a disaccharide.[11] Likewise, masses of diacetylated α-MSH peptide and phosphorylated CLIP are readily detected.

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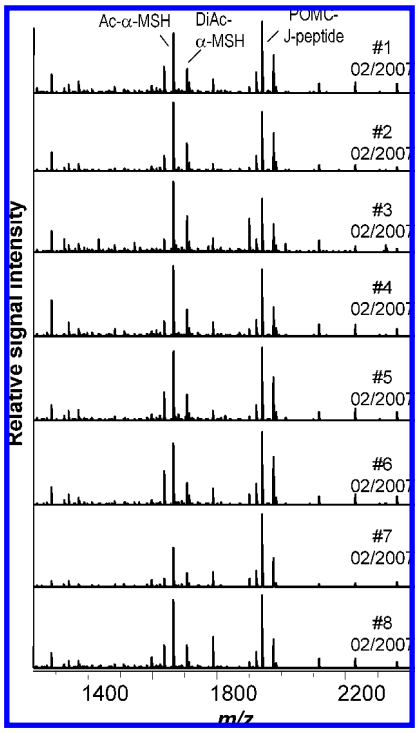

Figure 3. Long-term preservation effect of DHB on peptide extracts and robust reproducibility of peptide signals detected by MALDI MS in eight individual samples of mouse pituitary extracts prepared using DHB solution as an extraction solvent. Traces are labeled with respective animal identity number. Tissue samples acquired in June 2004 were stored in the original DHB extraction solution at +4 °C; MALDI samples were prepared and run in February 2007.

The lower occurrence of peptide and protein fragmentation in our experiments suggests that the DHB approach is a mild and effective extraction method that preserves the SP complement found within the appropriate brain region during the sampling and extraction procedure. Further, extraction of peptides with the DHB solution shows reproducibility among individual samples. In Figure 3, the peptide profiles of eight individual pituitary samples are compared. The texture of microwave-irradiated tissue is reported to change, which may hinder the precise isolation of small, well-defined brain regions.[11] With DHB extraction the sample is collected from an unmodified organ or tissue, thus allowing higher morphological sampling precision.

Preservation of Peptides Stored in DHB Solution. Earlier we reported that DHB treatment aids in microdissection of individual neurons by stabilizing cell membranes, deactivating endogenous proteolytic enzymes, and reducing high salt concentrations in samples of marine specimens, thus improving MALDI MS spectra quality.[18] We also demonstrated that samples of endogenous peptides cocrystallized with DHB can be stored in a freezer for years and reanalyzed when needed. A striking finding of this study is that the peptides extracted from a tissue sample into the DHB solution are stable over a period of years. Here we compare peptide profiles of eight individual pituitary extracts stored in saturated DHB aqueous solution for almost 3 years at +4 °C. These samples exhibit a remarkable stability of peak pattern, with the exception of a di- and mono-Ac-α-MSH (Figure 4). The relative intensity of the Ac-α-MSH peak increased after storage, but the di-Ac-α-MSH peak reduced after storage. In our experiment, peptide extracts were stored with the tissue, thereby representing continuous tissue incubation/extraction. It is not known whether extracts would be stable for so long if tissue samples were removed at some point during storage.

What makes DHB tissue extracts so long-lasting? Although the molecular mechanism is not well understood, it is likely that the preservation effect of the DHB on raw tissue is due to a combination of its antioxidative[38] and bacteriostatic properties in concert with the acidity of the solution. Benzoic acid derivatives substituted by hydroxy groups such as in DHB have active bacteriostatic properties[39] and are often used in the pharmaceutical industry (http://www.chemicalland21.com/lifescience/phar/2,5-DIHYDROXYBENZOIC%20ACID.htm). In addition, DHB, or gentisic acid, is a potent free-radical scavenger[38] with a minimal chelating effect.[40] A metabolite of aspirin, DHB is stable under normal conditions and is used as a stabilizer in diagnostic tissue

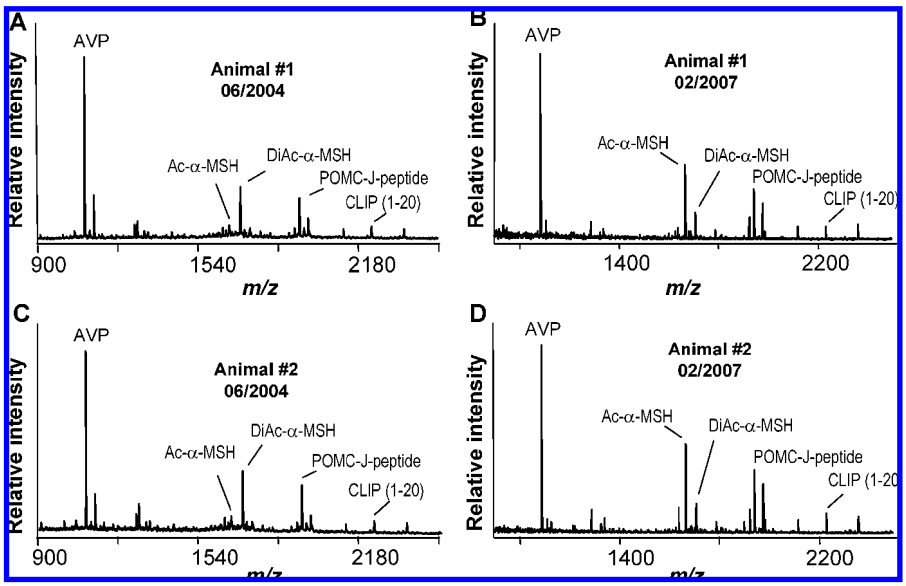

Figure 4. Representative MALDI MS spectra illustrate reproducible changes in signal intensity of di- and mono-Ac-α-MSH in mouse pituitary extracts with time: (A and C) freshly prepared extracts; (B and D) extracts after 3 years of storage at +4 °C.

imaging and radiolabeling.[41−43] Interestingly, in human plasma, levels of gentisic acid may reach 20 μM under aspirin therapy, which leads to a proven concentration-dependent inhibitory effect on oxidation of lipoproteins in human plasma.[40,44,45] It has been suggested that activated neutrophils can produce DHB via hydroxylation of salicylate by an unknown pathway in patients with inflammatory disorders.[46] Moreover, DHB is one of the most commonly occurring aromatic acids of plant tissue.[47] Due to the presence of a carboxylic group on the aromatic nucleus, gentisic acid has a surprisingly low toxicity, unlike other phenols.[48,49] According to a chemical vendor, Science Lab.com, DHB has an acute oral toxicity in mice (LD50: a dose causing death in 50% of the animals) of 4500 mg/kg (http://www.sciencelab.com/xMSDS-2,5-Dihydroxybenzoic_Acid-9923770) comparable to the LD50 of 4000 mg/kg for sea salt NaCl (http://www.sciencelab.com/msds.php?msdsId=9927593). DHB is also used in dermocosmetic formulations to stimulate the process of epidermal renewal and nontherapeutic skin treatment of wrinkles, fine lines, and blemishes of the skin (http://www.freepatentsonline.com/5766613.html). Another possible protective mechanism is formation of thermodynamically stable complexes with biomolecules containing iron or other metal catalysts of the free-radical oxidation process,[50] which may protect the SPs from side-specific oxidation.

We find that crystallization of DHB from the DHB tissue extracts during drying under vacuum dramatically reduces peptide signal intensities in the remaining volume of extract. In addition, DHB does not precipitate from saturated DHB-tissue extracts stored at +4 °C, although a regular saturated DHB solution does. We are not certain what the effect of the tissue is, but its presence may play a role in DHB's long-term preservation of peptides in tissue extracts.

Application of the DHB Extraction Method to the Analysis of Peptides from Other Tissues. To validate the new extraction method and expand its utility, we demonstrate two additional applications to previously characterized cells and tissue. First, we analyzed peptides present in the CNS of *Aplysia*; specifically, bag cell neuron (BCN) clusters located in the vicinity of the abdominal ganglion and situated on the pleuro−visceral connectives. Each cluster contains several hundred small (<50 μm) neurons that

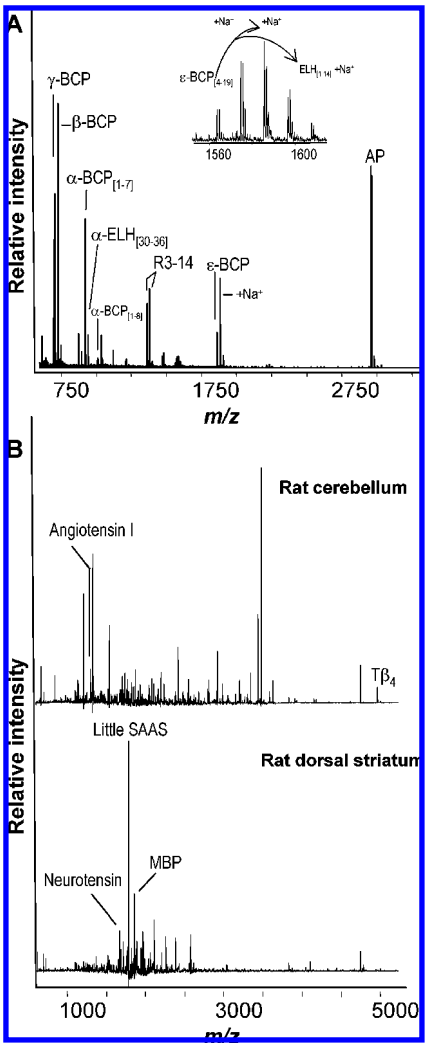

Figure 5. MALDI MS spectra of the DHB peptide extracts from various samples. (A) Bag cell neurons known to express egg-laying hormone precursor (P01362) in the *Aplysia* CNS. Peaks corresponding by mass to predicted peptides are labeled: BCP, bag cell peptides α, β, γ, and ϵ; AP, acidic peptide; ELH, egg-laying hormone. In addition, peptide II encoded by R3−14 prohormone (P01364) and expressed in R3−14 neurons adjacent to bag cell neurons is observed in its native and modified form (pyrrolidone carboxylic acid at the N-terminus). (B) Top: rat cerebellum. Peaks corresponding by mass to predicted peptides are labeled: Tβ_4, thymosin β 4. Bottom: rat dorsal striatum. Peaks corresponding by mass to predicted peptides are labeled: MBP, myelin basic protein-derived peptide.

are well characterized morphologically, functionally, and biochemically.[20,51−53] When using an extraction volume of DHB solution more than 100-fold larger than the tissue, numerous peptides known to be contained in BCNs are detected (Figure 5A). Notably, salt concentration in the *Aplysia* tissue/cell samples is 480 mM,[54] but minimal salt adducts are observed. An exception is ϵ-BCP, with a sodium adduct peak that is more intense than the protonated molecular ion peak in the spectrum, a situation reported previously using other sampling methods.[20]

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Second, we evaluated peptides from morphologically defined regions of the rat brain, the dSTR, and cerebellum, sampled with a biopsy punch from freshly prepared brain slices. As illustrated in Figure 5B, top and bottom, peaks corresponding to known peptides located in these brain regions, angiotensin,[55] neurotensin,[11] little SAAS,[11] and thymosin β,[13] and many putative peptide peaks are readily detected in the extract prepared from a minute amount of sample tissue. The different patterns of putative peptide peaks observed in dSTR and cerebellum suggest that the DHB extraction method preserves unique peptide content in the brain regions.

Interfacing the DHB Extraction Method with Liquid Chromatographic Separations. This approach is well suited to direct MALDI, but will it work with other MS approaches? Although not tested here, with the high number of peptides recovered in the tissue extracts, the importance of interfacing the extraction procedure with separation methods may be significant. The issue is the large excess of DHB relative to the peptide present in the extracts. During a reversed-phase liquid chromatography (LC) separation, DHB often comigrates with polar peptides of 500−1500 m/z.[56] A number of approaches, however, have been reported for purification of gentisic acid from biological samples such as plasma,[57] biological fluids,[58] and plants.[39,59] The most promising may be solid-phase extraction and/or LC methods based on ion-exchange and mixed-mode interactions. As one example, peptide extracts can be purified from DHB using TiO_2 sorbent, which has been shown to complex with DHB under acidic conditions.[60] Last, multidimensional LC should be effective; because of its pK_a of 3, DHB can be eliminated during ion-exchange separation according to the first dimension, while peptides (with pK_a typically in the range of 4−8) can be resolved in successive separations. Finally, with the advent of mixed-mode sorbents, purification and separation of the DHB peptide extracts may be achievable in a single step.

CONCLUSIONS

The efficacy of the described DHB extraction approach extends to the measurement of SPs from all chemically complex brain tissues tested in this study. Although a number of effective approaches are available to sample and extract SPs from tissue samples, this approach is simpler and has distinct advantages for extracting peptides from a variety of tissue samples ranging in size and chemical composition. The method not only allows effective recovery of numerous known peptides from *Aplysia* and mammalian brains in amounts sufficient for direct mass spectrometric detection but also preserves for years the extract and tissue samples without freezing. It is easily employed when the tissue sample is used for other purposes besides SP extraction such as functional tests of SP function in a brain slice or punch prior to measurement. A combination of technical simplicity, robustness, and reliability makes the new protocol amendable to the analysis of a wide variety of biological samples ranging from singe cells to brain regions.

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