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Identification of Antioxidative Peptides from *Lactobacillus plantarum* Lp6 Fermented Soybean Protein Meal

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Abstract: In this study, we previously reported that peptides fractions obtained by gel filtration chromatography from Fermented Soybean Protein Meal Hydrolysate (FSPMH) showed strong antioxidative activity. In this study we particularly focused on the fraction F2 which showed the highest antioxidant properties. The fraction F2 was subjected to reverse phase high performance liquid chromatography (RP-HPLC) for purification. The resultant peaks were collected separately as fractions and subsequently subjected to DPPH radical scavenging and Cu^{2+} chelating tests. The amino acid sequences of isolated peptides were then determined by liquid chromatography/tandem mass spectrometry (LC-MS/MS). The RP-HPLC fraction P1 showed stronger 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability and possessed relatively strong Cu^{2+} chelation ability compared to 5 other RP-HPLC fractions. The amino acid sequences indicated that the isolated peptides contained Histidine.

Key words: *Lactobacillus plantarum* Lp6, fermented soybean protein meal, peptide fractions, antioxidative peptides, radical scavenging

INTRODUCTION

Fermentation is one of the oldest techniques in food manufacture and preservation that contributes directly to many advantageous properties of products by biochemical modification of microorganisms (Je *et al*., 2005; Chukeatirote *et al*., 2006; Blanco *et al*., 2009). Over the past decade, peptides and protein hydrolysates prepared from common food proteins have emerged as new sources of natural antioxidants. Protein hydrolysates from different sources, such as zein protein (Kong and Xiong, 2006), fermented soybean protein (Amadou *et al*., 2009b), egg-yolk (Sakanaka and Tachibana, 2006) and yellowfin sole frame (Jun *et al*., 2004) have been shown to possess antioxidant activity. A typical enzymatic protein hydrolysate is a mixture of proteases, peptones, peptides and free amino acids (Chang *et al*., 2007). The levels and compositions of free amino acids and peptides were reported to determine the antioxidant activities of protein hydrolysates (Wu *et al*., 2003). The type of proteases used and the degree of hydrolysis also affect the antioxidant activity (Kong and Xiong, 2006).

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Many antioxidative peptides also have been identified from a variety of fermented food proteins, such as soybean protein meal (Amadou *et al*., 2009a) and royal jelly protein (Guo *et al*., 2009). Marcuse (1962) reported that several amino acids, such as Tyr, Met, His, Lys, Gly and Trp, are generally accepted as antioxidants despite their prooxidative effects in some cases. Kawashima *et al.* (1979) noted that some di- and tri-peptides containing aromatic amino acid residues, as well as peptides containing Tyr, Pro and His, showed strong antioxidant activity.

Soybean protein meal collected from soybean oil plants is a valuable protein source from which bioactive peptides can be produced during fermentation by microbial proteolytic enzymes (Granito *et al*., 2005). However, there is little information regarding the antioxidative properties of peptides isolated from *Lactobacillus plantarum* Lp6 fermented soy protein meal hydrolysate.

The objective of this study was to investigate the antioxidative activity of peptides isolated from *Lactobacillus plantarum* Lp6 fermented soy protein meal hydrolysate by DPPH radical scavenging and Cu^{2+} ion chelating activity and to determine the amino acid sequences of the purified peptide.

MATERIALS AND METHODS

The studies were carried out at State Key Laboratory of Food Science and Technology, Jiangnan University (Wuxi, China) during July, 2008 to December, 2009.

Materials

1, 1-diphenyl-2-picrylhydrazyl (DPPH) and protease were purchased from Sigma-Aldrich, Inc (Shanghai, China); the strain *Lactobacillus plantarum* Lp6 was obtained from the culture collection of Jiangnan University (Wuxi, China). Soybean protein meal (SPM) was of food grade and was obtained from Sun-Green Biotech Co. Ltd (Nantong, China). All other chemicals used were obtained from the Chemical Reagent Co., China and were of analytical grade.

Fermentation Method

The *Lactobacillus plantarum* Lp6 used was stored initially at 4°C and cultured for 18 h at 37°C in Man-Rogosa-Shape (MRS) broth prior to use for fermentation. A 0.025 mL of *L. plantarum* Lp6 was prepared in sterilized distilled water and then mixed with 25 g of soybean protein meal (10^7 colony forming units cfu g^{-1}) fortified with soluble starch (0.4 g g^{-1} of SPM) and/or protease (0.01 g g^{-1} of SPM) in polyethylene bag (140× 200 mm) and vacuum sealed. Also disodium phosphate (2 mg g^{-1}) was added to improve the activity of *L. plantarum* Lp6 and then solid-state fermentation was performed for 72 h at 37°C.

Preparation of Fermented Soy Protein Meal Hydrolysate Extract

Fermented Soy Protein Meal (FSPM) extract was prepared according to the method described by Ye *et al.* (2003). Five grams of FSPM were mixed with 50 mL of distilled water, homogenized for 1 min and incubated at 37°C for 60 min. The incubated mixture was centrifuged at 9600 rpm for 2 min and the residue was washed with 20 mL distilled water, centrifuged again at the same speed and time and the combined supernatant was freeze-dried and stored at -20°C until further use.

Isolation of Antioxidant Peptides

The fermented soybean protein meal hydrolysate (FSPMH) was subjected to fractionation using Sephadex G-15 gel filtration column (45×2 cm^2, Amersham Pharmacia

Biotech AB, Sweden). Seven fractions were collected, freeze-dried, stored at -20°C and used for the test of antioxidant activity where F2 was found to exhibit the highest activity which quite agreed with our earlier work (Amadou *et al*., 2010).

The fraction F2 with the highest antioxidant activity was dissolved in distilled water and further purified by reversed phase high performance liquid chromatography (RP-HPLC) C_{18} column (250×10 mm, KromTek Technologies Inc). The two solvent reservoirs contained the following eluents: (A) 0.1% (v/v) trifluoroacetic acid (TFA) (B) 0.1% (v/v) TFA in 80% (v/v) acetonitrile. The elution program consisted of a gradient system (0-100% B in 80 min) with a flow rate of 1.5 mL min^{-1}. The elution pattern was monitored by measuring the absorbance at 214 nm. All solvents were degassed just prior to use. All samples were centrifuged to remove aggregated protein. Each peak was manually collected. The collected antioxidant peptides were, concentrated and protein content was determined using Folin method (Folin, 1922) before the DPPH radical scavenging and Cu^{2+} ion chelating activity test.

DPPH Radical Scavenging Activity

The scavenging effect of FSPM extract on DPPH free radical was measured according to the method of Shimoda *et al.* (1992) with slight modification. Three milliliters of each sample solution 140.0 μg mL^{-1} were added to 3 mL of 0.1 mM DPPH dissolved in 95% ethanol. The mixture was shaken and left for 30 min at room temperature. The absorbance was measured at 517 nm. A lower absorbance represents a higher DPPH scavenging activity. The scavenging effect was expressed as shown in the following Eq. 1:

$$\text{DPPH scavenging activity (\%)} = \left(\frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}}\right)\times 100 \quad (1)$$

Metal Chelating Activity

The RP-HPLC fractions ability to chelate prooxidative Cu^{2+} was investigated according to the procedure of Wang and Xiong (2005) with slight modifications. In the chelation test, 1 mL of 2 mM $CuSO_4$ was mixed with 1 mL of pyridine (pH 7.0) and 20 μL of 0.1% pyrocatechol violet. After the addition of 1 mL of samples (140.0 μg mL^{-1}), the disappearance of the blue color, due to dissociation of Cu^{2+}, was recorded by measuring the absorbance at 632 nm at 5 min of the reaction. The Cu^{2+} chelating activity of the RP-HPLC fractions samples were calculated as in the following Eq. 2:

$$\left(\frac{\text{Blank absorbance} - \text{Sample absorbance}}{\text{Blank absorbance}}\right)\times 100 \quad (2)$$

Peptide Sequence Analysis

Molecular mass and peptide sequence of purified fraction was determined using a matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-TOF MS) (4700 proteomics Analyzer, Applied Biosystems, USA). All spectra were measured under the following conditions: MS: Reflector Positive, CID (OFF), mass rang (700-3200 Da), Focus Mass (1200 Da), Fixed laser intensity (6000), Digitizer: Bin Size (1.0 ns). MS/MS: 1 kV Positive, CID (ON), Precursor Mass Windows (Relative 80 resolution (FWHM)), Fixed laser intensity (7000) Digitizer: Bin Size (0.5 ns). A-Cyano-4-hydroxycinnamic acid (Aldrich, Steinheim, Germany) was used as a matrix. An amount of 0.5 mL of the purified peptides solution plus 0.5 mL matrix was deposited on the sample slide and left to dry at room temperature. The resulting spectra were analyzed and compared.

Statistical Analysis

An Analysis of Variance (ANOVA) of the data was performed and Tukey test with significant differences ($p<0.001$) was used to compare the means using SPSS version 13.0 (SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

Antioxidant Activity

The RP-HPLC chromatogram obtained after further separation of F2 fraction; about six different peaks were obtained (P1, P2, P3, P4, P5 and P6), which is an indication of the level of F2 purity (Fig. 1). Each peak was separately collected and subjected to several antioxidative activity tests. The scavenging effects of different fractions obtained by RP-HPLC on the DPPH radical and their effects on metal chelating activity are shown in Table 1, the DPPH radical scavenging activity of the RP-HPLC fractions ranged from 43.75 to 67.01% with RP-HPLC purified fraction P1 exhibit significantly ($p<0.001$) the highest activity and fraction P4 the lowest. The Cu^{2+} chelating activity varied between 11.42 to 47.92%. Fractions P6 showed the highest Cu^{2+} chelating activity (47.92%) followed by fractions P5 and P1 (34.95 and 34.41%), respectively (Table 1). The different DPPH radical scavenging patterns observed for the RP-HPLC purified fractions in this study may be likely related to the structure of peptides eluted at different stages of the purification (Zhu *et al*., 2008). DPPH is an oil-soluble free radical that becomes a stable product after accepting an electron or a hydrogen atom from an antioxidant. These antioxidants donate hydrogen to free radicals, leading to non-toxic species and therefore to inhibition of the propagation phase of lipid oxidation (Jao and Ko, 2002). Hydrolysis of proteins after fermentation resulted in the formation or exposure of high-affinity metal-binding groups such as, the imidazole and carboxylic groups which therefore increased the electrostatic and ionic interactions between

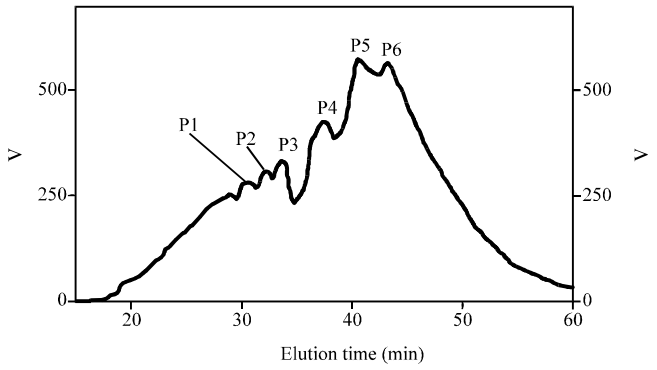


Fig. 1: HPLC chromatogram of F2 fraction

Table 1: Antioxidant activity of fermented soybean protein meal fractions

Sample	DPPH radical scavenging activity (%)	Cu^{2+} chelating activity (%)
P1	67.01 ± 1.77^{e}	34.41 ± 2.48^{bc}
P2	50.27 ± 0.56^{bc}	19.91 ± 3.13^{ab}
P3	53.65 ± 0.37^{cd}	21.99 ± 3.95^{ab}
P4	43.75 ± 0.74^{a}	11.42 ± 4.09^{a}
P5	46.76 ± 1.04^{ab}	34.95 ± 3.82^{bc}
P6	56.36 ± 0.67^{d}	47.92 ± 1.29^{c}

Values are Means±SD of three determinations. Column with different letters indicate statistical differences ($p<0.001$)

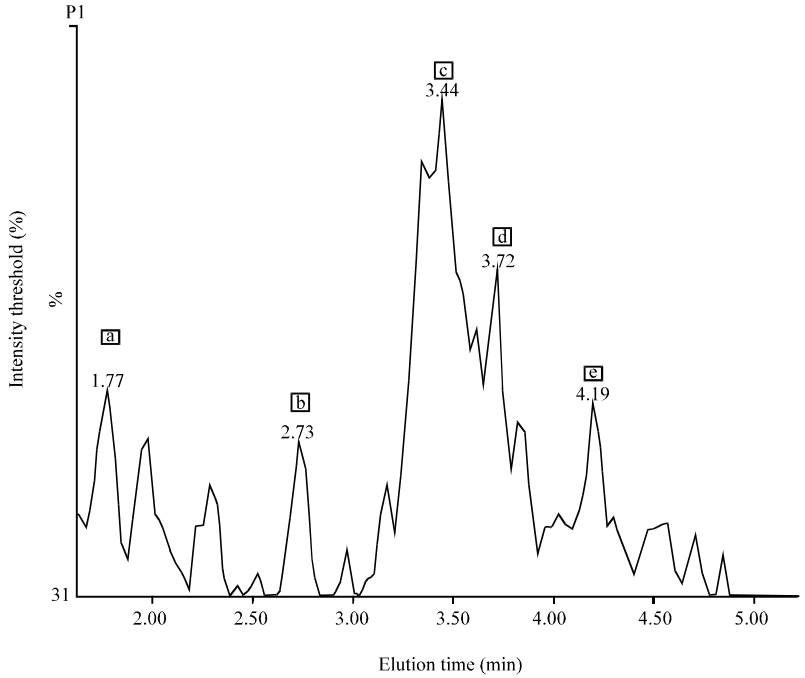


Fig. 2: Chromatogram of HPLC fraction P1 in Bio Lynx protein/peptide editor

the peptides and Cu^{2+}. Decker *et al.* (1992) showed that the specific peptide structure and amino acid side chain groups played an important role not only in terminating free radical chain reactions but also in chelating transition metal ions.

Isolation of Antioxidant Peptides and Determination of Amino Acid Sequences

Table 1 showed that P1 exhibited the highest antioxidant activity, consequently, the P1 RP-HPLC fraction was analyzed (Fig. 2) for amino acid sequences; and the chromatogram shown in Fig. 3a-e represent the matched peptides in Bio Lynx protein/peptide Editor (Mass Lynx V4.1). The amino acid sequences of the antioxidative peptide P1 fraction and their molecular weights are showing in a table included in the Fig. 3. Rajapakse *et al.* (2005) noted that the peptide isolated from fermented mussel sauce contained Gly, Phe and His, showed the highest exhibition of radical scavenging activity and metal chelating activity. In the free radical-mediated lipid peroxidation system, antioxidant activity of peptide or protein is dependent on molecular size and chemical properties such as hydrophobicity and electron transferring ability of amino acid residues in the sequence. Chen *et al.* (1998) and Guo *et al.* (2009) reported antioxidative peptides that were isolated from digested soybean protein and royal jelly proteins hydrolysate respectively, found that Histidine-containing peptides and some small peptides with 2 to 6 amino acid residues had strong antioxidant activity. Present results indicated that the antioxidant activity of the peptide fraction isolated from FSPMH using gel filtration and RP-HPLC contained Histidine in the amino acid sequences (Table encrypted in Fig. 3), which is an accordance with the findings of Chen *et al.* (1998), Rajapakse *et al.* (2005) and Guo *et al.* (2009). Our previous research on F2 fraction isolated using gel filtration also showed the highest Histidine content in amino acid analysis (Amadou *et al.*, 2010).

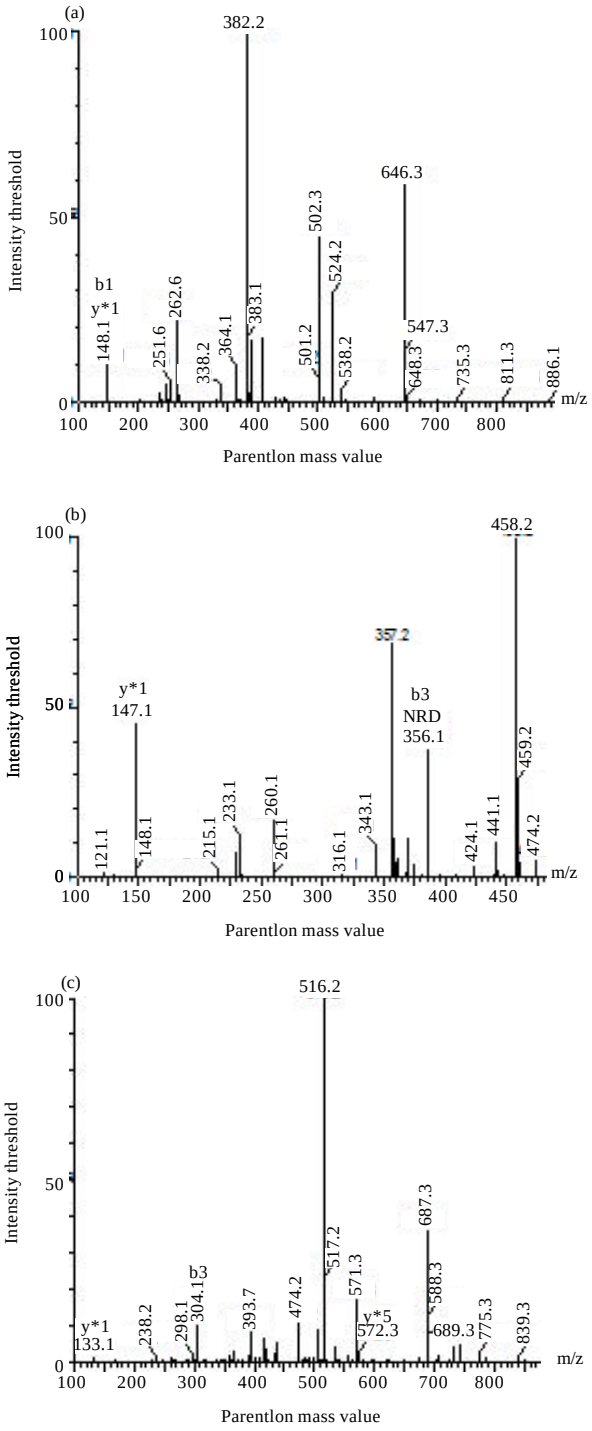
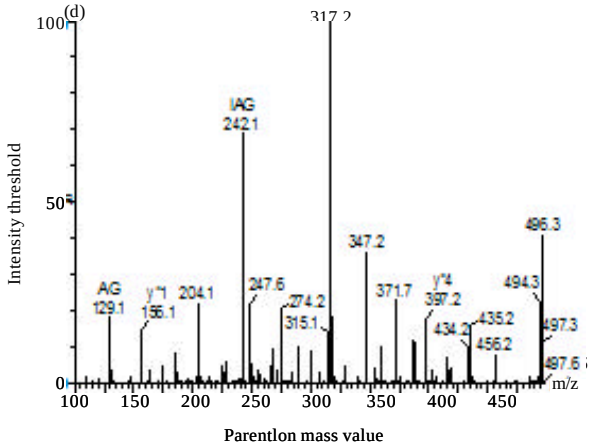
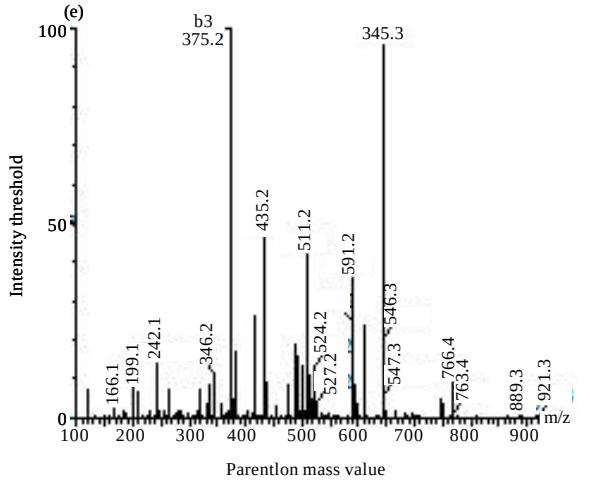


Fig. 3: Continued





	Score (%)	Mass	AA sequance
a	100	645.3 "0.3	Phe-Asp-His-Val-Glu
b	100	644.3 "0.3	Phe-Asn-His-Leu-Asp-His
c	100	495.3 "0.3	Val-lle-Ala-Gly-His
d	100	495.3 "0.3	Val-Leu-Ala-Gly-His
e	100	686.3 "0.3	Asp-Ser-Thr-Asr-His-Asn

Fig. 3: (a-e) ESI-MS^{E} fragmentation pattern of reversed-phase HPLC fraction P1 and its amino acid sequence/molecular weights.

CONCLUSION

Based on the findings obtained in this study, the peptide fractions obtained by RP-HPLC purification of the FSPMH fractionated extract (P1) exhibited strong antioxidative activity measured in terms of DPPH radical scavenging activity and metal chelating activity. The antioxidative capacity of peptides isolated from the *Lactobacillus plantarum* Lp6 fermented soybean protein meal hydrolysate related to their amino acid sequences. Results from this study indicated that it is feasible to produce natural antioxidants from soybean protein meal by fermentation and hydrolysis and different separation and enrichment techniques, which could be a means to adding values to the human nutrition in the prevention of chronic illnesses such as cancer and cardiovascular diseases.

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