Journal of Chromatography B, 879 (2011) 657–661

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Short communication

Application of matrix solid-phase dispersion methodology to the extraction of endogenous peptides from porcine hypothalamus samples for MS and LC–MS analysis

Xiaoming Cai, Chaoran Wang, Junyan Xu, Xingya Xue, Xiuli Zhang*, Xinmiao Liang

Key Lab of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, Liaoning, PR China

ARTICLE INFO

Article history:
Received 28 September 2010
Accepted 30 January 2011
Available online 21 February 2011

Keywords:
Endogenous peptides
Matrix solid-phase dispersion extraction
NanoLC–MS/MS
Peptide extraction
Porcine hypothalamus

ABSTRACT

In this study, we investigated a novel application of matrix solid-phase dispersion (MSPD) methodology for the extraction of endogenous peptides from porcine hypothalamus tissue samples. Several experimental factors of the MSPD procedure were examined. Finally, silica-based octadecyl was chosen as dispersing material and blended with 0.25 g porcine hypothalamus at a ratio of 5, and 10 mL of 60% acetonitrile with 0.2% formic acid in water was chosen as the extraction and elution solvent. This MSPD extraction method was compared to the classic acid extraction method. More peaks were observed in the MSPD extracts (74 ± 5) by MALDI-TOF MS than in acid extracts (34 ± 5). Moreover, 14 potential endogenous peptides were identified in the MSPD extracts after nanoLC–MS/MS analysis, while only 2 endogenous peptides in the acid extracts. These results indicated that MSPD could be employed as a simple and efficient method for the extraction of endogenous peptides from tissues.

1. Introduction

Endogenous peptides perform a crucial role in cell–cell communication and involve in a wide variety of animal behavioral mechanisms [1–3]. An efficient extraction of the peptides from complex samples is critical for experimental success in studies of endogenous peptides, since other components in samples can complicate analysis. The most commonly used approach for endogenous peptides is acid extraction, in which peptides are extracted from tissues by strong acid solutions following mechanical disruption and rapid heating process [3,4]. Although widely used, there are still many limits of this method, such as low efficiency for the extraction of acidic and very hydrophobic peptides, unsuitable for thermally and acid labile peptides, excessive time-consumption, and so on. Therefore, new extraction methods should be developed for endogenous peptides.

Matrix solid phase dispersion (MSPD) is an analytical process for the preparation, extraction and fractionation of compounds from solid, semi-solid and highly viscous biological samples [5–7]. It can perform extraction and clean-up stages simultaneously, and eliminates most of the complications associated with the classical liquid–liquid and solid phase extractions (SPE). In the MSPD process, the samples are architecturally disrupted by manually blending with solid support (takes about 30 s), producing a new sample matrix-solid support phase which can provide an enhanced surface area for subsequent extraction [5]. The mixture of sample and solid support is then packed as a column in a syringe and washed with different solvents. The clean-up step is conducted with a co-column. The elution from the column could be analyzed directly by LC/MS or MS/MS. MSPD simplifies the process and reduces the time for sample preparation, and has found favor in many applications [8–14]. However, the feasibility of using MSPD for endogenous peptides has not been tested yet.

So, here we propose the novel application of MSPD for the extraction of endogenous peptides from porcine hypothalamus samples. Several factors that affect the MSPD extraction were examined to obtain a suitable extraction condition. Then, the MSPD extraction method was compared with the commonly used acid extraction method. Finally, 14 potential endogenous peptides were identified in the MSPD extracts after nanoLC–MS/MS analysis, but only 2 were identified in the acid extracts.

2. Experimental methods

2.1. Sample collection and extraction

About 50 porcine hypothalami were obtained from a local slaughterhouse and mixed together, and then divided into 50 glass

* Corresponding author. Tel.: +86 411 84379521; fax: +86 411 84379539.
E-mail address: zhangxiuli@dicp.ac.cn (X. Zhang).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.01.038

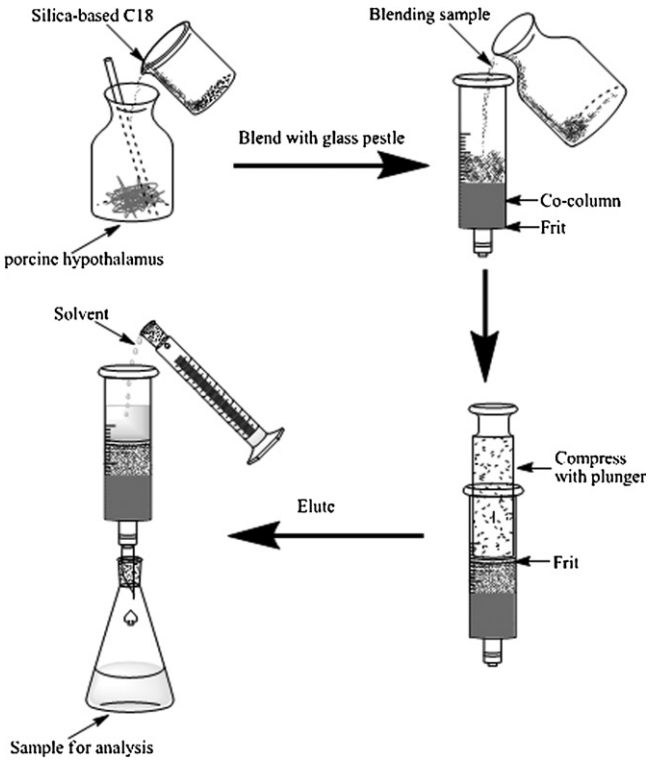

Fig. 1. MSPD extraction system and steps.

bottles equally (0.25 g/bottle). Each of these 50 units was stored at −80 °C until extraction.

For MSPD extraction (shown in Fig. 1), one unit of sample was removed from the freezer and blended with 1.25 g silica-based octadecyl adsorbent (Sipore, Dalian, China) in its glass bottle for 1 min with a glass pestle. Then, the mixture was transferred to a 10 ml SPE cartridge that contained 2.0 g of C18 (clean-up layer) at the bottom. Finally, a polyethylene frit was put at the top of the column and slightly pressed to remove air and avoid preferential channels. The column was washed first with 10 ml 0.2% formic acid (FA)/water to remove salt and impurities. Then, peptides were eluted from the column with 10 ml 60% acetonitrile (ACN)/0.2% FA. Non-polar components and matrix from the tissue sample were remained in the column. All of the adsorbent, containers, and solutions were cooled for more than 2 h before use.

For acid extraction, hypothalamus sample was removed from the freezer and prepared by reported method [15,16].

2.2. MALDI-TOF and nanoLC–MS/MS analysis

MALDI-TOF MS was performed on a Bruker AutoflexTM instrument (Bruker Co., Bremen, Germany) in linear mode. The instrument was equipped with a nitrogen laser (λ = 337 nm) and other settings were optimized for maximal sensitivity. 1 μL of extracts was spotted on a ground-steel MALDI target and crystallized by 1 μL dihydroxybenzoic acid (25 mg/mL). All mass spectra were obtained in the positive mode with a mass range of 500–4000 Da.

NanoLC–MS/MS analysis was performed on a nanoACQUITY UPLC system (Waters, MA) coupled to a Q-TOF Premier mass spectrometer. The peptide extract was concentrated (ACN removal) and diluted to 600 μL with 0.1% trifluoroacetic acid (TFA), then filtered through a 10-kDa molecular weight cutoff tube at 14,000 g for 45 min at 4 °C and rediluted (10 fold) by 0.1% TFA. 5 μL of this solution was loaded onto a symmetry C18 (5 μm) trap column (180 μm × 20 mm, Waters corporation) at a flow rate of 8 μL/min using 0.1% FA in water. Separation was performed on a house-packed C18AQ (5 μm, 120 Å) capillary column (75 μm × 150 mm). The mobile phase consisted of A (0.1% FA/water) and B (0.1% FA/ACN). Gradient elution was set as follows: 0–15 min, 98% A; 15–90 min, 98–65% A; 90–95 min, 65–20% A; 95–100 min, 20% A. The flow rate was 0.5 μL/min. The peptides were eluted into a nano-electrospray ionization Q-TOF MS and detected in positive mode. Data was collected by the data directed analysis MS method: MS survey was from *m*/*z* 200 to 1950 and with a scan time of 0.6 s/scan; intensity threshold for switching from MS scan to MS/MS was set at 20 ion counts; the top three ions of greatest intensity were selected for MS/MS study; MS/MS spectra were recorded in the range of 50–1990 Da and with a scan time of 0.9 s/scan; other settings were optimized for maximal sensitivity.

Table 1
Number of ion signals detected under different extraction conditions.

Tested MSPD extraction conditions	Total number of ions (mean ± SD)
Type of solid support	
Silica-based C18	63 ± 1
Silica-based C8	55 ± 1
Oasis HLB	60 ± 1
C18 mass/sample mass radio	
3:1	30 ± 3
5:1	73 ± 5
7:1	60 ± 2
9:1	60 ± 6
Elution solvent	
20% ACN (0.2% FA)	57 ± 0
40% ACN (0.2% FA)	60 ± 1
60% ACN (0.2% FA)	71 ± 1
80% ACN (0.2% FA)	65 ± 3
Elution volume	
5 mL	48 ± 3
10 mL	68 ± 1
15 mL	68 ± 2

2.3. Data processing

For each MALDI MS spectrum, a peak-picking algorithm (centroid, signal-to-noise ratio > 6) was applied by use of flexAnalysis software (Bruker Daltonics, Bremen, Germany), and files containing mass lists and peak intensities were exported to Excel file format for further analysis. The nanoLC–MS/MS data was processed by Micromass Proteinlynx 2.2.5. The resulting .pkl files were searched against the PIG_NCBI Database using Mascot version 2.1 (Matrix Science). Monoisotopic masses were used for the search. No enzyme was specified, and the variable modifications C-terminal amidation, N-terminal acetylation, and methionine oxidation were included in the search parameters. Peptide mass tolerance was set at 0.8 Da, and MS/MS tolerance was set at 0.5 Da.

3. Results and discussion

3.1. Selection of suitable MSPD extraction conditions for endogenous peptides

Several experimental factors of the MSPD procedure were examined to obtain suitable MSPD extraction conditions for peptides in porcine hypothalamus tissue. MALDI-MS was used for a quick characterization of the extracts in these optimization experiments. Finally, silica-based octadecyl was chosen as dispersing material and blended with 0.25 g porcine hypothalamus at a ratio of 5, and 10 mL of 60% ACN was chosen as the extraction and elution solvent. The tested factors, conditions and number of detected peaks in MALDI-TOF MS are shown in

Table 1. Two replicates were performed for each set of the experiment.

Firstly, three different types of dispersing materials (40–60 μm): silica-based octadecyl (C18) and octyl (C8), and reverse phase hydrophilic and hydrophobic copolymer (Oasis HLB, Waters), were tested. 0.25 g of porcine hypothalamus was blended with 1.75 g of dispersing material, and MSPD extraction was performed as described in Section 2. Although the number of ions in the extract of the C18 column (63 ± 1) is not much more than those from the C8 column (55 ± 1), the intensity of the ions in the 1500–3000 Da mass range was higher in the C18 extract (shown in Fig. 2). It may be due to the stronger van der Waals attractions between the peptides and C18 chains. Interestingly, the number of peptides in the MSPD extracts from the HLB column (60 ± 1) was similar to those from the C18 column, although Aristoteli et al. has reported that Oasis HLB can extract more peptides than the C18 support from the liquid samples [17]. It is possible that the polymer was too soft to disrupt the cell tissue when performing MSPD extraction. Therefore, C18 was chosen as the solid support for subsequent experiments.

Then, four different ratios of C18 material to hypothalamus mass: 3, 5, 7, and 9, were tested. As shown in Table 1, the best ratio was 5–1. The poor extraction efficiency at smaller ratio (3–1) may be due to the inadequate surface area of the support for the dispersion and absorption of peptides. When greater ratios (7–1 and 9–1) were used, the excess surface area and bonded phase on the support may have caused the excess adsorption of some peptides, reducing the number of ions.

We also tested four solvents with different polarity by mixed ACN and water at different volume ratios: 20/80, 40/60, 60/40 and 80/20, respectively. 0.2% FA (v/v) was added to the solvents for inactivating the proteolytic enzymes. Results (Table 1) showed that the MSPD extraction of peptides was not significantly affected by changes of the ACN/H_2O ratio. The highest extraction efficiency was

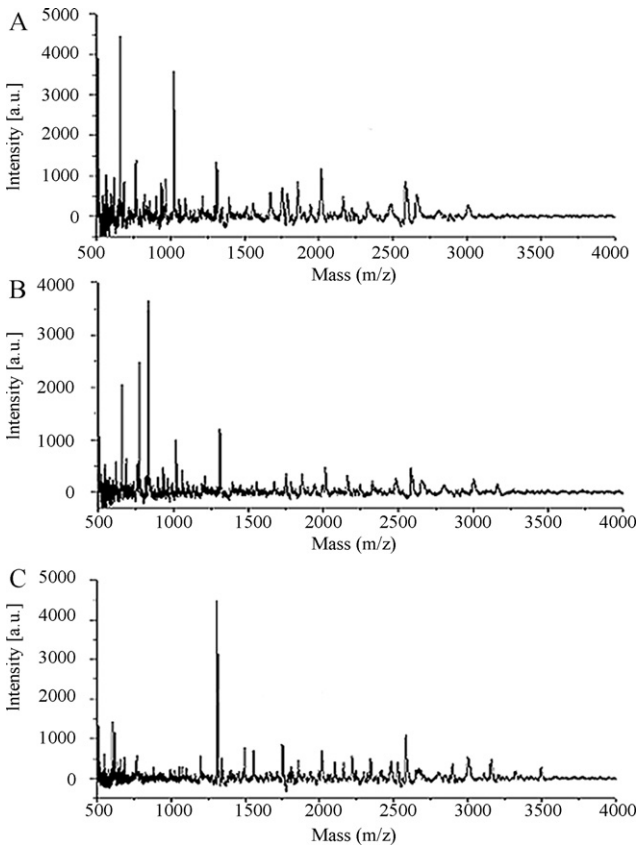

Fig. 2. MALDI-TOF MS analysis of peptide extracts obtained from the three different MSPD dispersing materials: A-C18; B-C8; C-HLB.

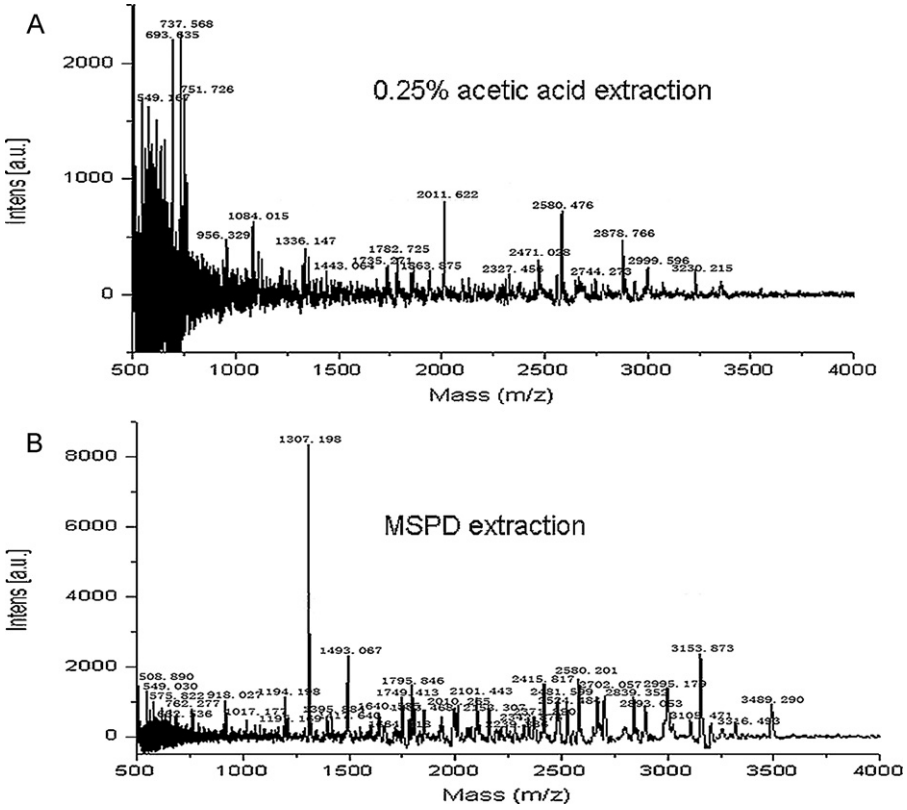

Fig. 3. MALDI-TOF MS analysis of the peptide extracts obtained from acid extraction and MSPD extraction.

Table 2
Peptides identified from known neuropeptide precursors.

Precursor	Peptide name	Peptide sequence	*m/z*	Mr (calc)	Mr (expt)	Delta	MSPD[a]	AE[b]
Hemoglobin β	LVV-hemorphin-7	L.VVYPWTQRF.F	598.3367	1194.6588	1194.6186	0.0403	Y[c]	N[d]
POMC	Corticotropin 145–151	G.KPVGK.K	528.3392	527.3319	527.3431	−0.0112	Y	N
POMC	j-peptide 108–134	R.EEEEVAAGEGPGPRGDGVAPGPRQD.K	826.0656	2475.1750	2475.1262	0.0488	Y	Y
POMC	NPP 89–117	R.RNGSSSGGGGGGGGAGQKREEEEVAAG.E	605.3169	2417.2385	2417.0916	0.1469	Y	N
POMC	Lipotropin 178–184	L.AGAPP.E	412.2402	411.2329	411.2118	0.0211	Y	N
POMC	Lipotropin 209–218	E.AEAAEKKD.E	431.2711	860.5276	860.4239	0.1037	Y	N
POMC	j-peptide 125–131	G.VAPGP.R	440.7677	439.7604	439.2431	0.5173	Y	N
POMC	NPP 54–61	L.SAETPV.F + acetyl (N-term)	644.8622	643.8549	644.3017	−0.4468	Y	N
POMC	j-peptide 109-134	E.EEEVAAGEGPGPRGDGVAPGPRQD.K	783.0442	2346.1108	2346.0836	0.0271	Y	Y
POMC	j-peptide 114-131	A.AGEGPGPRGDGVAPGP.R + amidated (C-term)	695.3577	1388.7008	1388.6797	0.0212	Y	N
secretogranin II	Secretoneurin 201-214	F.QELGKLTGPNNQ.K	649.8590	1297.7034	1297.6626	0.0408	Y	N
secretogranin II	Secretogranin-2 593-610	L.EYLNQEKAEKGREHIA.K	639.0068	1913.9986	1913.9595	0.0391	Y	N
DBI	DBI 1-27	M.SQAEFEKAAEEVKNLKTKPADDEML.F + acetyl (N-term)	955.1723	2862.4951	2862.3956	0.0994	Y	N
DBI	DBI 1-28	M.SQAEFEKAAEEVKNLKTKPADDEMLF.I + acetyl (N-term)	753.3870	3009.5189	3009.4641	0.0548	Y	N

[a] Samples obtained from matrix solid-phase dispersion extraction.
[b] Samples obtained from acid extraction.
[c] Peptide had been identified in the samples.
[d] Peptide had not been identified in the samples.

gotten when a solvent of medium polarity (60% ACN) is used as the elution solvent. The improved extraction by the 60% ACN solvent may be due to the better solubility of both hydrophobic and hydrophilic peptides in this medium polarity solvent.

Different volumes of the elution solvent were also tested to ensure efficient extraction and elution of the peptides from the MSPD column and co-column. The peptide extraction decreased when 5 mL of elution solution was used (48 ± 3), while peptide extraction was identical for volumes of 10 mL and 15 mL (68 ± 1 and 68 ± 2). The decreased number of detected ions in the MSPD extract with a 5 mL of elution solution was a result of inefficient extraction and elution of peptides.

3.2. Comparison of MSPD with acid extraction

The MSPD method for peptide extraction was compared to the conventional endogenous peptide extraction method, namely ice-cold acid extraction after boiling in hot water (hereinafter to be referred as acid extraction). With respect to time efficiency, the MSPD extraction process could be finished in about 10 min while over 1 h was needed to complete the acid extraction with a same amount of sample. Furthermore, the time needed for MSPD extraction did not significantly increase in response to an increase of sample amount, while time needed for the acid extraction did increase. So, it seems that the MSPD method would be effective for handling peptide samples for both analysis and preparation scale.

MALDI-TOF MS spectra of the peptide extracts obtained from the two extracts are shown in Fig. 3. It is clear that the MSPD method (74 ± 5, Fig. 3B) resulted in the extraction of a higher number of peptides than the acid extraction method (34 ± 5, Fig. 3A). The ion intensities of some peptides in the mass range of 500–800 Da were slightly higher in the acid extracts than the MSPD extracts. It may be because that many short peptides (about 5 residues in length) are hydrophilic and generally soluble in aqueous media, while these peptides would have weak interactions with the hydrophobic C18 chains.

NanoLC–MS/MS analysis was conducted to identify peptides in the MSPD and acid extracts. In total, 14 potential endogenous peptides were identified from the MSPD extracts, while only 2 possible endogenous peptides were identified from the acid extracts (Table 2). The nanoLC–MS/MS chromatography of the two extracts with identified peptides marked is shown in Fig. 4. It is possible that the identified peptides in this study was fewer than in previous studies involving rat (or mouse) hypothalami [2,16,18], this was due to postmortem degradation of endogenous peptides. After all, more time is needed after slaughter to obtain hypothalamus samples from pigs than from rats. Although a high number of endogenous peptides were not identified in

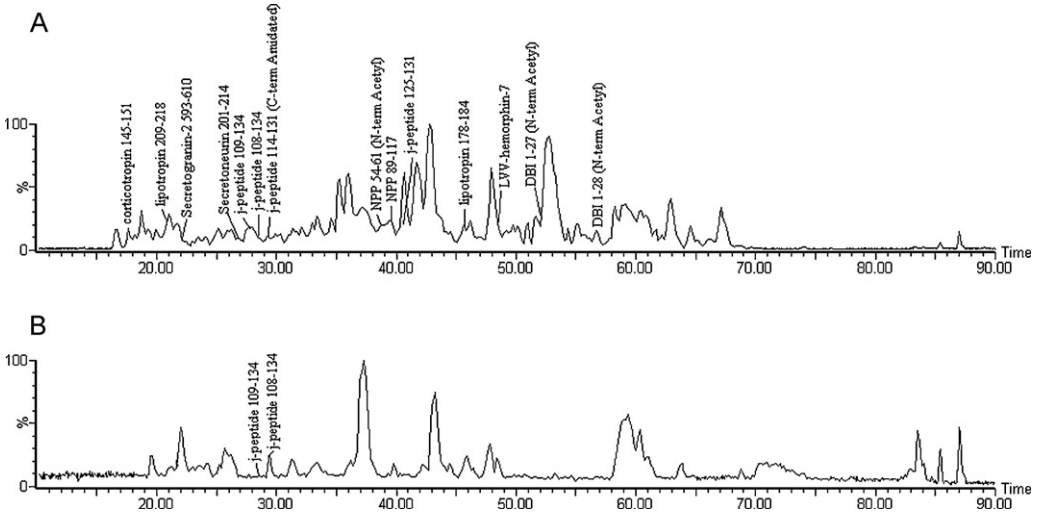

Fig. 4. Typical base peak chromatograms of the extracted endogenous peptides from the porcine hypothalamus tissue using (A) MSPD extraction and (B) acid extraction. The identity of peptides, as identified by using nanoLC–MS/MS, is indicated.

this study, enough peptides were identified for the purpose of comparing the two extraction methods. Of the 14 identified peptides, 13 were derived from secretory pathway precursors, and the LVV-hemorphin-7 from hemoglobin is an endogenous ligand for the angiotensin IV receptor. Interestingly, we identified two N-terminal acetylated peptides from the known neuropeptide precursor, diazepam-binding inhibitor (DBI). These novel acetylated N-terminal fragments of DBI were first identified in rat hypothalamus samples by Dowell et al. [16], and this was the second time that they were identified. It was worth mentioning that these two novel peptides from DBI could only be identified with 2D LC–MS/MS analysis in Dowell's study, but in our study they were identified with 1D LC–MS/MS analysis with an MSPD extraction. The MSPD method improved the extraction efficiency of the two DBI peptides and facilitated their identification. In the MSPD extracts, we also identified one novel N-terminal acetylated peptide and one new C-terminal amidation peptide from another neuropeptide precursor: proopiomelancortin. The most common modifications in bioactive neuropeptides are N-terminal acetylation and C-terminal amidation, and although the N-terminal acetylation cannot definitively indicate peptide activity, it may imply peptide bioactivity. After all, acetylation can affect peptide–receptor recognition and is necessary for the bioactivity of many peptides [16,19]. All of these results collectively infer that additional novel endogenous bioactive peptides could be identified through the use of MSPD as an extraction method.

4. Conclusions

In this study, we investigated the novel application of MSPD for the extraction of endogenous peptides from porcine brain tissue samples. A suitable MSPD extraction condition for peptides was obtained through several optimization experiments. The efficacy of the MSPD peptide extraction method was compared to the commonly used acid extraction method. It can be seen from the MALDI-TOF and nanoLC–MS/MS analysis results that a greater number of total peptides and potential endogenous peptides could be identified from the MSPD extracts. In addition, MSPD extraction required much less time than acid extraction. Therefore, the MSPD method could be effectively employed as a novel method for the extraction of endogenous peptides from tissues. Moreover, preliminary results show that more novel endogenous peptides from samples could be extracted with MSPD, although additional experimental confirmation is required. This may be because the MSPD extraction method involves different physical and chemical processes than other extraction methods. Finally, we believe that the MSPD method will also be effective for the direct extraction of endogenous peptides with special post-translational modifications from tissue samples by using dispersing materials that have special selectivity for these modified peptides.

Acknowledgements

This work was supported by Major National Sci-Tech Projects (2009ZX09301-012) and Project of National Science Foundation of China (20975100).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.01.038.

References

[1] P. Schulz-Knappe, H.D. Zucht, C. Heine, M. Jurgens, R. Hess, M. Schrader, Comb. Chem. High Throughput Screening 4 (2001) 207.
[2] F.Y. Che, X. Zhang, I. Berezniuk, M. Callaway, J. Lim, L.D. Fricker, J. Proteome Res. 6 (2007) 4667.
[3] M. Svensson, K. Skold, P. Svenningsson, P.E. Andren, J. Proteome Res. 2 (2003) 213.
[4] F.Y. Che, J. Lim, H. Pan, R. Biswas, L.D. Fricker, Mol. Cell. Proteomics 4 (2005) 1391.
[5] S.A. Barker, J. Biochem. Biophys. Methods 70 (2007) 151.
[6] S.A. Barker, J. Chromatogr. A 885 (2000) 115.
[7] A.L. Capriotti, C. Cavaliere, P. Giansanti, R. Gubbiotti, R. Samperi, A. Lagana, J. Chromatogr. A 1217 (2010) 2521.
[8] R. Dassanayake, H. Wei, R.C. Chen, A. Li, Anal. Chem. 81 (2009) 9795.
[9] L.A. Berrueta, M. Fernanandez-Armentia, A. Bakkali, A. Gonzalo, M.L. Lucero, A. Orjales, J. Chromatogr. B 760 (2001) 185.
[10] S. Wang, H. Mu, Y. Bai, Y. Zhang, H. Liu, J. Chromatogr. B 877 (2009) 2961.
[11] H. John, M. Eddleston, R.E. Clutton, F. Worek, H. Thiermann, J. Chromatogr. B 878 (2010) 1234.
[12] M. Michel, B. Buszewski, J. Chromatogr. B 800 (2004) 309.
[13] B. Shao, H. Han, X.M. Tu, L. Huang, J. Chromatogr. B 850 (2007) 412.
[14] Y. Yang, B. Shao, J. Zhang, Y.N. Wu, J. Ying, J. Chromatogr. B 870 (2008) 241.
[15] A.F.M. Altelaar, S. Mohammed, M.A.D. Brans, R.A.H. Adan, A.J.R. Heck, J. Proteome Res. 8 (2009) 870.
[16] J.A. Dowell, W. Vander Heyden, L. Li, J. Proteome Res. 5 (2006) 3368.
[17] L.P. Aristoteli, M.P. Molloy, M.S. Baker, J. Proteome Res. 6 (2007) 571.
[18] A. Bora, S.P. Annangudi, L.J. Millet, S.S. Rubakhin, A.J. Forbes, N.L. Kelleher, M.U. Gillette, J.V. Sweedler, J. Proteome Res. 7 (2008) 4992.
[19] F.L. Strand, F.L. Strand, Neuropeptides: Regulators of Physiological Processes, in: Cellular and Molecular Neuroscience Series, The MIT Press, Cambridge, MA, 1999.