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Short communication

Rapid identification of bioactive peptides with antioxidant activity from the enzymatic hydrolysate of *Mactra veneriformis* by UHPLC–Q-TOF mass spectrometry

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ABSTRACT

Analysis of peptide components of protein hydrolysates is often difficult due to the lack of suitable analytical methods. In the present study, an UHPLC–Q-TOF MS/MS method was developed and used to identify peptides derived from the protein hydrolysate of *Mactra veneriformis*. The peptide sequences were deduced by *de novo* sequencing based on MS/MS fragmentation data. A total of 21 peptides, four nucleobases, and one nucleoside were identified from the hydrolysate using this method. These peptides were chemically synthesised and showed antioxidant activity in radical scavenging assays. This method is suitable for quick, sensitive, and accurate analysis of complex protein hydrolysates.

1. Introduction

Bioactive peptides derived from protein hydrolysates have broad applications in cosmetics, food additives, nutraceuticals, and pharmaceuticals. They are usually short peptides containing a sequence of 2–20 amino acids derived from the parent protein. These peptides exhibit a variety of biological functions including angiotensin-I-converting enzyme (ACE) inhibition (Murray & FitzGerald, 2007), antioxidant (Liu, Wang, Duan, Guo, & Tang, 2010), antimicrobial (Salampessy, Phillips, Seneweera, & Kailasapathy, 2010), anti-diabetic (Li, Zheng, Tang, Xu, & Gong, 2011), and immune modulatory (Shahidi, Han, & Synowiecki, 1995) properties.

Nowadays, proteins in a mixture can be identified using shotgun proteomics technology (Wolters, Washburn, & Yates, 2001). After the proteins are digested by specific proteases, the peptides generated are characterised by tandem mass spectrometry (MS/MS). The patent proteins are identified by matching the MS/MS spectra of their proteolytic peptides with those projected from a sequence database. The Edman degradation and MS/MS are two methods used to identify bioactive peptides (Koyama et al., 2013; Liu et al., 2010). The Edman degradation method requires highly pure samples and is not suitable for the analysis of complex mixtures such as protein hydrolysates. In contrast, high resolution LC–MS/MS is able to rapidly resolve peptide components in a complex mixture (Köcher, Swart, & Mechtler, 2011). In the present study, we used ultra-high performance liquid chromatography (UHPLC)-coupled MS/MS to rapidly identify peptides in the protein hydrolysate of *Mactra veneriformis*. The MS spectra of peptides were acquired under the collision-induced dissociation (CID) mode, and the amino acid sequences of the peptides were deduced by *de novo* sequencing based on MS fragmentation data.

The marine bivalve *M. veneriformis* is commercially cultured along the coasts of China, especially in Jiangsu province. Our previous studies showed that *M. veneriformis* flesh samples contain bioactive polysaccharides, nucleosides, and fatty acids (Liu et al., 2012; Wang, Zhang, Di, Liu, & Wu, 2011). *M. veneriformis*

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hydrolysates also contain proteins and peptides, but their sequences have not been resolved due to the lack of a suitable analytical method. We herein report the identification of bioactive peptides in the *M. veneriformis* hydrolysate using UHPLC–MS/MS.

2. Materials and methods

2.1. Materials

The *M. veneriformis* was collected from the Maojia aquaticultural regions of Jiangsu province of China in June 2010, and was examined by Prof. Xihe Wan (Institute of Oceanology and Marine Fisheries, Jiangsu) prior to processing. After collection, shells were discarded and the flesh stored and transported at −20 °C.

2.2. Chemicals and reagents

Trypsin, papain, neutrase, and pepsin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DPPH, ascorbic acid, pyrogallol, thiobarbituric acid (TBA), 2-deoxy-D-ribose, ethylene diamine tetraacetic acid (EDTA), and hydrogen peroxide (H_2O_2) were purchased from Shanghai Chemical Reagent Company (Shanghai, China). The hydroxyl, superoxide, and DPPH radical scavenging assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other reagents were from Sigma Chemical Co. All reagents were of analytical grade.

2.3. Preparation of M. veneriformis hydrolysates

M. veneriformis flesh was cut into small pieces and decocted twice (1 h each time) in boiling water to remove polysaccharides and other water-soluble components. Furthermore, the flesh was hydrolysed with trypsin, papain, neutrase, and pepsin under optimal conditions (Table S1, Supplementary material). After the hydrolysis was completed, the mixture was heated in a boiling water bath for 10 min to inactivate the enzyme. Dialysis membrane (10 kDa molecular weight cut-off) was used to remove polysaccharides and other substance with molecular weight greater than 10 kDa from the hydrolysates. Finally, the hydrolysates were lyophilized to powder and stored at −20 °C.

2.4. Radical scavenging assay

2.4.1. Hydroxyl radical scavenging assay

Test samples (0.1 ml) were added to phosphate buffer (0.4 ml, 50 mmol/l, pH 7.5) containing 0.2 mmol/l EDTA, 10 mmol/l H_2O_2, 60 mmol/l 2-deoxy-D-ribose, 2 mmol/l ascorbic acid, and 1 mmol/l $FeCl_3$. After the mixture was incubated at 37 °C for 1 h, HCl (8 mol/l, 1 ml) was added to stop the reaction. Subsequently, 1 ml of 1% thiobarbituric acid (TBA) was added into the reaction tubes, which were placed in boiling water for 15 min. Then the absorbance at 532 nm was determined. The scavenging activity was calculated using the formula ·OH scavenge = $(A_0 - A)/A_0 \times 100\%$, where A_0 and A represent the absorbance at 532 nm of the blank control and the sample, respectively.

2.4.2. DPPH radical scavenging assay

An aliquot (1 ml) of purified peptide was mixed with 1 ml of methanol solution containing 1 mmol/l DPPH radicals. The mixture was allowed to stand for 40 min in the dark, and the absorbance at 517 nm was determined. The scavenging activity of peptides was calculated using the formula DPPH scavenge = $(A_0 - A)/A_0 \times 100\%$, where A_0 and A represent the absorbance at 517 nm of the blank control and the sample, respectively.

2.4.3. Superoxide ($O_2^{\cdot-}$) radical scavenging assay

The superoxide ($O_2^{\cdot-}$) scavenging assay was performed using the pyrogallol autoxidation method. Eighty microlitres of the peptide samples was mixed with 80 μl of 50 mmol/l Tris–HCl buffer (pH 8.3) containing 1 mM EDTA in a 96-well microplate. Then 40 μl of 1.5 mmol/l pyrogallol in 10 mmol/l HCl was added. The mixture was allowed to stand at room temperature for 4 min. The rate of $O_2^{\cdot-}$-induced polymerisation of pyrogallol (ΔA/min) was determined based on the increase in absorbance at 420 nm. The scavenging activity of peptides was calculated using the formula $O_2^{\cdot-}$ scavenging activity = $(\Delta A_0 - \Delta A)/\Delta A_0 \times 100\%$, where ΔA_0 and ΔA represent the increase in absorbance at 420 nm of the blank control (Tris–HCl buffer) and the sample, respectively. Glutathione was used as a positive control.

2.5. Fractionation of hydrolysates

The hydrolysates were reconstituted with distilled water and separated on a Sephadex G-25 gel filtration column (2.0 cm × 100 cm; GE Chemicals, Uppsala, Sweden) using distilled water as the eluting solvent at a flow rate of 0.4 ml/min. The absorbance at 260 nm was recorded. Five elution fractions (F1–F5, 4 ml each) were collected and lyophilized. Their radical scavenging activities were determined after reconstitution with water.

2.6. UHPLC–MS/MS analysis

The two most active fractions collected from the Sephadex G-25 gel filtration column (F3 and F4) were subjected to analysis on a Waters ACQUITY UHPLC system coupled to a Synapt Mass Quadrupole Time-of-Flight Mass Spectrometer. Five microlitres of sample was loaded onto an ACQUITY BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm), and the column was run using a linear gradient of water–acetic acid (100:0.5, v/v) (eluent A) and methanol (eluent B) as follows: 0–3 min, 5% B; 3–15 min, 5%–30% B; 15–17 min, 30% B; flow rate, 0.3 ml/min. The MS spectra were acquired under the positive electrospray ionisation and CID mode. Collision energy in the range of 10–35 eV was tested for each pre-selected precursor ion and optimised based on the quality of ion fragmentation. Argon was introduced as a collision gas at a pressure of 10 psi. Peptide sequencing was performed using the Biolynx software by analysing ion peaks in the m/z range of 50–2000.

2.7. Peptide synthesis and hydroxyl radical scavenging assay

Twenty-one peptides identified in F3 and F4 were chemically synthesised using the solid phase method and purified by HPLC by GenScript Corporation (Nanjing, China). These chemically synthesised peptides were tested for their hydroxyl radical scavenging activities as described in Section 2.4.1. The IC_{50} values were calculated using the GraphPad software.

2.8. Statistical analysis

The data are presented as mean ± S.D. Each experiment was conducted in quadruplicates.

3. Results and discussion

3.1. M. veneriformis flesh hydrolysates and their antioxidant activities

In the present study, *M. veneriformis* flesh hydrolysates were produced by incubating under optimal conditions with trypsin, papain, neutrase, and pepsin, respectively. The antioxidant activities of the hydrolysates were evaluated in radical scavenging

assays. Our results revealed that the trypsin hydrolysate had higher radical scavenging activities than all other hydrolysates (Fig. S1). Therefore, the trypsin hydrolysate was selected for further analysis.

3.2. Radical scavenging activities of the elution fractions of the trypsin hydrolysate

The trypsin hydrolysate was separated on a Sephadex G-25 gel filtration column (Fig. 1A) to generate five fractions. The weight percentages of fractions 1–5 (F1–F5) after lyophilization were about 9%, 8%, 69%, 11%, and 2%, respectively. F3 and F4 exhibited more potent activities than the other fractions in the hydroxyl, superoxide, and DPPH radical scavenging tests (Fig. 1B).

3.3. Determination of peptides in F3 and F4 by UHPLC–ESI-MS/MS analysis

F3 and F4 were subjected to UHPLC–ESI-MS/MS analysis and their total ion chromatograms (TICs) are displayed in Figs. 2A and 3A, respectively. Ion peaks of significant intensity in the TICs of F3 and F4 were identified and their intensity, retention time, and m/z ratio were recorded. Figs. 2B and 3B are stacking charts of ions with an m/z ratio in the range of 398–990 and 113–318, respectively. These ions were fragmented with optimised collision energy in the MS/MS analysis. A total of 21 peptides were identified based on the MS/MS data (Table 1). These peptides were *de novo* sequenced using the Biolynx software (Tables 1 and S2). Additionally, four nucleobases and one nucleoside were detected based on their retention time, UV spectra, and MS fragmentation pattern (Tables 1 and S3).

Advances in LC–ESI-MS/MS analytical techniques have made peptide analysis in highly complex mixtures possible. In the present study, we used UHPLC for rapid peptide separation with excellent resolution. In the subsequent MS/MS analysis, the translational energy in CID was optimised to obtain the most informative fragmentation data. As previously described (Serdler, Zinn, Boehm, & Lehmann, 2010), amino acids were identified based on mass differences of consecutive fragmentations and peptide sequences were deduced accordingly. For instance, the ion m/z 908.42 was identified as an octapeptide with the sequence of YSEMPPGK (Fig. 4A) based on its y ion series of m/z 147.11, 204.13, 398.24, 529.28, 658.32, and 745.35 and b ion series of m/z 251.10, 380.15, 511.19, and 762.31. In another case, inosine was identified based on the ion peaks with m/z ratios of 269.08 $(M+H)^+$ and 137.05 $(M+H-ribose)^+$, and its retention time and UV absorbance at 248 nm (Fig. 4B).

There is a certain degree of ambiguity in peptide identification using MS/MS. For example, it is difficult to distinguish amino acids that have the same or very similar molecule weights, such as Gln and Lys, and Leu and Ile. With improved resolution, accuracy, and sensitivity, it is possible to distinguish Gln and Lys by MS analysis, but not Leu and Ile, which have the exact same molecular weight. Fortunately, amino acid identification in the present study was not affected by these inherent limitations of MS analysis.

After peptide components of F3 and F4 were identified, these peptides were chemically synthesised and evaluated for hydroxyl radical scavenging activity. Of the 21 peptides synthesised, 20 displayed detectable activity. The peptide TDY showed the highest activity with an IC_{50} value of 140.2 μM (Table S4) followed by peptides LDY, WDDMEK, WHNVSGSP, LYEGY, and MEMK, in that order. Antioxidant peptides are usually short peptides consisting of fewer

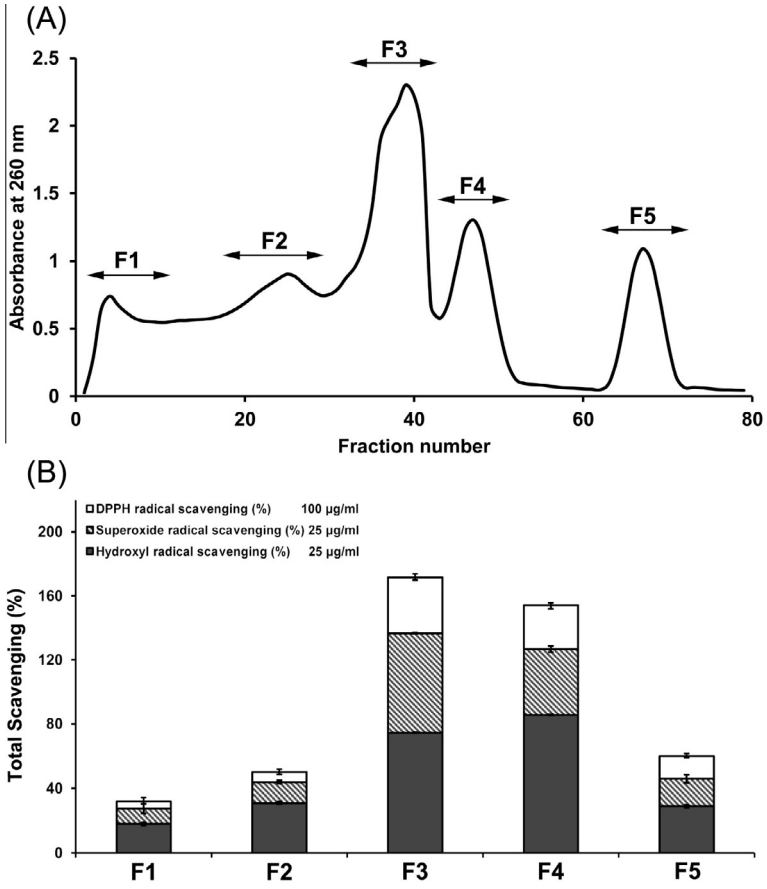

Fig. 1. (A) Elution diagram of the trypsin hydrolysate of *M. veneriformis*. The hydrolysate was eluted on a Sephadex G-25 gel filtration column with distilled water at a flow rate of 0.4 ml/min; (B) hydroxyl, superoxide, and DPPH radical scavenging activities of fractions 1–5 (F1–F5) from Sephadex G-25 chromatography ($n = 4$).

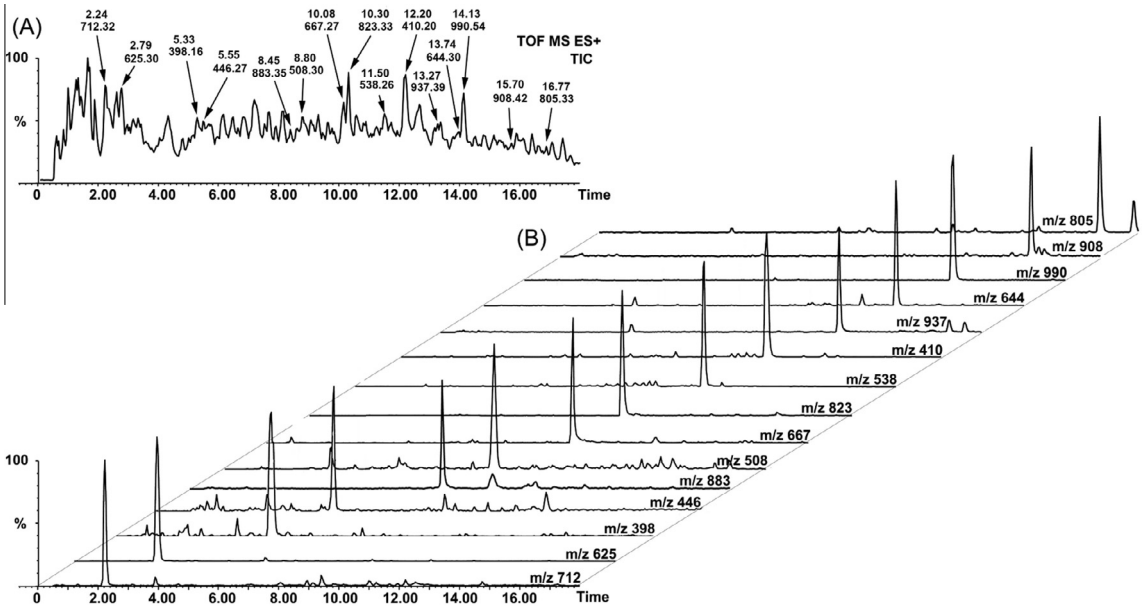

Fig. 2. UHPLC–MS analysis of F3. (A) TIC of F3; (B) TIC of the monitored peaks.

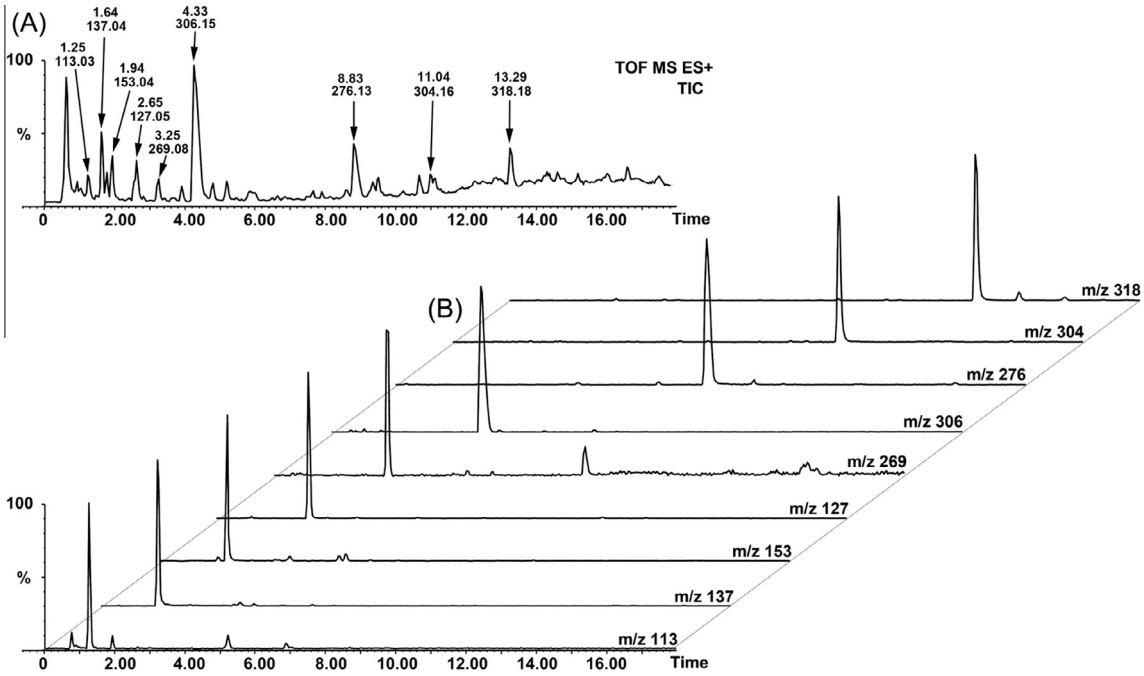

Fig. 3. UHPLC–MS analysis of F4. (A) TIC of F4; (B) TIC of the monitored peaks.

than 20 amino acid residues. These peptides are often able to form chelates with metals or act as hydrogen/electron donors, which make them capable of terminating the radical chain reaction or preventing the formation of free radicals (Ren et al., 2008). Also, antioxidant peptides often contain hydrophobic amino acids, which play an important role in their antioxidant activity (Mendis, Rajapakse, Byun, & Kim, 2005). In the present study, we found that the peptides identified in the trypsin hydrolysate of *M. veneriformis*, which also showed antioxidant activity are rich in proton-donor amino acids (e.g. Tyr, Phe, Gly, Glu, Thr, and Asp) and hydrophobic amino acids (e.g. Tyr, Ser, Phe, Val, Leu/Ile, Pro, Ala, and Gly). Take the peptide TDY as an example, the hydroxyl group of Thr, the phenolic hydroxyl group of Tyr, and the carboxyl group of Asp can all act as a proton-donator, which are probably responsible for the potent free radical scavenging activity of this peptide. Besides the amino acids, the nucleobases and nucleoside detected in F3 and F4 may also contribute to the antioxidant activities of these two fractions (Gudkov, Shtarkman, Smirnova, Chernikov, & Bruskov, 2006).

4. Conclusion

In summary, we developed a rapid UHPLC–Q-TOF MS/MS method suitable for peptide identification in complex mixtures like protein hydrolysates. This method was successfully used to identify

Table 1
Peptides, nucleobases, and nucleoside in F3 and F4 identified by UHPLC–MS/MS.

Fraction	Categorisation	Retention time (min)	$(M+H)^+$	Analyte
F3	Hexapeptide	2.24	712.32	HMPVTK
	Pentapeptide	2.79	625.30	TERGY/TEFTK
	Tripeptide	5.33	398.16	TDY/NHK
	Tetrapeptide	5.55	446.27	LVSK
	Octapeptide	8.77	883.35	WHNVSGSP
	Pentapeptide	8.80	508.30	ALSAF
		10.08	667.27	FDEQE
	Hexapeptide	10.30	823.33	WDDMEK
	Tetrapeptide	11.50	538.26	MEMK
	Tripeptide	12.20	410.20	LDY
	Octapeptide	13.14	937.39	YWVTSGPK
	Pentapeptide	13.74	644.30	LYEGY
	Nonapeptide	14.13	990.54	VAMVPPFET
	Octapeptide	15.72	908.42	YSEMPPGK
	Hexapeptide	16.89	805.33	FHNMEK
F4	Nucleobase	1.25	113.03	Uracil
		1.64	137.04	Hypoxanthine
		1.94	153.04	Xanthine
		2.65	127.05	Thymine
	Nucleoside	3.25	269.08	Inosine
	Dipeptides	4.33	306.15	TW
		8.83	276.13	AW
		11.04	304.16	VW
		13.29	318.18	LW

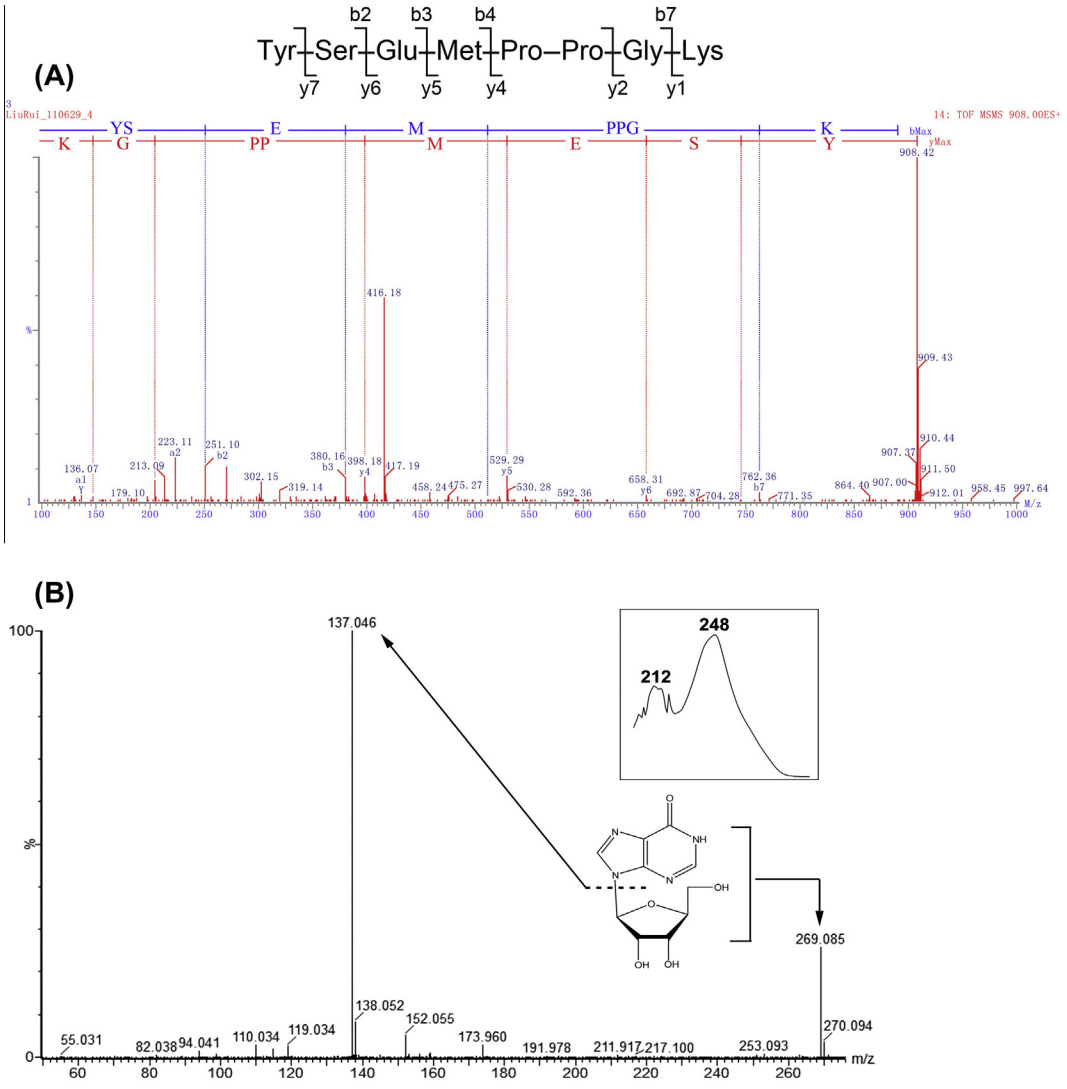

Fig. 4. UHPLC–MS/MS analysis of ion peaks m/z 908 and m/z 269. (A) A peptide with the sequence of YSEMPPGK was identified from ion m/z 908; (B) inosine was identified from ion m/z 269.

antioxidant peptides in the trypsin hydrolysate of *M. veneriformis*. This method is quick, sensitive, and effective, and may be used to identify bioactive peptides from other complex sources.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.06.113.

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